Efficacy of interventions and role of raw colostrum feeding programs in the transmission of *Mycobacterium avium* subsp. *paratuberculosis*

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Patrick Pithua

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Scott J Wells (Co-Advisor) and Sandra M Godden (Co-Advisor)

June, 2009

Acknowledgements

I am forever grateful to Drs. Scott J Wells and Sandra M Godden for offering me the opportunity to pursue graduate studies at the University of Minnesota. I thank you very much. I would also like to extend my sincere appreciation to the other members of my PhD examination committee including Dr. Michael Oakes, Dr. Srinand Sreevatsan, Dr. John Fetrow and Dr. Peter Davies. I learnt at least something new from each one of you and I will take all the good advice received with me wherever I will go.

I am particularly thankful and indebted to my family for their unconditional love and support. To my mother Christine, you are the best mum in the world and I would not trade your place for anything. To Uncle George, you too are the best uncle one could have in the world. To all my friends, I say thank you for always being tolerant towards me.

Dedication

This dissertation is dedicated to my family including Maranda, Edgar, Philip, Joseph, Christine, Harriet, Candida, Eric, Samuel, Tutu, Atat Edica, Uncle George, and Susan.

Abstract

This thesis described the role of raw bovine colostrum feeding programs and natural nursing practices in the transmission of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and the efficacy of commercially available colostrum replacement products in preventing MAP transmission, additional to their effect on production and longevity performance outcomes. Incidences of fecal excretion of MAP by calves following natural exposure were also evaluated.

Calves fed CR (vs MC) had a lower risk of MAP infection when the serum ELISA (HR = 0.474, *P* = 0.081), bacterial fecal culture (HR = 0.572, *P* = 0.076) or both test combinations (HR = 0.559, *P* = 0.056) were used to define MAP status of study cohorts, suggesting that MC could be an important vehicle by which calves become exposed to MAP within hours following birth, and that CR feeding programs may be an effective management tool for use in dairy herds in a Johne's disease control effort.

From birth-to-54 months of follow-up, risk of death (HR = 1.22, *P* = 0.17), culling (HR =1.01, *P* = 0.95), and death and/or culling (HR = 1.1, *P* = 0.61) event outcomes did not significantly differ between groups (CR vs MC). Similarly there were no significant differences between groups (CR vs MC) with respect to the risk of death (HR = 1.22, *P* = 0.46), culling (HR =1.01, *P* = 0.98), and death and/or culling (HR = 1.05, *P* = 0.85) event outcomes when only heifers that entered the lactating herd (period from first calving date to 54 months of age) were considered. Feeding CR (vs MC) had no significant effect on age at first calving (*P* = 0.34), number of breedings per conception in the first (*P* = 0.83) and second (*P* = 0.32) lactations respectively, and calving-to-conception intervals in the first (*P* = 0.7) and second (*P* = 0.21) lactations, respectively. Considering the milk yield outcome, feeding CR (vs MC) significantly (*P* = 0.02) decreased first lactation milk by 429 kg, although there were no significant effects of feeding CR (vs MC) on second lactation (*P* = 0.18) and lifetime milk yields (*P* = 0.5), respectively.

Risk of MAP infection was not significantly different between groups of calves that ingested MAP DNA positive colostrum (vs MAP DNA negative colostrum) when the serum ELISA (HR = 0.74, *P* = 0.65), bacterial fecal culture (HR = 0.92, *P* = 0.85) or both test combinations (HR = 0.82, *P* = 0.65) were used to define MAP status of study

cohorts, suggesting lack of an added risk of MAP infection associated with ingesting MAP DNA positive raw colostrum by Holstein calves. This finding contradicted several other reports which seem to provide evidence in support of colostrum as a possible early vehicle by which calves get exposed to MAP in infected herds.

Cows that were fecal culture positive were significantly more likely to have detectable MAP in their colostrum (OR =2.02 , $P < 0.001$) and teat skin (OR =1.87 , $P = 0.008$) compared with fecal culture negative cows with the population attributable fraction estimates for exposure for each of the latter outcomes being 18% and 19.5%, respectively.

Finally, MAP was not recovered from fecal samples collected between 1-to-90 d of age and tested using the sedimentation bacterial culture method suggesting that the calves studied did not excrete detectable levels of MAP in feces following natural exposure.

Table of Contents

Acknowledgements i

Dedication ii

Abstract iii

Table of Contents v

List of Tables vi

List of Figures viii

List of Abbreviations ix

Chapter 1: Introduction and a general review of the epidemiology of Johne's disease in dairy herds 1

Chapter 2: Efficacy of feeding plasma-derived commercial colostrum replacer for the prevention of transmission of *Mycobacterium avium* subsp. *paratuberculosis* in Holstein calves 17

Chapter 3: Effect of a plasma-derived colostrum replacement feeding program on adult performance and longevity in Holstein cows 42

Chapter 4: Experimental validation of a nested PCR targeting the genetic element ISMAP02 for detection of MAP in bovine colostrum 66

Chapter 5: Lack of association between ingestion of *Mycobacterium avium* subsp. *paratuberculosis* DNA positive raw colostrum and the risk of infection in Holstein calves 80

Chapter 6: *Mycobacterium avium* subsp. *paratuberculosis* DNA detection in colostrum and teat skin associated with Mycobacterial fecal excretion: Implications for prevention of transmission in Holstein calves 97

Chapter 7: Lack of evidence for detectable fecal excretion of *Mycobacterium avium* subsp. *paratuberculosis* in calves born in a high Johne's disease prevalence herd 118

General conclusions 125

References 130

List of Tables

Table 1. Descriptive statistics and results of logistic regression models adjusted for herd effect to compare the absolute risk of developing MAP infection at various ages in 236 cows fed CR at birth and 261 cows fed raw bovine MC at birth. 37

Table 2. Results of final Weibull models adjusted for random herd effect to evaluate the risk of developing MAP infection in 261 cows fed MC at birth and 236 cows fed CR at birth. 38

Table 3. The number of cows enrolled per herd and number of cows with positive test results for MAP in the CR and MC groups, respectively, and herd estimates for effect of CR versus MC on the hazard of developing MAP infection on the basis of both diagnostic test outcomes. 39

Table 4. Unconditional comparison of number of calves fed CR vs MC at birth with respect to risk of death, and culling at various stages during the follow-up period up to 54 months of age. 60

Table 5. Final proportional hazards models evaluating time-to-removal from the herd for any reason between cows fed CR vs MC at birth, from birth and from date of first..... 61

Table 6. Unconditional comparison of milk yield and breeding performance indices between cows fed CR vs MC at birth. 62

Table 7. Final models evaluating breeding performance and milk yield indices of cows fed CR vs MC at birth. 63

Table 8. The colony counts associated with MAP concentration for each dilution of the cell suspension used to inoculate colostrum samples. 77

Table 9. The limits of detection at each concentration of incocula for the detection of Mycobacterium avium subsp. paratuberculosis in colostrum using the nested PCR of the target gene ISMAP02..... 78

Table 10. The total number of known infected colostrum samples based on the sonicated and unsonicated MAP strain K-10 cell suspensions as the inocula correctly detected as positive for MAP DNA using the a nested PCR of the target element ISMAP02. 79

Table 11. Unconditional comparison of the number of MAP positive events observed in Holstein cows attributable to ingestion of MAP DNA positive colostrum (exposed) vs MAP DNA negative colostrum (unexposed) at birth. 95

Table 12. Final proportional hazards models describing the association between ingesting MAP DNA positive colostrum (vs MAP DNA negative colostrum) and time-to-MAP positive diagnosis (hazard). 96

Table 13. The general characteristics of four Minnesota dairy herds enrolled in 2005 for a study of the association between positive MAP fecal shedding status and risk of detecting MAP in colostrum and teat swabs collected from Holstein cows around calving time. 113

Table 14. The comparison of number of colostrum samples and teat swabs in which MAP was detected, and the distribution of other variables in a study of the association between positive MAP fecal shedding status and risk of detecting MAP in colostrum and teat swabs collected from Holstein cows around calving time in four Minnesota dairy farms in 2005..... 114

Table 15. Final logistic-regression models estimating the association between MAP fecal shedding status and risk of detecting MAP in colostrum and teat swabs collected from Holstein cows around calving time for fecal culture positive cows. 115

Table 16. Logistic regression models representing the association between MAP fecal shedding category, and the risk of detecting MAP in colostrum and teat swabs around calving time for fecal culture positive cows. 116

Table 17. Population attributable fraction (PAF) estimates for the risk of MAP detection in colostrum and teat swabs attributable to a positive MAP fecal shedding status around calving time..... 117

List of Figures

Figure 1. Distribution of estimated true MAP prevalence of 12 herds participating in a study to evaluate the effect of feeding of CR versus MC to heifer calves at birth on subsequent development of MAP infection. 40

Figure 2. Weibull hazard functions for the analysis of interval until a positive test outcome for MAP, as determined by use of a serological ELISA (A), bacterial culture of fecal samples (B), or a combination of both diagnostic tests (C), in cows fed CR (solid lines) or raw bovine MC (dotted lines) at birth..... 41

Figure 3. Herd adjusted survival plots for the analysis of time-to-death and/or culling outcome from date of birth (A) and first calving (B) respectively, for cows fed CR (solid lines) or MC (dotted lines) at birth. 64

Figure 4. Herd adjusted survival plots for the analysis of time-to-conception outcome in first (A) and second (B) lactation period for cows fed CR (solid lines) or MC (dotted lines) at birth. 65

List of Abbreviations

AFC	Age at first calving
AIC	Akaike information criterion
CR	Plasma-derived commercial colostrum replacer
FPT	Failure of passive transfer
HR	Hazard ratio
JD	Johne's disease
MAP	*Mycobacterium avium* subsp *paratuberculosis*
MC	Maternal colostrum
OR	Odds ratio
PCR	Polymerase chain reaction
S/P	Sample-to-positive ratio
US	United States

Chapter 1: Introduction and a general review of the epidemiology of Johne's disease in dairy herds

The agent

The subspecies *Mycobacterium avium paratuberculosis*, which causes paratuberculosis or JD in ruminants (cattle), belongs to a group of related microorganisms in the genus *Mycobacteria.* This genus includes bacteria that are acid-fast, aerobic, non-motile, non-spore forming, and slow growing. A mycobactin requirement for growth is characteristic of MAP.[1] A strict intracellular pathogen, MAP is also known to survive in the environment for finitely long periods of time. This can be attributed to the fact that MAP is resistant to many physical conditions. For example, MAP remained viable in soil for 11 months, [2] in water for 17 months, [3] and frozen at –14° C for 12 months. [3] Urine on the other hand is bactericidal [3]but MAP can nevertheless survive in slurry for 252 days.[4]

Frequency and distribution

Johne's disease is present worldwide.[5-17] In the US, national and regional surveys have been undertaken to establish the burden of the disease in dairy and beef herds. The latest national JD study (involving environmental fecal sample testing using bacterial culture methods) conducted in 2007 by the US Department of Agriculture's (USDA) National Animal Health Monitoring System (NAHMS) revealed that ≥ 68% of dairy herds in the US are infected with MAP.[18]

State level studies have also been conducted to confirm frequency and distribution of MAP infections in individual states. For example a herd prevalence of 9.4 % was reported in California dairy herds.[19] In Missouri 40 % of beef herds were reported to be infected with MAP.[20] There is marked variation of MAP prevalence within herds. A study conducted in 33 New York State dairy herds reported a within-herd prevalence ranging from 0.7% to 28.2 % based on fecal cultural testing for MAP. [21] In Colorado, a within-herd prevalence of seropositive cows ranging from 0 % to 7.8 % was reported.[22]

While these surveys confirm that JD is endemic in US dairy and beef herds, the burden of JD at the state and national levels is grossly underestimated due to methodological reasons.

Transmission

Calves are thought to be most susceptible to MAP infection within the first 6 months of birth.[23-25] In a recent meta-analysis [25]of studies targeting age-related susceptibility to MAP infection, significant differences of susceptibility to MAP infection between calves < 6 months old and adult cattle, and in calves aged between 6 and 12 months old and adult cattle were observed suggesting an age related resistance to MAP infection.[25, 26] Therefore, JD control programs have focused mainly on preventing MAP transmission to calves.[25, 27-31] However, it must be recognized that while success in the control of JD depends on breaking MAP transmission cycles, the patterns of MAP transmission within cattle herds are still not fully understood.[31, 32]

The most important source of MAP for within-herd transmission is feces (manure) from infected adult cattle [24, 30, 31, 33] with transmission to calves mainly occurring vertically (i.e. dam-to-calf). Studies show that asymptomatic cows can excrete MAP in their colostrum and milk.[34, 35] Thus, feeding raw colostrum and milk from infected adult cattle is thought to play a significant role in transmitting MAP to calves.[34-36] Transmission of MAP from one generation of cattle to the next occurs primarily through the fecal-oral route, [25, 30, 30, 31, 31, 37] following ingestion of MAP in either water or feed contaminated with infected feces, or when calves are permitted to suckle at teats, and udders of dams soiled by infected feces present in the farm environments.[27, 28, 38, 39] The significance of the fecal-oral route in the transmission of MAP is well recognized as reflected in most JD control recommendations. [27, 29-31, 33]

Recent experimental evidence showing that infected calves and young stock (< 2 years old) can excrete MAP in feces and successfully transmit MAP to other calves horizontally (i.e. calf-to-calf) [24, 40, 41] underscores the potential significance of MAP infected calves in the maintenance of JD within-herds, even if management control

programs targeting perinatal transmission through the fecal-oral route, [30, 31, 37] colostrum or milk, [34-36] and environmental [42] sources were to be successful.

Other mechanisms of MAP transmission have been documented. For example, MAP has been detected in tissues collected from fetuses in cows clinically and subclinically infected with JD suggesting a congenital transmission route.[43-45]Whittington and Windsor[46] estimated that 9% (95% CI, 6%-14%) of fetuses from cows subclinically infected with JD, and 39%(95%CI, 20%-60%) of fetuses from clinically affected cows were infected with MAP at any one point in time through a meta-analysis of studies[43-45] targeting in utero infection of calves by MAP. Though the latter prevalence may have been underestimated due to methodological reasons, the evidence for in utero transmission[43-45] and its frequency[46] underscores the significance of this transmission route in the maintenance of JD infections within-herds, even if control programs targeting perinatal transmission of MAP through the fecal-oral and other routes [34-36, 39] were to be successful.

Evidence of dissemination of MAP to sexual organs of naturally infected bulls has also been reported in several studies. [47, 48] In one such study, [48] MAP was isolated from the semen, testes, epididymis, and seminal vesicles of breeding bulls suggesting that subclinically infected bulls shed MAP in semen which when used during insemination, could potentially infect the uterine environment. However the importance of this potential mode of transmission in maintenance of MAP infection within herds remains unknown.[30]

Between herds, transmission of infection is thought to be mostly facilitated by addition of replacement heifers or bulls from herds that are infected with MAP.[22, 31, 38, 49] However, screening to exclude infected replacements as recommended[31] is not effective due to imperfect sensitivity of the available screening tests for individual infected animals and herds.[50-53]

Risk factors for presence of MAP in dairy cattle

Most JD risk factor studies have been undertaken using cross sectional survey methods.[21, 22, 36, 38, 49, 54-60] Such studies suffer from a number of limitations. First, producers' response to survey questions may be biased by their previous knowledge of

presence of disease in their herds and management control measures that are in place, or should be adopted. Second, within a given geographic region, management practices may be uniform such that use of comparable control herds with regards to husbandry practices is impractical. Third, surveys do not take into account the long incubation period of JD[32] and the dynamic nature of management practices on most dairy farms.[29] Consequently the management practices recorded during cross sectional surveys may not reflect the risk at time of infection.

These studies have nevertheless been useful in identifying risk factors associated with JD, thus guiding areas of emphasis when implementing JD control programs. But, given the fact that such studies cannot demonstrate cause-and-effect relationships between potential risk factors and prevalence of JD in dairy herds, [21, 55, 57] it remains unknown whether implementation of control programs based on decisions derived from them are effective. Clinical trials designed to substantiate herd management interventions that can change the status of herds from infected to non-infected have been lacking.

Herd factors

Number of cows in the herd (herd-size) and presence of cows manifesting clinical signs of JD are the most consistent factors associated with JD positive herd status.[21, 22, 59] Larger herds are more likely to be infected by MAP compared to smaller herds.[22, 38, 54, 59, 61] Conditions that favor the perpetuation of MAP infection seem to exist in large herds. For example, large herds are more likely to purchase replacement heifers that may be subclinically infected by MAP from external sources compared to smaller herds. Open herds have been positively associated with presence of JD. [22, 38, 49]

Herds with a few cases of advanced clinical disease may have a large percentage of subclinically infected animals. Subclinically infected animals excrete significant amounts of MAP in feces thereby contaminating the environment of farms which may in turn act as a source of infection for uninfected animals in the herd.[62] Exposure of calves to manure of adult cattle and other calves has been positively associated with increased risk of MAP infection.[24, 40] Group housing of preweaned calves, group housing of post weaned calves, group housing for periparturient cows, frequency of cleaning cow barns,

using same housing cleaning equipment for both young and adult stock, and spreading manure on crop fields from which forage fed to cattle is later harvested may expose susceptible animals to MAP infected fecal material.[21, 38, 49, 54] Management at calving has been examined and prompt separation of calves from their dams has been associated with reduced risk of MAP infection in herds.[56] However, it should be recognized that while it is assumed that MAP infected calves are not infectious until ≥ 2 years old and that segregating youngstock from adult cattle is sufficient in breaking the MAP transmission cycles in JD infected herds, [27, 29-31] recent evidence of fecal excretion of MAP by calves and young stock (< 2 years old) and successful horizontal (i.e. calf-to-calf) transmission of MAP [24, 40, 41] underscores the potential significance of MAP infected calves in the maintenance of JD within-herds, even if the latter interventions targeting cow-to-calf transmission were to be successful.

Animal factors

Cows in their second parity or higher (i.e. older cows) are more likely to test positive for JD during their first month of lactation than those in their first parity (i.e. younger cows).[60, 61, 63] This is probably a reflection of the long incubation period of MAP infection. Breed predisposition to JD has been examined and herds that are predominantly Jersey in composition are more likely to be infected with MAP than those herds in which other breeds are predominant. [56, 60] A recent English study [61] found a significantly higher prevalence of MAP infection in dairy breeds of cattle in comparison with suckler beef breeds. No plausible explanation exists in support of these observations. However increased level of exposure (due to high within herd MAP prevalence) rather than increased genetic or breed susceptibility might be reason for the apparently higher prevalence of infection in the latter breeds. In a recent study the majority of Jersey cows originated from herds with high prevalence of JD suggesting that effect of herd may have confounded the apparent effect of breed. [60]

Cows born to seropositive dams are more likely to test positive for MAP antibodies compared with herd mates born to seronegative dams,[61, 64] a reflection of possible congenital[43-45] or perinatal transmission from dam-to- daughter through the fecal-oral

route, or ingestion of colostrum or milk contaminated with MAP. [34-36] Cows fed colostrum from multiple sources were more likely to test positive for MAP than those fed colostrum collected from their own dams at birth.[36] Additionally, allowing calves to suckle alien (foster) cows increased the risk of MAP infection in calves two-fold compared with feeding milk replacer.[36]

Environmental factors

Mycobacterium avium susbp. *paratuberculosis* is ubiquitous in the environment surrounding many infected dairy farms.[39] This can be attributed to the fact that MAP is excreted in feces of subclinical and clinically infected cows, and the fact that MAP is resistant to a variety of physical conditions. For example, MAP was found to survive in shaded environments for up to 24 weeks.[65] Additionally MAP remained viable in soil for 11 months, [2] in water for 17 months,[3] and after being frozen at –14° C for 12 months. [3] Urine was bactericidal [3]although MAP still survived in slurry for up to 252 days.[4]

While there is no clear information on the importance of environmental contamination in transmission and maintenance of MAP infection within-herds, several studies have demonstrated variation of within herd prevalence of JD across regions [38, 54, 60]suggesting effects of local environmental factors. The relationship between certain soil characteristics and occurrence of JD in cattle and sheep has been reported. Recent studies have shown a positive association between prevalence of JD infected dairy herds and soil acidity. [58, 66, 67] In Australia, sheep raised on soil with a high organic-and-clay content were more likely to be affected by ovine JD compared with sheep grazed on high sand-and-nitrogen content soils.[68] These observations suggest that the environment may constitute an important critical control point for MAP. Knowledge of factors responsible for MAP survival is critical for understanding role of the environment in transmission of JD and how this can be manipulated for control purposes.

Diagnosis

Major developments in MAP diagnostic technology have occurred in the past decade. Commercially available diagnostic tests for MAP in the US include tests based

on bacterial culture for direct identification of MAP from clinical specimens (mostly feces), tests based on detection of DNA probes specific to MAP through amplification using PCR, and tests for detection of antibodies generated against MAP present in serum or milk.[51, 69] The performance of some of these tests under various specimen sampling strategies has been examined under US husbandry conditions [52, 70-75] and recently, recommendations on how to use these tests in practice was described.[69] These recommendations were premised on a general understanding among experts to the effect that the current tests for diagnosis of MAP should be used for well defined and specific purposes (e.g. testing for screening or eradication purposes),given their imperfect nature.[51, 53, 69]

However, it must also be recognized that while developments in MAP diagnostics capabilities over the years have been impressive, a major setback has been the inability to perform perfect test validation studies on culture, [70, 76, 77] antibody, [52, 53, 78] and DNA probe based tests[75, 76, 79-82] respectively, due to the lack of a cheap, quick, and reliable Gold standard (i.e. ideal reference test), and the variable incubation periods of MAP infections in cattle.[53]In a recent review, [53] details on the ideal MAP diagnostic test evaluation methodology and the errors to be avoided during MAP test validation studies were discussed.

Tests based on culture of the organism

The primary limitation of the fecal culture based test for MAP is that the sensitivity for detection of MAP using this method is low in spite of the high costs associated with its routine use[51] (approx $25/ sample at the Minnesota Veterinary Diagnostic Laboratory, St. Paul, MN). Additionally, compared with MAP antibody based tests or test based on genetic probes, the solid media fecal culture tests have a very slow turnaround time ranging between 12 and 16 weeks, although the turnaround time is quicker for liquid culture based systems.[51]

Several studies [70, 76, 77] have reported sensitivity of fecal culture tests that varied between 23% and 74% depending on the stage of JD infection (i.e. subclinical or clinical disease status). However, specificity of the culture based test is generally high,

approximating 100 % in situations where PCR amplification techniques are used to confirm the genetic identity of the isolate. [51, 53, 83] Given these characteristics, the bacterial culture technique is best suited for detection of MAP fecal shedders that should be removed from the herd to avoid transmission of infection within herds. It is therefore indicated when confirmation of infection or herd JD eradication (within herd prevalence < 5 %) are the goals for testing.[69, 83]

It should be noted that although the so-called "pass-through" phenomenon [84] (i.e. animals naturally ingesting and shedding MAP in their feces while apparently escaping pertinent infection by MAP !) is thought to compromise the specificity of bacterial culture tests (resulting in false-positive results) [53], available evidence[84] in support of the nature of the "pass-through" phenomenon remains wanting. Influence (if any at all) of the "pass-through" phenomenon with respect to fecal culture test outcome interpretations is therefore unknown. Further investigations are needed to unambiguously demonstrate the reality with respect to presence of "pass-through" cows in JD infected herds.

Tests based on antibody detection

The enzyme linked immunosorbent assay (ELISA) is the most commonly used and frequently evaluated technique. [53, 69] Advantages of the antibody assays include their rapid turnaround time and the relative low costs of testing serum or milk clinical specimens.[51, 69] However, a major problem affecting antibody assays as diagnostic tools is that antibody responses to MAP appears late in the course of infection.[51] Thus, the antibody tests are not adequately sensitive for early detection of the disease in cattle.

Dargatz and others [52] reported a serological antibody test sensitivity (with fecal culture used as reference test) that ranged from 15.4 % to 88.1 % depending on clinical stage and MAP fecal shedding level of the affected animal. The average sensitivity of this test was estimated to be 50 % (95 % CI: 45.9 % - 54.1 %). [52] A recent study of a milk ELISA yielded a sensitivity of 61%. [53, 78]

Although reported specificity for both serum and milk ELISAs can be described as good (range, 88% to 100%), [52, 53, 78, 85, 86] the specificity of these tests may be compromised by non-specific reactions with antigens that present similar epitopes as

MAP(e.g. *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, or *Mycobacterium terrae*) resulting in false positive results for MAP.[87] Nonetheless serum and milk ELISAs are attractive for MAP herd screening and surveillance programs [69, 78] although producers may occasionally use these test outcomes for undertaking culling decisions in herds endemically infected with MAP. Under these circumstances (given the imperfect specificity of the ELISA tests), it is recommended that a producer confirm MAP infection in an ELISA positive cow by performing fecal culture or PCR testing prior to implementing a culling decision.[69] In the event that confirmatory diagnosis using the latter tests cannot be undertaken, producers may perform a second or repeat ELISA and implement their culling decision based on the magnitude of the S/P[a], such that cows with high-to-very high S/P are culled immediately. Presence of clinical signs additional to a positive ELISA test outcome may be sufficient evidence for implementing a culling decision.

Tests based on amplification of unique genetic probes

The genetic elements *IS900* [88, 89] and *ISMAP02* [90, 91] are considered specific to MAP and are detectable in clinical specimens by PCR amplification.[51, 76, 79, 81, 92] Several studies have been conducted to evaluate sensitivity and specificity of MAP specific DNA probes for detecting MAP present in fecal samples of subclinically infected cattle. [75, 76, 79-82] Estimated sensitivities were 62 %, [79] 33.5%, [76] 23%, [75] and 70%, [82] respectively. In a recent study, sensitivity was shown to improve with number of MAP organisms being excreted in feces. [75]

Estimated specificity for these tests were 97 %, [79] 100%, [76] 99.7%, [75] and 88.3%,[82] respectively. Although the main advantage of tests based on amplification of unique MAP genetic elements is the fact that results are obtained within 3 days (vs a minimum of 12 weeks for fecal culture tests), [75, 76, 79]false positive results are common due to laboratory generated sample contamination. [51, 80] Further evaluations of these tests for diagnosing MAP infections using milk and colostrum clinical specimens are also warranted.

[a] HerdChek®, IDEXX Laboratories, Inc, One IDEXX Drive, Westbrook, ME

Economic effects of Johne's disease

Several studies [93-96] have been undertaken to establish direct costs of JD to the dairy industry at both herd and national levels. However, a number of limitations plague studies on the economic impact of JD. First, cause-and-effect relationships cannot be inferred from these studies [93-96] because of their cross sectional observational study design. Second, losses due to JD vary with production and management systems, burden of disease (i.e. prevalence), and methods of loss estimation (i.e. not standardized). Third, the long incubation period of the disease makes it difficult to segregate losses associated with MAP etiology given the fact that the shortened productive life of dairy cows may be due to reasons unrelated to either subclinical or clinical JD. Consequently, subclinically infected animals may be misclassified, and losses either underestimated or not attributed to MAP infection.

Notwithstanding the above limitations, consistent economic losses attributable to JD at the herd level have been in the form of decreased milk production, reduced fertility, poor feed utilization, decreased herd longevity, increased culling and mortality rates. [33, 93-95, 97-99]

In Michigan, a 10 % increase in herd prevalence of JD was associated with a 33 kg (73 lbs) decrease in mean weight of culled cows translating into a loss of $ 1150 annually in the herds studied. Similarly, mortality attributed to JD was three times higher in infected herds compared to negative herds. The latter was translated into an average loss of $ 3000 associated with increased costs of replacements and lost slaughter value.[95]

A 1996 study [96] conducted by the US. Department of Agriculture's (USDA) National Animal Health Monitoring System (NAHMS) estimated that JD infected herds lost $ 100/cow in inventory when compared to uninfected herds. These losses were attributed to increased replacement costs and reduced milk production. In heavily infected herds with clinically affected animals, losses were ≥ $ 200/cow attributed to a marked reduction in milk production (≥ 1543 lb or 700 kg/cow), greater incidence of culling, lower cull cow values, and increased mortality rates. [96] Across the US, average annual cost of JD was estimated at $ 24.50/cow in lost productivity translating into ≥ $ 225 million in losses to the dairy industry annually. Important to note is the fact that these

losses may have been underestimated since their computation was based on a national apparent JD prevalence ≥ 21%, [38] compared with an apparent prevalence of ≥68% in 2007.[18]

Cows infected with JD are more likely to be susceptible to other diseases including mastitis than their uninfected herd mates.[33] In one Minnesota study, [98]cows manifesting signs of JD were three times more likely to be diagnosed with pneumonia compared with their otherwise healthy or subclinically infected herd mates. Further costs associated with JD include the cost of implementing control practices (e.g. testing costs). For example, a cost benefit analysis [100] for the implementation of a JD control program at the herd-level estimated a mean net present value (NPV) (defined here as the difference between the cost of an investment and the discounted present value of all future earnings from that investment) of –$14/cow if the cost of testing were to be borne by the producers.

Factors hindering herd control of Johne's disease

While advances in knowledge of possible determinants of JD,[21, 22, 38, 49, 54-57, 59, 60, 66, 67, 101-105] and concerted efforts towards herd prevention and control have been made over the years,[106, 107] JD remains endemic in the US causing major economic losses to the dairy industry.[18, 31, 95, 96]

Several factors hinder efforts to control and ultimately eliminate JD from dairy herds. The long incubation period of the disease means that subclinically infected cows can excrete large quantities of MAP in their feces resulting in heavy environmental contamination, and subsequent exposure of large numbers of susceptible animals to infection. [32, 33, 37] The situation is further compounded by the fact that the current diagnostic tests have imperfect sensitivity for early detection of infected cattle in affected herds.[50-53, 76, 78, 83, 108-110] *Mycobacterium avium* subsp. *paratuberculosis* has been isolated from a variety of wildlife species including rabbits, deer, and even earthworms(*Oligochaeta, Lumbricidae*).[111-117] Evidence for the presence of MAP in wildlife species underscores the possible significance of inter-species transmission[113, 114, 116, 118] of MAP in the maintenance of JD infections within-herds, even if JD management control programs targeting perinatal transmission of MAP through the fecal-oral,

colostrum/milk,[34-36] and environmental contamination[39] routes were successful. Additionally, MAP is known to be resistant to a number of physical conditions, [2, 3, 65, 66, 101, 119-121] surviving in the environment for finitely long periods. Though environmental management including manure waste management aimed at controlling the spread of MAP within and between dairy farms is commonly recommended, [27, 29-31, 33, 57] the significance of MAP present in the environment in the transmission of the disease remains unknown.

Herd management measures being applied on farms for JD control purposes[25, 30, 31, 106, 107] have been based on extrapolation of knowledge gained from either cross-sectional surveys,[21, 22, 38, 49, 54-57, 59, 60, 66, 67, 101-105] or simulation modeling studies.[122-124] Cross-sectional studies are best used to construct hypotheses and not to demonstrate cause-and-effect relationships. As such, there are no guarantees that disease control decisions based on cross-sectional study findings can succeed when implemented in the field. While simulation modeling studies [122-124] emphasize the need to implement all necessary herd management strategies to achieve a lowering of the within herd JD prevalence to levels that are economical attractive over a 20 year period, no long-term prospective controlled field based clinical trials have been undertaken to validate the efficacy of the most frequently recommended herd level management control measures. Therefore, there is no evidence to substantiate the notion that specific herd management measures for the control of JD are efficacious, practical, and cost-effective. It is also unknown whether suggested control measures optimize productivity and profitability at the herd level.

Finally, due to the complex nature of MAP transmission patterns and the uniqueness of dairy herds, no single approach to control can be applied to all farms. This means aspects to control efforts must be designed to meet specific needs of producers given their unique circumstances.[29] The process of eradicating JD from farms is long-term in nature and can take many years depending on the JD burden, control measures adopted and compliance with the implementation of recommended interventions.[32, 122-124]

Herd level control strategies

Herd level options for JD control may include treatment, vaccination, testing-and-culling or removal of infected animals, and husbandry or herd management interventions.[30, 31]

While the lack of an approved drug (there is no known treatment that can cure an infected animal) specific for the disease in cattle, [125] makes the treatment option for JD control impossible, results from recent studies[126-129] suggest a promising future with the use of monensin. Treatment with monensin sodium was shown to decrease pathologic lesions associated with MAP infections in calves and adult cows, respectively.[126, 127] Similarly, cows fed monensin sodium were found to excrete significantly less MAP in feces than placebo treated cows, and were less likely to test positive for JD antibodies on a milk-ELISA test. [130, 131] These findings suggest that treatment with monensin might reduce fecal excretion of MAP by infected cows consequently limiting the environmental load of MAP with the effect of decreasing risk of MAP infections due to environmental exposure.

Simulation models have indicated that the test-and-cull strategy does not eliminate JD from the average herd (size $\geq$ 100 cows) and should only be applied as an impetus for improved calf hygiene [122-124, 132] or when eradication of the disease (often in herds with very low prevalence of disease i.e. $\leq$ 5%) is the goal.[69]

While availability of vaccines for JD is limited and their use generally restricted in the US, [31] preliminary experimental and field studies[133-135] have shown that vaccination against MAP lessens severity of the disease and slows progression to the clinical state in affected animals. However, vaccination against MAP as a control strategy while economically attractive does not seem to reduce prevalence of JD under current US production and management systems, according to simulation modeling studies. [123]

Based on findings from simulation modeling studies,[122-124] the most effective JD control measures are considered to be those based on herd management practices (i.e. improved calf-hygiene and contract heifer rearing) aimed at breaking the transmission cycle of MAP in dairy herds.[31, 32] The focus on the calf (heifer) is premised upon the understanding that calves are most susceptible to infection during the first 6 months of

birth.[31] In a recent meta-analysis [25]of studies targeting age-related susceptibility to MAP infection, significant differences of susceptibility to MAP infection between calves < 6 months old and adult cattle, and in calves aged between 6 and 12 months old and adult cattle were observed suggesting an age related resistance to MAP infection.[25, 26] Therefore, the goal is to limit exposure of calves to putative sources of MAP infection and to prevent transmission of MAP from dam-to- calf.[27, 30, 31] However, while there is no evidence to substantiate the efficacy, practicality, and straight forwardness of implementating specific management interventions, an Australian study[32] and two Minnesota reports[106, 107] provided evidence suggesting that participation in a JD control program (with emphasis on management interventions) reduces the incidence of clinical disease, fecal excretion, and seroprevalence of MAP over time.

In several reviews of herd control of JD, the most recommended strategies for breaking MAP transmission cycles were discussed[27, 29-31, 33] including calving in clean maternity pens, prompt separation of calves from their dams after birth, avoidance of natural nursing, separate housing for calves and adult cattle, maintaining closed herds, avoiding spreading manure to crop fields, disinfection of sick cow pens, and removal of animals with clinical JD. Recent evidence showing that infected calves and young stock (< 2 years old) can excrete MAP in feces and successfully transmit MAP to other calves horizontally [24, 40, 41] suggests the need for individual housing of calves in infected herds. However, the efficacies of the preceding interventions have not been fully evaluated in controlled clinical trials.

Detection of MAP in colostrum and milk collected from MAP infected cows[34, 35, 136] has led to the suggestion that feeding raw bovine colostrum or milk may be one of the earliest means by which susceptible calves become exposed to MAP. Nielsen and others indicated in a recent report that the practice of feeding calves colostrum collected from multiple donors was significantly associated with affected calves testing positive for MAP in the future compared with herd mates fed colostrum collected from their own dams.[36] Additionally, herds in which calves were fed colostrum collected from known MAP infected cows were 87 times as likely to be infected with MAP as herds in which such colostrum feeding practices are not the norm. [105] As such, feeding programs that

encourage use of colostrum from uninfected cows or alternatively, feeding commercial colostrum replacer products, and pasteurized colostrum [29, 31, 33, 137-141] while discouraging the feeding of pooled colostrum have been recommended to producers.[27] Similar recommendations have been made with respect to milk feeding programs including feeding commercially available milk replacers or pasteurized milk instead of raw milk (or unpasteurized raw waste milk).[33] However, the transmission of MAP through raw colostrum and/or milk feeding programs is not fully understood. Additionally, the efficacies of the preceding colostrum management interventions have not been fully evaluated using controlled clinical trials. It is the lack of such evidence that inspired the work presented in this thesis.

Objectives

The goal of this thesis is to describe the role of colostrum in the epidemiology of JD and evaluate the evidence for calf excretion of MAP following natural exposure. To achieve this, the following general objectives were defined.

Objective 1: To evaluate the role of raw colostrum feeding programs in the transmission of MAP in calves.

Objective 2: To describe the efficacy of plasma derived colostrum replacement products as a possible management tool for the prevention of transmission of MAP in calves.

Objective 3: To compare the effects of the two colostrum feeding programs (i.e. colostrum replacement product vs raw colostrum) on future performance and survival of calves in the herd.

Objective 4: Describe the extent of colostrum and teat contamination by MAP attributable to excretion of MAP by infected cows and its implication for potential exposure of calves to MAP infection under natural farm settings.

Objective 5: Describe the patterns of fecal excretion of MAP by calves and its implication for horizontal (calf-to-calf) transmission of the disease under natural farm settings.

Descriptive outline

Chapter 2: Is a description of a clinical trial conducted to determine the efficacy of feeding a commercially available plasma colostrum replacement product (vs raw colostrum) for preventing transmission of MAP in calves.

Chapter 3: Is a description of the effect of a commercial plasma-derived colostrum replacement product (vs raw colostrum) on performance (i.e. milk production and reproductive performance) and long-term survival of calves in the herd (i.e. longevity).

Chapter 4: Describes an experimental validation of a nested PCR for detecting MAP DNA in colostrum.

Chapter 5: Is a description of the risk of MAP infection in calves fed colostrum positive for MAP DNA compared with herd mates fed MAP DNA negative colostrum.

Chapter 6: Describes the relationship between fecal excretion of MAP by cows around calving time and the risk of detecting MAP DNA in colostrum and teat swabs collected from the cows.

Chapter 7: Is a description of fecal excretion of MAP by young calves born in a heavily infected herd and therefore naturally exposed to MAP and potential for calf-to-calf transmission of MAP.

Chapter 8: Is a description of the conclusion of findings of chapters 2 to 7 and how these findings relate to the role of colostrum in the transmission dynamics of MAP within herds.

Chapter 2: Efficacy of feeding plasma-derived commercial colostrum replacer for the prevention of transmission of *Mycobacterium avium* subsp. *paratuberculosis* in Holstein calves

P. Pithua et al. /JAVMA 234(2009) 1167-1176

Abstract

Objective: To estimate the relative risk of Johne's disease (JD) infection in calves fed plasma derived colostrum replacement (CR) product vs raw bovine maternal colostrum (MC).

Study Design: Randomized controlled clinical trial.

Animals: 497 heifer calves born in 12 JD endemic commercial Holstein dairy farms located in Minnesota and Wisconsin.

Procedures: Every calf was separated from its dam within 30 to 60 minutes after birth and systematically assigned to be fed raw MC (control group, n = 261 calves) or CR (treatment group, 236 calves). The calves were monitored to adulthood and tested for *Mycobacterium avium* subspp *paratuberculosis* (MAP) infection using an ELISA test for serum antibodies against MAP and the bacterial culture test for MAP in feces at approximately 30, 42, and 54 months of age. Weibull regression models were used to evaluate the effect of feeding CR (vs raw bovine MC) on the risk of developing JD infection.

Results: Calves fed CR at birth were less likely (hazard ratio = 0.559) to become infected with MAP (as determined by use of an ELISA, bacterial culture, or both tests), compared with the likelihood for calves fed MC at birth.

Conclusions and Clinical Relevance: This study revealed that feeding CR reduced the risk of MAP infection in Holstein calves born in JD endemic herds, which implied that feeding raw bovine MC may be a source of MAP for calves. Plasma colostrum replacement products may be an effective management tool for use in dairy herds attempting to reduce the prevalence of JD.

Introduction

Paratuberculosis (ie, JD) is caused by infection with MAP. Halting transmission of MAP within dairy farms by use of cost-effective and efficacious tools is an important factor to consider when developing JD control programs. Cattle are considered most susceptible to infection during the first month after birth. [23, 27, 28, 38] Although manure from infected adult cattle is considered the most important source of MAP, [38]with transmission occurring mainly via the fecal-oral route, MAP organisms can also be excreted in milk and colostrum of JD infected cows.[34, 35] Thus, raw bovine MC contaminated with MAP may contribute towards transmission of MAP within dairy farms.[35]

Feeding adequate- or good-quality MC is essential for preventing failure of passive transfer (defined as a calf serum IgG concentration < 10 g/L within 24 hours after birth), a condition that increases the risk of morbidity and death in young stock. Colostrum replacers, derived from either bovine plasma (serum) extracts or lacteal derivatives, are marketed as a convenient alternative to MC in situations in which quantity or quality (or both) of MC is compromised. Colostrum replacers for calves are intended to provide ≥ 100 g of IgG/dose as well as other nutrients, including vitamins, minerals, fats, lactose and protein. [137] It has been suggested that CR products may be useful management tools to assist in the control of infectious disease agents (eg, *E. coli*, *Salmonella* spp, *Mycoplasma* spp, bovine leukemia virus, and MAP) that may be transmissible from adult cows to calves through ingestion of raw MC. Therefore, programs that encourage use of CR products for calves have been recommended to producers.[28, 38] However, to our knowledge, there is no evidence to suggest that implementation of a program for feeding CR products to calves on dairy farms will yield a beneficial effect of reducing the transmission of MAP and therefore reducing the risk of JD in calves, compared with results after feeding raw MC.

The objective of the study reported here was to investigate the effects of feeding CR vs raw bovine MC on the risk of transmission of MAP, and development of subclinical MAP infection in Holstein dairy calves.

Materials and Methods

Animals

Holstein calves on 12 commercial dairy farms (herds 1 through 12, respectively) located in western Minnesota (n = 9 farms) and eastern Wisconsin (3 farms) were used in the study. Herd participation was entirely voluntary. Criteria for herd inclusion were producer willingness to comply with study protocols, participation in the Minnesota (or Wisconsin) Dairy Herd Improvement Association (DHIA) milk production records system, and evidence that JD was endemic in the herd. Endemic status was initially assigned on the basis of a history of culling cattle because of JD during the year preceding the start of the study, although confirmatory testing to determine herd infection status was subsequently performed. Before the start of the study, producers completed a questionnaire describing their routine colostrum management programs and general herd information. The study was approved (protocol No. 0704A06362) by the Institutional Animal Care and Use Committee of the University of Minnesota.

Study design

A prospective clinical trial study was conducted to evaluate the risk of MAP infection in heifer calves fed either raw MC (control group, n = 261 calves) or CR (treatment group, 236 calves) product at birth. Heifer calves born between July and October 2003 were enrolled in the study. Samples and data collection were completed in December 2007.

Each enrolled heifer calf was separated from its dam within 30 to 60 minutes after birth and was not allowed the opportunity to suckle its dam. By use of a systematic allocation procedure (ie, alternating such that a calf was assigned to one group and the subsequent calf was assigned to the other group), calves were assigned to be fed raw MC (control group) or a single dose of CR[a] (treatment group). The MC fed to a calf originated from its dam, although there were instances in which an insufficient quantity of MC was available from a dam, which necessitated that refrigerated colostrum from other dams

[a] Secure®, American Protein Corp, Ames, IA

was fed instead. The CR contained 125 g of IgG/dose and was mixed with 2 L of warm water, as per manufacturer's instructions, prior to feeding.

Calves in 11 of the 12 participating herds were fed 3.8 L of MC at the first feeding, whereas calves in the other herd were fed 1.9 L of MC during the first feeding. Calves in 5 herds were routinely provided a single meal of MC followed by a commercial milk replacer thereafter. In the other 7 herds, calves were routinely provided a second meal consisting of 1.9 L of MC within 8 to 12 hours after the first colostral feeding. In those herds, calves assigned to the treatment group were fed a second meal consisting of 1.9 L of a commercial milk replacer supplemented with a single dose of a commercially available colostrum supplement product [b](CS) within 8 to 12 hours after the first CR meal was fed. In contrast to CR, CS products are intended to provide < 100 g of IgG/dose when fed to calves, and are not formulated to replace MC. The CS product used in this study contained 45 g of IgG/dose, and both CR and CS products originated from the same manufacturer. Preweaning diets (up to 56 days of age) for all calves consisted of commercial milk replacer and unrestricted amounts of water and calf starter pellets.

Preweaning samples and data collection

For each enrolled calf, calf managers recorded the identification of the dam, identification of the calf, birth date, number of calves born (singletons or twins), type of colostrum fed (CR or MC), calving score, and interval from birth until feeding of colostrum. Paired 20 mL samples of MC fed to each calf in the MC group was collected into sterile sample tubes that were subsequently labeled with the respective identification of the calf and farm identification prior to freezing at –20^{o}C for subsequent analysis to determine contamination with MAP. The intent was to establish whether calves were exposed to MAP via MC.

The short-term objective of this study was to evaluate the effect of feeding CR vs MC on serum IgG and total protein concentrations in young (1-to- 8 day old) calves and morbidity and mortality risk for calves between birth and weaning. Findings for the short-term objective of this study have been reported elsewhere.[142]

[b] Lifeline®, American Protein Corp, Ames, IA

Post weaning samples and data collection

Calves were weaned at approximately 56 days of age and monitored until adulthood. Calves were tested for MAP infection at approximately 30, 42, and 54 months of age. Data on calves were not officially collected by the investigators between weaning and the first testing event at 30 months of age. However, records of deaths, sale of animals, or culling events during this period were retrieved from commercially available dairy management software[c] installed in participating farms. Alternately, some of the death and culling events were accessed from records maintained manually by the producers. Despite this effort, some heifers were lost from the study during this period. These heifers were censored at the last known date present within the herd (weaning date). There were no losses to follow-up in the period between the first testing events (30 months of age) until conclusion of the study (54 months of age). Prior to the start of testing of the adult study cows for MAP infection, producers completed a questionnaire describing details of their routine JD management programs.

During each sampling event at approximately 30, 42, and 54 months of age, 10 mL of blood was collected from the coccygeal vein of each cow by use of a 20-gauge, 1-inch needle into a 16 X 100-mm blood collection tube. Approximately 10 g of feces was collected manually from the rectum of each cow by use of a disposable plastic examination sleeve and stored in sterile plastic sample containers prior to shipment to the Minnesota Veterinary Diagnostic Laboratory located at the University of Minnesota, College of Veterinary Medicine for processing and testing.

Serologic testing for MAP antibodies

Serum derived from blood samples was tested for antibodies against MAP by use of a commercially available serologic ELISA test kit.[d] Seropositive results for MAP were indicated by use of an optical density value. For each sample, values for MAP antibodies were reported as an S/P. Samples with an S/P < 0.25 were classified as having negative results, whereas those with an S/P ≥ 0.25 were classified as having positive results for

[c] Dairy Comp 305®, Valley Agricultural Software, Tulare, CA
[d] HerdChek®, IDEXX Laboratories, Inc, One IDEXX Drive, Westbrook, ME

MAP antibodies. In a validation study, [52] sensitivity of this test varied from 15.5% to 88.1%, depending on the clinical stage and amount of MAP fecal shedding of each infected cow.

Bacterial culture for MAP

The 72-hours sedimentation culture method for fecal samples was used to culture MAP from fecal samples. The detailed protocol for this method has been described elsewhere.[38] Briefly, fecal samples (2 g) were mixed with 40 mL of sterile distilled water for 30 minutes and then placed on a horizontal surface and allowed to settle for 1 hour. An aliquot (5 mL) was aspirated from the top of the suspension and transferred into a 50-mL tube that contained 35 mL of 0.9% hexadecyl pyridinium chloride. The tube was thoroughly mixed (manually shaken), and the suspension was allowed to settle for 72 hours. Sediment (2 mL) was then collected from the bottom of the tube and mixed with 0.1 mL of 50 mg of amphotericin B rehydrated in 10 mL of sterile distilled water. Four tubes of Herrold's egg medium containing Mycobactin J and control tubes of Herrold's egg medium without Mycobactin J were each inoculated with 0.2 mL of the suspension. Caps were loosely applied to the tubes, and tubes were then incubated in a horizontal position at 37^{o}C for 1 to 3 weeks. When there was a dry appearance on the surface of the media at that time, caps were then tightened and tubes were incubated for an additional period. Beginning at approximately 6 weeks after start of incubation, tubes were examined on a weekly basis to detect growth of MAP. Results were reported as negative, positive–low shedder (1 to 10 colonies/tube), positive–moderate shedder (10 to 50 colonies/tube), positive–high shedder (51 to 100 colonies/tube), or positive–very high (> 100 colonies/tube) per g of fecal sample.

Estimation of herd MAP infection prevalence

The initial evidence used to indicate that selected herds had MAP infection was based on the recollections of the producers' with regard to culling of cows infected with MAP during the year preceding the start of the study. To confirm that these herds were infected with MAP at the conclusion of the study in 2007, the investigators estimated the

true within-herd prevalence of MAP for each herd. The apparent prevalence of MAP infection in each herd was first determined by testing 30 cows (other than those in the study cohort) that were in their second or greater lactation; these cows were randomly selected from each herd during the last sampling event conducted at 54 months for the detection of antibodies against MAP. Blood samples were collected as described previously and tested by use of a commercial ELISA kit [d] with an estimated specificity of 98.6% and sensitivity of 50%. [52]For each herd, true prevalence estimates for MAP were calculated from the estimated apparent prevalence by use of the following equation: [143]

$$p\,(MAP) = (AP - [1 - Specificity])/\,(1 - [\{1 - Specificity\} + \{1 - Sensitivity\}])$$

where p (MAP) was the true MAP prevalence and AP, the apparent MAP prevalence for each herd, respectively.

The Minnesota Veterinary Diagnostic Laboratory maintained an electronic database with records of test results for all samples submitted by veterinary clinics serving many dairies in Minnesota and western Wisconsin. Test records for MAP infection (serologic ELISA [based on testing ≥ 30 cows and ≥ 2 MAP-positive outcomes/record]) were retrieved from the database for each of the 6 herds (herds 2, 3, 5, 9, 11, and 12) in which the estimate of apparent MAP prevalence (as determined on the basis of results for 30 randomly tested cows at the last sampling event) was 0%. These test records were used to estimate apparent prevalence prior to calculating true prevalence estimates for these herds.

Disease outcome definitions

The primary outcome for this study was subclinical MAP infection indicated by a positive test result on the serologic ELISA test or bacterial culture test for MAP in feces. A third outcome variable was derived by applying a parallel diagnostic test interpretation criterion to both test outcomes (ie, cows were considered infected with MAP when they had positive test results for either the serologic ELISA, bacterial culture of feces, or both diagnostic tests).

Statistical analysis

Descriptive and multivariable analyses were conducted by use of standard statistical software.[e] For all analyses, values of $P \leq 0.05$ were considered significant.

The proportion of cows with positive test results for MAP at each sampling event (approx 30, 42, and 54 months of age) and the cumulative proportion positive for MAP (all months combined) was summarized. The difference in the risk of MAP infection between treatment groups (CR vs MC) was tested by fitting logistic regression models to the data while adjusting for random effects of herd (n = 12).

For each cow in the study, months of observation was calculated from the time of enrollment (calf birth date) to the sampling date when the first positive test result for MAP was established or when the study concluded at approximately 54 months of follow-up monitoring, at which time all surviving cows were censored. Cows that died or were culled for any reason during the 54-month follow-up period were censored at the date of their death or culling. Cows lost during the follow-up monitoring period between weaning and the first testing event (approx 30 months of age) were assumed to have exited the study at weaning and were therefore censored from the analysis at date of weaning (approx 56 days after birth).

Three separate Weibull hazard regression models were fit to the data to evaluate the effect of feeding CR vs MC on the hazard of a positive MAP diagnosis (as determined on the basis of the serologic ELISA, bacterial culture of feces, or both tests) while adjusting for herd effect by inclusion of an inverse-Gaussian shared-herd frailty term.[144, 145] The Weibull distribution provides a variety of monotonically increasing or decreasing shapes of the hazard function determined by the estimated parameter, ρ. For $\rho = 1$, it implies a constant hazard function. For $\rho < 1$, it suggests a monotonically decreasing hazard function, whereas $\rho > 1$ is indicative of a monotonically increasing hazard function.[145]

In the study reported here, the Weibull distribution was chosen to model the hazard of MAP infection in CR (vs MC) treatment groups for 2 reasons. The first is that it provided a good fit to the data in comparison to other known hazard distributions (eg, exponential, Gompertz, log-normal, and log-logistic), as determined on the basis of the

[e] Stata® Corp, College Station, TX

AIC computed for each model with treatment group (CR vs MC) as the only predictor. Detailed description for computing the AIC has been reported elsewhere, [145]and per this criterion, the best-fitting model is the one that yields the smallest AIC value. The second reason was the long incubation period for MAP infections in cattle (assumed to be ≥ 2 years) meant that with the passage of time, the hazard rate of detecting infection increases. Because the diagnostic tests used in the study reported here have a sensitivity that increases with progression of JD from a subclinical to a clinical state, [51, 52]in our opinion, the Weibull hazard distribution provided the most suitable description for the natural course of MAP infection in cattle for the characteristics of the tests used to detect MAP infections in the study.

Similarly, the choice between Gamma and inverse-Gaussian distributions to model the shared herd frailty effect was based on their respective fit for the data, with a better fit indicated by a higher maximized log-likelihood of each full model (ie, models with the frailty terms included) and how well the shape of the frailty distributions fit the theoretic description of the herd effect. The Gamma frailty effect assumes that with passage of time, the effect of model variables on the population hazard decreases in favor of the frailty effect (ie, the herd effects would completely dominate in the long term). On the other hand, an inverse-Gaussian frailty effect assumes that the effect of model variables decrease with time, although they never completely disappear. [144]

The goodness-of-fit of the final models was evaluated by generating plots of cumulative hazard function against Cox-Snell residuals with a cumulative hazard approximating a 45^{o} line with a zero intercept, suggesting a good fit of the model to the data.[143, 145]

The possibility of a treatment-herd interaction was examined, but there was no evidence for such an interaction. Nonetheless, the data were stratified by herd, and estimates of the treatment effect (CR vs MC) on the hazard of MAP infection within each herd was calculated to examine whether individual herd-level responses to feeding of CR were consistent with the average population-wide estimates from the herd-adjusted models.

Power analysis

Power refers to the probability that a significant treatment effect of CR (vs MC) against MAP infection of a certain magnitude will be found when it truly exists.[143] In the study reported here, we tested the null hypothesis that calves fed MC at birth were at no greater risk of being infected with MAP than were calves fed a CR product at birth. The sample size needed to test this hypothesis was not determined a priori because the initial study[142]was designed to compare rates for failure of passive transfer and preweaning health (morbidity and deaths) among calves fed MC vs CR. Therefore, we conducted a post hoc power analysis to determine whether the sample of heifer calves enrolled provided the study reported here with sufficient power to enable rejection of the null hypothesis or to mitigate a type II error. The variables for this analysis were estimated from the data and included the observed treatment effect estimated from the model for positive outcomes to the serologic ELISA, bacterial culture or both tests, a 2-sided $\alpha \leq 0.05$, and the observed cumulative incidence of MAP infection (serologic ELISA, bacterial culture, or both) among study cohorts.

Results

Herd description, JD management, and herd infection status

Number of cattle in participating herds ranged from 190 to 1,500 cows (median, 594 cows). Fifty-two percent of dairy herds in Minnesota [146]and 54% of dairy herds in Wisconsin[146]are within this range for number of cows in the herd. Lactating cows were housed in free stalls at all participating dairies. Mean milk production (rolling herd average) for each farm ranged between 9,773 and 13,636 kg/y (21,500 and 30,000 lb/y), with a median of 11,727 kg/y (25,800 lb/y). Bulk-tank somatic cell count ranged between 180,000 and 280,000 cells/mL (median, 240,000 cells/mL).

Interval from birth to feeding of the first colostral meal ranged from 36 minutes to 138 minutes (median, 81 minutes). Colostrum was bottle fed to calves in 6 herds, whereas the remaining 6 herds fed colostrum to calves via an esophageal tube feeder. Calves were raised on-site at 6 farms, whereas the other 6 farms transported young (1-to-

3 day old) calves to be raised off-site at the facilities of 1 of 2 professional heifer growers.

Distribution of estimates for true within-herd MAP prevalence determined on the basis of testing 30 randomly selected cows at the last sampling event in 2007 by use of a serum ELISA or past ELISA test records (herds 2, 3, 5, 9, 11, and 12) obtained from the Minnesota Veterinary Diagnostic Laboratory database was summarized (Figure 1). All participating herds were infected with MAP, with the burden of infection varying among herds. The estimated true MAP prevalence for each herd ranged between 0.43% and 64% (median, 8%).

Of the 9 farms in Minnesota, 8 (herds 4, 5, 6, 7, 8, 9, 10, and 11) were participants in the Minnesota Voluntary JD Control Program. Only 1 (herd 12) of the 3 farms enrolled from Wisconsin reportedly participated in a similar voluntary JD control program. Testing strategies for MAP varied among study herds and were not mutually exclusive because some of the herds reported use of > 1 MAP testing strategy. Five herds (herds 1, 2, 4, 10, and 12) reported that they tested only cattle that had clinical signs of JD, whereas 1 herd (herd 7) reported that they tested only cattle purchased from external sources. Four herds (herds 5, 6, 7, and 8) reported that they tested cattle only at the end of lactation. Whole-herd testing for MAP was reported for 2 herds (herds 8 and 9). In 5 herds (herds 1, 4, 5, 10, and 11), the decision to cull a cow suspected of being infected with MAP was based on both a positive diagnostic test result and detection of clinical signs of JD. On the other hand, 6 herds (herds 2, 3, 6, 8, 9, and 12) based their culling decisions on only clinical signs of MAP, whereas only 1 herd (herd 7) culled MAP suspect cows solely on the basis of positive diagnostic test results for MAP.

Descriptive analyses

A total of 497 Holstein heifer calves born between July and October 2003 were enrolled in the study; 261 of these received MC, and 236 received CR. The slight imbalance in number of calves enrolled between the MC vs CR groups was attributed to the fact that early during the study, personnel on some participating dairies who were

working weekend shifts only fed MC to newborn calves. This was not likely to have introduced a bias for the study findings.

The number of cattle lost to follow-up monitoring between weaning and the first testing event (30 months of age) was more than twice as high in the CR group (12/236 [5.1%]), compared with that in the MC group (6/261 [2.3%]), although these values did not differ significantly ($\chi^2 = 3.33$; P = 0.07). Cumulative incidence for losses during the follow-up period was 20 of 497 (4.0%). By assuming that the calves lost to follow-up monitoring were censored from the study at weaning, bias attributable to a loss to follow-up monitoring, which would have disproportionately affected the CR group, may have been avoided in this analysis.

The proportion of cows in the MC and CR groups that had positive results for MAP during the first, second, and third testing events were determined (Table 1). The cumulative proportion of cows with positive results for MAP by use of the ELISA test was 25 of 497 (5.0%), with 16 of 261 (6.1%) in the MC group vs 9 of 236 (3.8%) in the CR group. Similarly, the cumulative proportion of cows with positive results for MAP on the basis of results of bacterial culture of feces was 46 of 497 (9.3%), with 29 of 261 (11.1%) in the MC group vs 17 of 236 (7.2%) in the CR group. When the positive outcomes for the serologic ELISA and bacterial culture were combined via a parallel test interpretation criterion, the cumulative proportion of cows with positive test results for MAP was 49 of 497 (9.9%), with 31 of 261 (11.9%) in the MC group vs 18 of 236 (7.6%) in the CR group.

Multivariable logistic regression analysis

Results from the logistic regression models adjusted for random herd effects and comparing the risk of MAP infection in CR vs MC groups during the first, second, and third testing events were summarized (Table 1). When a positive outcome for the serologic ELISA was considered the only test criterion for interpreting MAP infection status, cows fed CR at birth were 55% (OR = 0.488; *P* = 0.118) less likely to be infected with MAP, compared with the likelihood for cows fed MC at birth. Similarly, when a positive outcome for bacterial culture was considered the only test criterion for

interpreting MAP infection status, cows fed CR at birth were 46% (OR = 0.538; *P* = 0.067) less likely to be infected with MAP, compared with the likelihood for cows fed MC at birth. When positive outcomes for the serologic ELISA and bacterial culture were combined via a parallel test interpretation criterion, cows fed CR at birth were 48% (OR = 0.523; *P* = 0.051) less likely to be infected with MAP, compared with the likelihood for cows fed MC at birth.

Multivariable time-to-MAP positive model analysis

The AIC was computed for each hazard distribution. For the ELISA model, Weibull (AIC= 174), exponential (AIC = 199), Gompertz (179), log-normal (171), and log-logistic (174) were calculated. For the bacterial culture model, Weibull (AIC = 251), exponential (305), Gompertz (259), log-normal (246), and log-logistic (250) were calculated. For the both- tests model, Weibull (AIC = 257), exponential (318), Gompertz (266), log-normal (253), and log-logistic (257) were calculated.

In the analysis for the study reported here, the inverse-Gaussian shared-herd frailty distribution resulted in models with a better fit than that for the Gamma shared-herd frailty distribution (log likelihood for the serologic ELISA, –71.446 vs –72.249 for inverse-Gaussian and Gamma shared herd frailty, respectively; bacterial culture, –111.731 vs –111.691 for inverse-Gaussian and Gamma shared herd frailty, respectively; and combination of serologic ELISA and bacterial culture, –113.418 vs –113.461 for inverse-Gaussian and Gamma shared herd frailty, respectively) Thus, the inverse-Gaussian shared-herd frailty models were preferred. Evaluation of the shapes of plots of cumulative hazard function against Cox-Snell residuals suggested that the final models provided relatively good fits for the data (results not provided).

Predicted hazard functions for each final model comparing CR vs MC groups were plotted (Figure 2). In all cases, a hazard of close to 0 was detected up to approximately 30 months, which reflected the duration of the follow-up monitoring period when no MAP testing was implemented. The hazard rate of MAP infection gradually increased in both groups up to approximately 54 months, but the increase was more rapid in the MC group, which suggested that cows in the MC group had a greater likelihood of developing MAP

infection with the passage of time, compared with the likelihood for cows in the CR group.

Results for the Weibull regression models adjusted for the shared-herd frailty effect determined on the basis of records for 497 heifer calves in 12 herds were summarized (Table 2). The estimated shape parameters for the models were as follows: ELISA, ρ = 3.54; bacterial culture, ρ = 3.65; and both tests, ρ = 3.77. These values suggested a monotone increasing hazard of MAP infection for both the CR and MC groups. The shared-herd frailty effects were significant ($P < 0.001$) in all 3 models, which suggested the existence of true unmeasured herd effects.

The models in this study accounted for herd as a random-effect variable (frailty) and the fixed effects for treatment (CR vs MC) were interpreted as population average estimates (ie, treatment effect estimates had herd specific independent interpretations).[147] Cows in the CR group had a 53% (HR = 0.474; $P = 0.081$) reduction in the hazard of MAP infection (as determined on the basis of results for the ELISA), compared with the likelihood for cows in the MC group. For cows that had positive results for the bacterial culture test, a 43% (HR = 0.572; $P = 0.076$) reduction in hazard of MAP infection was detected for the CR group, compared with the likelihood for the MC group. When outcomes for the ELISA and bacterial culture were combined via a parallel test interpretation criterion, a 44% (HR = 0.558; $P = 0.056$) reduction in the hazard of MAP infection was detected for cows in the CR group, compared with the likelihood for cows in the MC group.

Estimates of individual herd treatment effects

Estimates of the treatment effect (CR vs MC) on MAP infection (modeled for serologic ELISA, bacterial culture, or both) stratified on the basis of herd was determined (Table 3). Cows in 4 herds (herds 7, 9, 10, and 11) did not have a failure event (ie, no positive test results for MAP in study cattle during the 54-month study period). Therefore, the effect of treatment within these 4 herds could not be estimated. There was a significant protective effect of feeding CR for herd 5 (HR = 0.24; $P < 0.007$). Although numeric estimates of treatment effect revealed that sometimes treatment was protective

(herds 3, 4, and 6) or not protective (herds 1, 2, and 8), there was no significant difference between treatment groups for these herds. These herd-stratified data should be interpreted with caution because no overall herd-by-treatment interaction was detected and because stratification of the data by herd would have significantly reduced the analytic power for each estimate of within-herd treatment effect.

Power analysis

Variables for the power analysis included the observed treatment effect estimated from the model for outcome of the ELISA, bacterial culture, or both tests (Table 2), a 2-sided $\alpha \leq 0.05$, and the observed cumulative incidence (49/497 [9.9%]; Table 1) of MAP infection (ELISA, bacterial culture, or both tests) among study cohorts. This analysis revealed that a sample of this size (n = 497 heifer calves [assuming calves were evenly allocated between groups]) provided the current study with a statistical power ≥ 80% to enable the rejection of the null hypothesis at $P \leq 0.05$ if the true reduction in hazard of MAP infection had been 51% (HR =0.487) in the population of cows fed CR vs MC at birth (compared with the 44% [HR = 0.558] risk reduction estimated from the current data).

Discussion

To our knowledge, the study reported here is the first prospective, long-term evaluation of the role of MC in the epidemiology of MAP and the efficacy of a CR product for prevention of MAP transmission in preweaned Holstein dairy calves. These results provide evidence that feeding a CR reduced the risk of MAP infection in Holstein calves.

A major strength of the study was its design (ie, a prospective clinical trial conducted in 12 herds with JD). This study provided us with the ability to identify cause-and-effect relationships between the 2 colostrum-feeding programs (CR vs MC) and risk of MAP infection, thus providing dairy producers with evidence needed to judge the performance of alternative colostrum feeding programs for the prevention of MAP transmission within commercial dairy herds. An additional strength of the study was the

fact that data analysis by use of Weibull and inverse-Gaussian shared-herd frailty models allowed for the adjustments of herd effects, thus enabling the possible generalization of study results to a wider population of herds with management systems similar to those included in this study because the estimates of the fixed effects of treatment (CR vs MC) had a population average interpretation [147](ie, HR estimates calculated on the basis of the hazard of MAP infection among cows in the CR group vs hazard of MAP infection among cows in the MC group were not herd specific and were therefore interpreted independent of the herd effects).

We were not able to test the dams for MAP infection prior to birth of each calf. The possibility of in utero MAP transmission has been reported [44, 45]; thus, some calves in the study may have been infected prior to receiving their first colostral meal. However, we are confident that the potential risk of MAP transmission to calves through in utero exposure was balanced between groups (CR vs MC) by way of systematic allocation of calves to the treatment groups at the time of enrollment. Therefore, the potential for in utero MAP transmission is unlikely to have biased the results of this study.

Herd was included in the final models as a random-effect variable, but initial adjustment for herd as a fixed-effect variable yielded treatment-effect estimates (ELISA or bacterial culture [or both tests] model, b = –0.603 [P = 0.049] and –0.582 [P = 0.056] for fixed-effects herd adjustment and random-effect herd adjustment, respectively) that did not alter the final conclusions derived from the herd random-effects models. Because the fixed-herd effects were not of interest in this study (and because there were a large number of herds [n = 12 herds], which may have caused parameter estimates to become unstable) and were considered nuisance parameters meant to adjust for within herd clustering,[148] we decided to model herd as a random-effect variable, although herds that participated in the study were a convenience (not a random sample) sample from an underlying population of dairy herds in Minnesota and Wisconsin.

Although adjusting for herd-level clustering is a necessity in a study such as this, a potential limitation of adjusting for herd effects as a random variable is that it tends to amalgamate the effect of treatment in larger herds (herds that enrolled more calves in the study), which yields average population-effect estimates with less variation and making it

difficult to discern those herds likely to benefit (or not benefit) from the intervention. For this reason, although there was no evidence for a treatment-herd interaction in this study, we analyzed the data after stratifying by herd to examine treatment responses for each herd. Heterogeneity of treatment response was detected at the herd level. For example, there was a numeric increase in risk of MAP infection in cows fed CR at birth, compared with the risk for infection in cows fed MC at birth, in 4 herds, but this apparent increase was not significant. We initially suspected that these herds may have made a conscious effort to test cows at the end of lactation and then withheld feeding of colostrum from MAP-positive dams at the time of enrollment. The consequence of this could have been a potential reduction in risk of MAP infection among calves enrolled in the MC group, thus biasing the treatment effect toward the null. However, investigations by the authors yielded no evidence to suggest that testing of adult cows for MAP was undertaken in study herds with the intended purpose of withholding the feeding of suspect colostrum from the calves of that herd. Additionally, a numeric reduction in risk of MAP infection in cows fed CR at birth, compared with the risk for cows fed MC at birth, was detected in 3 herds, but this probable protective effect was significant in only 1 herd (herd 5).

Possible explanations for the variability in treatment response among herds included random error, although it is entirely possible that a CR-feeding program provided little to no added protection to Holstein calves against MAP infection in some herds but offered absolute benefits in others; stratification of the data by herd, which may have resulted in a type-II error (ie, the relatively small sample size [mean, 43 calves enrolled/herd; range, 14 to 83 calves enrolled/herd]) within herds may have compromised the statistical power to detect a significant treatment effect and increased the variability in treatment response at the herd level); and a short observation period for time-at-risk, relative to the long incubation period of MAP infection (which is believed to range from 24 to 72 months from exposure to disease onset). The cumulative median time-at-risk for cows in these herds was approximately 41 months (39 months for the CR group vs 42 months for the MC group). Infection with MAP in cattle is believed to have a prolonged course, with clinical manifestation of the disease occurring at $\geq$ 24 months of age after exposure of calves to MAP within the first 6 months of birth. In view of this

limitation, and given that the mean ± SD censoring rate per herd was approximately 98 ± 3.0% between birth and 54 months of the follow-up period, it is possible that several MAP-infected cows were censored from these herds for reasons unrelated to the treatment prior to being tested or before having a positive result for MAP during testing. Moreover, the diagnostic tests used to detect MAP infection in this study have limited and unreliable sensitivity during the early phases of the disease in cattle.[51, 52] Consequently, the risk of MAP infection within herds may have been underestimated, which may have further exacerbated the type-II error for herd-level treatment effects. The lack of a significant treatment effect with regard to the risk of MAP infection in some herds may have also been attributable to insufficient concentrations of MAP in the MC to result in effective transmission and consequent establishment of infection in calves. The latter may have been a result of a low within-herd burden of JD for some herds (herds 1, 2, 6, 7, and 10) and therefore a reduction in overall risk of MAP infection. These factors, in addition to the limited period of exposure to MC (calves were fed MC once or twice within the first 24 hours after birth) and limited quantity of MC fed (2 or 4 L) to calves in these herds, it is more likely that calves in the MC group may not have been infected despite possible exposure to MAP-containing raw MC.

Notwithstanding the aforementioned explanations, the variability and inconsistency in the treatment response among herds should have been expected. In the opinion of the authors, the variable responses to the treatment among herds suggested that we managed to include diverse herd populations in the study. If there truly were herd-level circumstances that may have affected the efficacy of a CR product, the authors were unable to identify in this study the factor or factors that may have predicted herds with a positive beneficial effect or herds without any apparent beneficial effect. Future studies should investigate this issue. However, given the significant reduction in analytic power resulting from stratification of the data by herd, results of this stratified analysis should be interpreted with caution.

All 12 herds enrolled in the study were confirmed to be infected with MAP and were medium (100 to 499 cows) to large (≥ 500 cows) dairy herds. However, these herds were not randomly selected from the entire population of dairy herds in Minnesota and

Wisconsin. Study herds were representative of 52% and 54% of Minnesota and Wisconsin dairy herds, respectively, as determined on the basis of herd size.[146] As such, caution must be used when extrapolating results to smaller (< 100 cows) dairy herds.

The post hoc power analysis conducted for the study reported here revealed that a sample of this size (n = 497 heifer calves [assuming calves were evenly allocated between groups]) provided the current study with a statistical power ≥ 80 % to enable the rejection of the null hypothesis at $P \leq 0.05$ if the reduction in the hazard of MAP infection had been 51% (HR = 0.487) in the population of cows fed CR vs MC at birth (instead of the 44% [HR = 0.558] hazard reduction estimated from the present data). Although post hoc analysis is a less appealing method in comparison to methods that estimate sample size and the associated statistical power a priori, in the opinion of the authors, this exercise may provide information for other investigators attempting to address a similar concern.

Despite the variable responses to feeding of CR among the herds, our findings revealed that the average population estimate of the treatment effect against MAP infection in this study nonetheless provided reasonable indications of the probable benefits of CR feeding programs that may accrue in some herds. In the study reported here, the incidence of MAP infection was higher in calves fed MC at birth, compared with the incidence in calves fed CR at birth. For all herds, it was estimated that feeding CR instead of MC reduced the risk for MAP infection in calves by 44%. The most plausible explanation for the protective effect of feeding CR at birth is that the CR product did not contain infective MAP, whereas fresh bovine MC did serve as an important source of transmission for MAP in Holstein calves, as suggested in an earlier study. [35] Although the study reported here revealed that CR feeding programs may have a role in JD control programs, the overall cost-benefit of adopting this management tool has yet to be determined. Analyses are being conducted to evaluate the effect of feeding CR vs MC on survivorship in the herd, milk production, and reproductive performance, as well as overall cost-benefit of adopting CR vs MC feeding programs.

Because the findings of this study suggested that feeding raw MC from herds endemically infected with JD may represent a risk factor for MAP transmission, these

results indicate the need to investigate other management strategies for reducing transmission of MAP through maternal colostrum. First, it would seem sensible to advise producers to withhold feeding colostrum from MAP test-positive dams to heifer calves. Also, research is ongoing to evaluate whether on-farm heat treatment of MC may provide an alternative method of controlling MAP transmission through colostrum. Laboratory studies[140, 149] have determined that heat treatment of bovine colostrum at 60°C for 60 minutes is sufficient to eliminate MAP and other important pathogens in colostrum without damaging important colostral immunoglobulins. Furthermore, a field study [141] revealed that calves fed heat-treated colostrum (60°C for 60 minutes) had enhanced passive transfer of IgG. Research is being conducted to investigate the short- and long-term impact of feeding heat-treated colostrum on health, productivity, and risk for MAP infection in adult dairy cows.

The study reported here revealed that feeding CR at birth (vs feeding raw MC at birth) reduced the risk of MAP infection in Holstein calves in JD-endemic dairy herds. Results of this study also suggested that feeding of raw bovine MC may represent a risk factor for MAP transmission in Holstein calves.

Acknowledgements

Supported by the Minnesota Rapid Agricultural Response Fund, the American Protein Corporation Incorporated, and the USDA-CSREES NRI Competitive Grants Program (No. 1716-669-6304).

Table 1. Descriptive statistics and results of logistic regression models adjusted for herd effect to compare the absolute risk of developing MAP infection at various ages in 236 cows fed CR at birth and 261 cows fed raw bovine MC at birth.

Variable	CR		MC				
	No. Positive	%	No. positive	%	OR*	95% CI	*P* value†
Test outcome at 30 months of age							
ELISA	4	1.69	9	3.45	0.540	0.160-1.823	0.321
Bacterial culture	8	3.40	13	4.98	0.581	0.222-1.520	0.269
Both diagnostic tests‡	8	3.40	13	4.98	0.581	0.222-1.520	0.269
Test outcome at 42 months of age							
ELISA	3	1.27	4	1.53	0.735	0.122-4.436	0.737
Bacterial culture	6	2.54	6	2.30	1.066	0.336-3.389	0.913
Both diagnostic tests	6	2.54	7	2.68	0.941	0.310-2.858	0.915
Test outcome at 54 months of age							
ELISA	2	0.84	3	1.15	0.438	0.084-2.277	0.326
Bacterial culture	3	1.27	10	3.83	0.317	0.086-1.174	0.085
Both diagnostic tests	4	1.69	11	4.21	0.371	0.115-1.197	0.097
Test outcome at all ages							
ELISA	9	3.81	16	6.13	0.488	0.199-1.198	0.118
Bacterial culture	17	7.20	29	11.1	0.538	0.277-1.045	0.067
Both diagnostic tests	18	7.63	31	11.9	0.523	0.272-1.003	0.051

*The OR for CR vs MC was adjusted for herd as a random-effect variable. †Values were considered significant at $P \leq 0.05$.‡Cows were considered infected with MAP when they had positive test results for either the serologic ELISA, bacterial culture of feces, or both diagnostic tests.

Table 2. Results of final Weibull models adjusted for random herd (herd-frailty*) effect to evaluate the risk of developing MAP infection in 261 cows fed MC at birth and 236 cows fed CR at birth.

Test	Treatment	*b*	SE (*b*)	HR	95% CI	*P* value†
ELISA						
	CR	–0.746	0.428	0.474	0.205 –1.096	0.081
	MC	Referent	—	—	—	—
Bacterial culture						
	CR	–0.558	0.314	0.572	0.309 –1.059	0.076
	MC	Referent	—	—	—	—
Both diagnostic tests‡						
	CR	–0.582	0.305	0.559	0.307 –1.016	0.056
	MC	Referent	—	—	—	—

*Frailty variances were suggestive of true existence of within-herd clustering of data with the following values for each model: ELISA outcome, 2.47 ($P < 0.001$); bacterial culture outcome, 1.54 ($P < 0.001$); and both tests outcome ($P < 0.001$).

b= Coefficient. — = Not applicable.

See Table 1 for remainder of key.

Table 3. The number of cows enrolled per herd and number of cows with positive test results for MAP in the CR and MC groups, respectively, and herd estimates for effect of CR versus MC on the hazard of developing MAP infection on the basis of both diagnostic test outcomes.

	CR			MC					
		MAP positive events			MAP positive events				
Herd*	No. of calves enrolled	No.	%	No. of calves enrolled	No.	%	HR	95% CI	*P* value†
1	27	1	3.70	34	1	2.94	1.587	0.099-25.42	0.744
2	35	2	5.71	48	2	4.17	2.209	0.311-15.68	0.428
3	28	3	10.7	23	5	21.7	0.413	0.099-1.731	0.227
4	11	2	18.2	14	3	21.4	0.828	0.137-5.000	0.837
5	27	5	18.5	23	12	52.2	0.237	0.083-0.679	0.007
6	16	3	18.8	23	5	21.7	0.905	0.294-5.188	0.773
7	7	0	—	12	0	—	—	—	—
8	35	2	5.71	32	2	6.25	1.045	0.147-7.421	0.965
9	8	0	—	6	0	—	—	—	—
10	30	0	—	21	0	—	—	—	—
11	9	0	—	8	0	—	—	—	—
12	10	1	10	10	0	—	—	—	—

*Herds 7, 9, 10, 11, and 12 were confirmed to have cattle infected with MAP. Herds 7, 9, 10, and 11 had no cows with positive results when tested for MAP during the study; therefore, effect of treatment could not be estimated for these herds. Herd 12 had 1 of 10 cows in the MC group with a positive results when tested for MAP during the study, but 0 of 10 cows in the CR group had a positive result when tested for MAP during the study; therefore, effect of treatment could not be estimated for herd 12.

See Tables 2.1 and Table 2 for remainder of key.

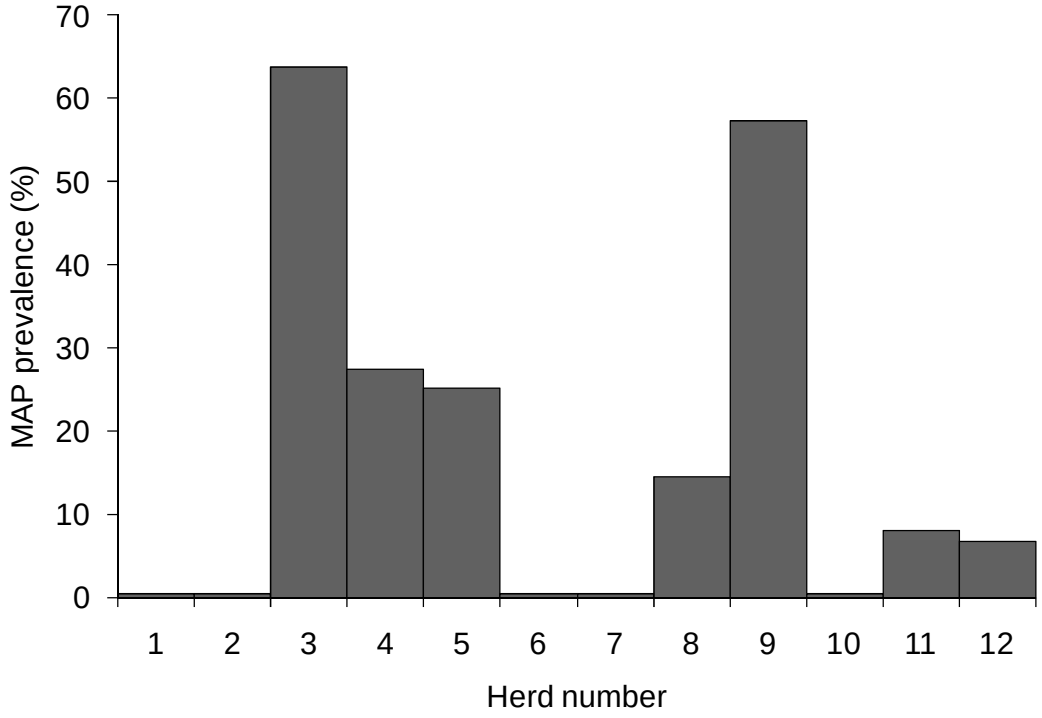

Figure 1. Distribution of estimated true MAP prevalence of 12 herds participating in a study to evaluate the effect of feeding of CR versus MC to heifer calves at birth on subsequent development of MAP infection.

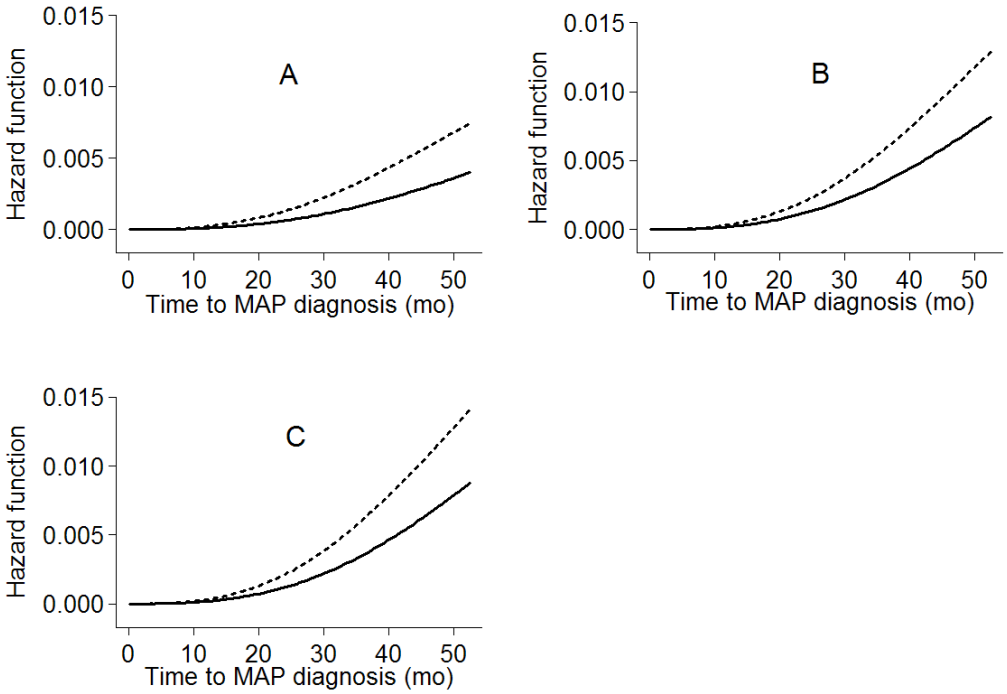

Figure 2. Weibull hazard functions for the analysis of interval until a positive test outcome for MAP, as determined by use of a serological ELISA (A), bacterial culture of fecal samples (B), or a combination of both diagnostic tests (C), in cows fed CR (solid lines) or raw bovine MC (dotted lines) at birth.

Chapter 3: Effect of a plasma-derived colostrum replacement feeding program on adult performance and longevity in Holstein cows

Abstract

The objective of this study was to evaluate longevity, milk production, and breeding performance in adult Holstein cows fed either a colostrum replacement product (CR) or raw bovine maternal colostrum (MC) within the first 1 h of birth.

A total of 497 Holstein heifer calves born in 12 commercial dairies located in Minnesota and Wisconsin were separated from their dams within 30 to 60 minutes after birth and systematically assigned to be fed either CR or MC. The calves were then observed from birth up to adulthood (approximately 54 months of age) during which time mortality and culling events plus actual milk yield and breeding performance data were collected. Time-to- death, time-to-culling, time-to-death and/or culling, time-to-a first calving event, and time-to-conception intervals were evaluated using proportional hazards survival analysis models. Number of inseminations per conception and lifetime milk yield (up to 54 months of age) was evaluated using generalized linear models.

Cows fed CR at birth were no different than cows fed MC with respect to overall risk of death, culling, death and/or culling (from birth to 54 months of follow-up and from first calving to 54 months of age, respectively), lifetime milk yield, and breeding performance.

Introduction

Failure of passive transfer, defined as calf serum IgG levels < 10 g/L in 1-to-2 d old calves, has been shown to have detrimental effects on daily weight gain while increasing the risk of morbidity and mortality in calves during the preweaning period. [150, 151] Failure of passive transfer has also been negatively associated with long-term performance including decreased milk yield and longevity in lactating adult cows. [150, 151] Feeding an adequate volume of clean, high quality maternal colostrum (MC) soon after birth is necessary to reduce the incidence of FPT in calves. [152, 153]

Colostrum replacement products, derived from either bovine plasma (serum) extracts or lacteal derivatives are formulated to provide ≥ 100 g of IgG/dose additional to a variety of nutrients including vitamins, minerals, fats, lactose, and protein. [137, 138, 154] They are marketed as a convenient alternative to MC to be fed to calves in situations in which quality- or -quantity (or both attributes) of MC are compromised for the possible prevention of FPT.

Studies reporting on the efficacy of various commercial CR products to prevent FPT have shown mixed results. In a previous study, Swan and others[142]reported observing a 93% FPT rate among calves fed one plasma-derived colostrum replacement product (CR) vs 28% in calves fed MC. Other studies, especially those feeding higher masses of IgG in CR products have reported better efficacy at preventing FPT. [155, 156] Despite the high FPT risk in CR fed calves, Swan and others [142]reported that preweaning morbidity and mortality risk was not different between CR vs MC-fed calves. Testing those same calves in adulthood for infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) revealed that feeding CR (vs MC) after birth resulted in a 44% reduction in risk of MAP infection.[154] Therefore additional to its potential role in preventing FPT in calves in the short-term, CR products may be useful management tools with the potential for assisting in preventing transmission of pathogens that are potentially transmissible to calves through ingestion of MC, including MAP.[138, 153, 154].

Ingestion of large volumes (≥ 4 L vs ≤ 2 L) of high quality MC by calves has been positively associated with increased milk yield and longevity in the adult lactating cows.[151] However, while CR feeding programs have the potential to prevent FPT and to play a role in JD control programs, respectively,[142, 154] their effect on longevity (i.e. the probability of not being removed from the herd for any reason including death, sale, or culling up to a particular age), milk yield, and breeding performance in adult lactating cows had not previously been described.

The objective of the present study was to describe the effect of CR vs MC calf feeding programs on subsequent survival in the herd (longevity), milk yield, and breeding performance in the lactating adult cow during the first 54 months of life.

Materials and Methods

Herd selection and description

Herds to be included in the present study were selected based on criteria described in detail in previous reports.[142, 154] Briefly, convenience samples of 12 commercial Holstein herds were recruited to participate in the study, 9 of these were located in south-eastern Minnesota while the remaining 3 herds were located in western Wisconsin. The size of participating herds ranged between 190 and 1,500 cows (median, 594 cows). Fifty-two percent of Minnesota and 54% of Wisconsin dairy herds fall within these categories on the basis of herd-size, respectively. [146] Mean milk production (rolling herd average) for each farm ranged between 9,773 and 13,636 kg/y (21,500 and 30,000 lb/y), with a median of 11,727 kg/y (25,800 lb/y). Bulk-tank somatic cell count ranged between 180,000 and 280,000 cells/mL (median, 240,000 cells/mL). Lactating cows were housed in free stalls in all participating herds and all herds practiced synchronized artificial insemination breeding programs.

All participating herds were infected with JD, with the burden of infection varying among herds. The estimated MAP prevalence for each herd ranged between 0.43% and 64% (median, 8%). [154] Of the 9 farms in Minnesota, 8 were participants in the Minnesota Voluntary JD Control Program. Only 1 of the 3 farms enrolled from Wisconsin reported participating in a similar voluntary JD control program.

Six of the 12 study farms routinely fed colostrum to calves through a bottle while the remaining six herds fed colostrum to calves using a tube. Calves were raised on-site in six farms while the other six farms transported calves at 1-to-3 d old to be raised off-site at the facilities of a professional heifer grower.

Study design

A prospective clinical trial study was conducted to evaluate longevity, milk production, and reproductive performance in heifer calves fed either raw MC (control group, n = 261 calves) or CR (treatment group, n = 236 calves) product at birth. Heifer calves born between July and October 2003 were enrolled in the study. Samples and data

collection were completed in December 2007 when study animals were approximately 54 months of age.

Each enrolled heifer calf was separated from its dam within 30 to 60 minutes after birth and was not allowed the opportunity to suckle its dam. By use of a systematic allocation procedure (ie, alternating such that a calf was assigned to one group and the subsequent calf was assigned to the other group), calves were assigned to be fed raw MC (control group) or a single dose of CR[a] (treatment group). The MC fed to a calf originated from its dam, although there were instances in which an insufficient quantity of MC was available from a dam, which necessitated that refrigerated colostrum from other dams was fed instead. The CR contained 125 g of IgG/dose and was mixed with 2 L of warm water, as per manufacturer's instructions, prior to feeding.

Calves in 11 of the 12 participating herds were fed 3.8 L of MC at the first feeding, whereas calves in the other herd were fed 1.9 L of MC during the first feeding. Calves in 5 herds were routinely provided a single meal of MC followed by a commercial milk replacer thereafter. In the other 7 herds, calves were routinely provided a second meal consisting of 1.9 L of MC within 8 to 12 hours after the first colostral feeding. In those herds, calves assigned to the treatment group were fed a second meal consisting of 1.9 L of a commercial milk replacer supplemented with a single dose of a commercially available colostrum supplement product [b] (CS) within 8 to 12 hours after the first CR meal was fed. In contrast to CR, CS products are intended to provide < 100 g of IgG/dose when fed to calves, and are not formulated to replace MC. The CS product used in this study contained 45 g of IgG/dose, and both CR and CS products originated from the same manufacturer.

Preweaning diets (up to 56 days of age) for all calves consisted of commercial milk replacer and unrestricted amounts of water and calf starter pellets.

[a] Secure®, American Protein Corp, Ames, IA
[b] Lifeline®, American Protein Corp, Ames, IA

Records and samples

For each enrolled calf, farm staff recorded the identification of the dam, identification of the calf, birth date, number of calves born (singletons or twins), type of colostrum fed (CR or MC), calving score, and interval from birth until feeding of colostrum. Illness or death events were also monitored and recorded.

Paired 20 mL samples of MC fed to each calf in the MC group was collected into sterile sample tubes that were subsequently labeled with the respective identification of the calf and farm identification prior to freezing at –20^{o}C for subsequent analysis to determine contamination with MAP. The intent was to establish whether calves were exposed to MAP via MC.

The short-term objective of this study was to evaluate the effect of feeding CR vs MC on serum IgG and total protein concentrations in young (1 to 8 d old) calves and morbidity and mortality risk for calves between birth and weaning. Findings for the short-term objective of this study have been reported elsewhere. [142]

Calves were weaned at approximately 56 d of age, monitored until adulthood, and tested for MAP infection at approximately 30, 42, and 54 months of age. The objective was to estimate the relative risk of MAP infection in calves fed plasma derived CR vs MC. Findings for this objective have been reported elsewhere.[154]

Data collection

Prior to weaning study calves, death events were manually recorded by the producers and associated professional heifer grower as described previously.[142] Data on calves were not officially collected by the investigator between weaning and the first MAP testing event at 30 months of age. However, records of deaths, sale of animals, or culling events during this period were accessed from records maintained manually by the producers. Despite this effort, some heifers were lost from the study during this period. These heifers were censored at the last known date present within the herd (weaning date). There were no losses to follow-up in the period between the first MAP testing events (30 months of age) until conclusion of the study (54 months of age).

For heifer calves that entered the milking herd (following a first calving event), records of death, sale, or culling events were directly retrieved from the herd management computer software [c] programs installed on each study farm.

Data on milk yield and breeding performance indices were retrieved from the Minnesota and Wisconsin DHIA on a trimonthly basis until the study was concluded at 54 months of follow-up.

Longevity indices

For the analysis of longevity, the outcomes (indices) of interest included death for any reason, evaluated from birth up to 54 months of follow-up, and from date of first calving up to 54 months of age for the subgroup of heifers that entered the milking herd, respectively; culling for any reason, evaluated from birth up to 54 months of follow-up, and from date of first calving up to 54 months of age for the subgroup of heifers that entered the milking herd, respectively; and death and/or culling events outcome, evaluated from birth up to 54 months of follow-up, and from date of first calving up to 54 months of age for the subgroup of heifers that entered the milking herd, respectively.

The time-to-death and time-to-culling event outcome variables for each heifer calf enrolled in the study was calculated from her birth date, to the date of either death or culling respectively, or when the study came to conclusion at 54 months of follow-up, at which point all heifers that remained alive or had not yet been culled, were censored from the analysis. For heifers that had a first calving event, time-to-death and time-to-culling event outcomes were calculated from date of first calving to the date of their death or culling respectively, or when the study came to conclusion at 54 months of follow-up, at which point all cows remaining in the study were censored. The combined time-to-death and/or culling event outcomes were similarly calculated for each heifer from her birth date, to the date of death and/or culling, or when the study came to conclusion at 54 months of follow-up, at which point all heifers that remained alive and/or had not yet been culled, were censored from the analysis. For heifers that had a first calving, combined time-to-death and/or culling event outcomes were calculated for each heifer

[c] Dairy Comp 305®, Valley Agricultural Software, Tulare, CA

from her first calving date, to the date of either death and/or culling, or when the study was concluded at 54 months of age at which point all heifers that remained alive and/or had not yet been culled, were censored from the analysis.

Analysis of longevity data

In this and subsequent analyses, summary statistics and multivariable analyses were completed using standard statistical software. [d] In all instances, statistical significance was considered for values of $P \leq 0.05$.

The proportion of animals experiencing death (yes/no), culling (yes/no), and combined death and/or culling (yes/no) events outcome were calculated for each group (CR vs MC). Differences in the risk of death, culling, and combined death and/or culling events outcomes recorded from birth up to 54 months of follow-up between groups (CR vs MC) were unconditionally tested using the χ^2-statitistic. Similar analysis was repeated for all heifers that had a first calving event with the starting point for analysis being the date of first calving for each heifer with follow-up ending at 54 months of age when the study was concluded.

Herd adjusted survival plots were produced to describe the distribution of time-to-death and/or culling intervals since the date of birth for each calf and for heifers that entered the milking herd, since the date of first calving.

Six separate proportional hazards models[157] were used to evaluate the effect of feeding a CR (vs MC) on outcomes including time-to-death from birth up to 54 months of follow-up, time-to-death from first calving up to 54 months of age, time-to-culling from birth up to 54 months of follow-up, time-to-culling from first calving up to 54 months of age, time-to-death and/or culling events combined from birth up to 54 months of follow-up, and time-to-death and/or culling events combined from first calving up to 54 months of age. Since cows in the same herd were more likely to be similar (vs cows in other herds) with respect to study outcomes, robust estimates of variance [158]were applied to all models to adjust for the within-herd cluster effects. The proportional hazards model assumption[157] was evaluated in all 6 models by plotting a non-zero slope of scaled

[d] Stata® Corp, College Station, TX

Schoenfeld residuals for the treatment group (CR vs MC) variable against time at risk associated with each model. A formal statistical test was performed in which non-significant ($P > 0.05$) findings indicated fulfillment of the proportional hazards assumption. The goodness-of-fit of the final models was evaluated by generating plots of cumulative hazard functions against Cox-Snell residuals with a cumulative hazard approximating a 45^{o} line with a zero intercept, suggesting a good fit of the model to the data.[143, 145, 159]

Breeding performance indices

Description of the effect of treatment (CR vs MC) on breeding performance was restricted to data recorded in the first and second lactation periods. Breeding performance indices of interest included age at first calving, likelihood of first calving events (i.e. birth-to-first calving interval), number of breedings per conception, and calving-to-conception intervals for study cows that conceived.

Age at first calving (AFC) is the interval between birth and freshening defined for all heifers that had a first calving event. In this study, AFC was calculated for all animals from their date of birth to the recorded date at first calving. Records of heifers that never had a first calving event either due to death or removal from the herd (or any other reason) were included in the analysis as censored observations for the evaluation of likelihood (or risk) of a first calving event outcome.

Number of breedings per conception was considered for only cows confirmed pregnant during the first and second lactation periods, respectively. Cows known to have been bred but failed to conceive within a given lactation were excluded from this analysis.

Calving-to-conception interval (i.e. days open) was calculated from date of the first and second calving events, to the date of the last insemination that resulted in a conception for all cows during the first and second lactation periods. For cows culled or those that died, the observation time was calculated from their calving dates to the reported date of death or removal from the herd. If such cows were not confirmed

pregnant at the time of their culling or death, then their records were censored during the analysis since their calving-to-conception intervals remained undefined.

Analysis of breeding performance data

Proportion of heifers with a first calving event, mean AFC, mean number of breedings, mean number of breedings per conception, and mean calving-to-conception interval were summarized. Differences in the distribution of the preceding variables between treatment groups (CR vs MC) were unconditionally tested using Pearson χ^2-test for categorical outcomes, Wilcoxon rank-sum (Mann-Whitney) test for continuous and count outcomes with skewed distributions, and *t*-tests for continuous outcomes with normal distributions (i.e. AFC only), respectively.

A proportional hazards model [157] was used to describe the effect of feeding CR (vs MC) on AFC (i.e. risk/hazard of a first freshening event). Adjustment for herd effect, evaluation of the proportional hazards assumption, and test for model fit were completed using the approaches described in the previous section (*See* analysis for longevity data).

Effect of treatment (CR vs MC) on first and second lactation number of breedings per conception was evaluated using two separate generalized linear models. Number of breedings per conception was assumed to follow a Poisson distribution. Effect of treatment (CR vs MC) on number of breedings per conception was described using the logit link function. Robust estimates of variance were applied to both models to adjust for the cluster effects of herd.

Herd adjusted survival plots were produced to describe the distribution of calving-to-conception intervals between groups (CR vs MC) while considering censored events for cows that failed to conceive. Effect of feeding CR (vs MC) on first and second lactation calving-to-conception intervals were evaluated using two separate proportional hazards models.[157] Adjustment for herd effect, evaluation of the proportional hazards assumption, and test for model fit were completed using methods described in the previous section (*See* analysis of longevity data).

Milk yield index

Actual milk produced per lactation (i.e. first and second lactations, respectively) and lifetime milk yield (up to 54 months of age) were the outcomes of interest. Data on lactation length (days) for each cow in the study was retrieved. For cows that died or were culled before completing a particular lactation or being dried-off, overall milk yield reported up to the last day prior to death or culling in that lactation was considered.

Analysis of milk yield data

Differences in unconditional (crude) means between CR (vs MC) groups for first and second lactation, and overall lifetime (up to 54 months of follow-up) milk yields were tested using two-sample *t*-tests (assuming equal variance for the means).

Three separate generalized linear models were used to describe the effect of feeding CR (vs MC) on first-and-second lactations, and on overall lifetime milk yield (i.e. up to 54 months of follow-up) respectively. Frequency distribution plots (Figure not shown) indicated that the milk yield outcomes (first-and-second lactations plus lifetime milk yields, respectively) followed a normal (Gaussian) distribution pattern. Treatment (CR vs MC) was assumed to affect milk yields linearly. An identity link function was therefore used to describe the relationship between feeding CR (vs MC) on milk yield. Independent variables forced into each model included treatment (CR vs MC), calving season (winter, January to March; spring, April to June; summer, July to September; and fall, October to December), AFC (0 = AFC $\leq$ 24 months and 1 = AFC > 24 months), and lactation length (days). Robust estimates of variance were applied to all models to adjust for the within herd cluster effects.[143, 160] Except for the group (CR vs MC) and lactation length variables, all other variables that did not satisfy a Wald-test $P \leq 0.05$ cut-point were dropped from the final models.

Results

Four hundred and ninety-seven Holstein heifer calves born between July and October 2003 were enrolled in the study, 261 of these received MC, and 236 received

CR. One hundred and ninety-eight of the 261 (76%) heifers enrolled in the MC group had a first calving event vs 165 of the 236 (70%) heifers in the CR group.

The imbalance in number of calves enrolled between the MC vs CR groups was attributed to the fact that early during the study, personnel on some participating dairies who were working weekend shifts only fed MC to newborn calves. It is unlikely that this error introduced a bias in the study findings.

Number of heifers lost to follow-up monitoring between weaning and the first MAP testing event (30 months of age) was twice as high in the CR group (12 of 236 or 5.1%) vs the MC group (6 of 261 or 2.3%), although these values did not differ significantly ($\chi^2 = 3.33$; $P = 0.07$). Cumulative incidence for losses during the follow-up period was 20 of 497 (4.0%). By assuming that the calves lost to follow-up monitoring were censored from the study at weaning, potential bias attributable to a loss to follow-up, which would have disproportionately affected the CR group, may have been avoided in this analysis.

Descriptive summary for longevity outcomes

The proportion of cows that experienced death, culling, and death and/or culling events from birth to 54 months of follow-up monitoring, and from date of first calving up to study conclusion at 54 months of age respectively, are presented(Table 4).

From birth up to 54 months of follow-up, 113 of 497 (23%) calves died in total, with 55 of 261 (21%) belonging in the MC group vs 58 of 236 (25%) in the CR group. Similarly, the cumulative proportion of animals culled for any reason between birth and 54 months of follow-up monitoring was 149 of 497 (30%), with 81 of 261 (31%) in the MC group vs 68 of 236 (29%) in the CR group. Death and/or culling events between birth and 54 months of follow-up monitoring were recorded in 262 of 497 (53%) animals in total, with 136 of 261 (52%) in the MC group vs 126 of 236 (54%) in the CR group. No significant differences in unconditional risks of death, culling, death and/or culling were observed between groups (CR vs MC) over this period (Table 4).

From point of first calving up to 54 months of age, the cumulative number of death events was 30 of 363 (8%), with 15 of 198 (8%) occurring in the MC group vs 15 of 165

(9%) occurring in the CR group. Similarly, the cumulative proportion of animals culled for any reason between first calving and 54 months of age was 118 of 363 (33%), with 64 of 198 (32%) in the MC group vs 54 of 165 (33%) in the CR group. Death and/or culling events between first calving and 54 months of age were recorded in 148 of 363 (41%) animals overall, with 79 of 198 (40%) in the MC group vs 69 of 165 (42%) in the CR group. Similarly, no significant differences in unconditional risks of death, culling, death and/or culling were observed between groups (CR vs MC) over this period (Table 4).

Survival plots describing the distribution of time-to-death and/or culling intervals between treatment groups (CR vs MC) from the point of birth (i.e. birth date) and first calving (i.e. for heifers that had a first calving event) respectively, are presented (Figure 3). No difference in the length of the time-to-death and/or culling intervals between groups (CR vs MC) was observed during both periods.

Multivariable models analysis for longevity outcomes

Results from the proportional hazards models for the effect of feeding CR (vs MC) on time-to-death, time-to-culling, time-to-death and/or culling events from birth up to 54 months of follow-up monitoring, and from the first calving date up to 54 months of age is presented (Table 5). In all cases, statistical testing indicated no evidence suggesting violation of the Cox proportional hazards assumption. Similarly, examination of the shapes of the plots of the cumulative hazard function vs Cox-Snell residual suggested that the final Cox models provided relatively good fits for the data (Figures not shown).

Hazard ratio (HR) comparing death events in the CR (vs MC) groups from birth up to 54 months of follow-up monitoring was 1.22 (95% CI = 0.92, 1.62; $P = 0.17$). Similarly, HR comparing culling events in the CR (vs MC) group from birth up to 54 months of follow-up monitoring was 1.01(95% CI = 0.63, 1.64; $P = 0.95$). For death and/or culling events, HR comparing CR (vs MC) group was 1.1 (95% CI= 0.77, 1.58; $P = 0.61$) from birth up to 54 months of follow-up monitoring (Table 5).

Considering only animals that had a first calving event reported, HR estimate for the risk of death in the CR (vs MC) groups was 1.22 (95% CI = 0.72, 2.04; $P = 0.46$) from the point of first calving up to 54 months of age. For the culling events outcome,

HR for the risk of culling in the CR (vs MC) group from point of first calving up to 54 months of age was 1.01 (95% CI = 0.62, 1.64; *P* = 0.98). For death and/or culling event outcome risk, HR estimate comparing CR (vs MC) groups was 1.05 (95% CI = 0.67, 1.64; *P* = 0.85) from the point of first calving up to 54 months of age (Table 5).

Descriptive summary for breeding performance outcomes

One hundred and ninety-eight of the 261 (76%) heifers enrolled in the MC group had a first calving event vs 165 of the 236 (70%) heifers in the CR group. This difference in percent of first calving events was not statistically significant (Table 6).

Unconditional (crude) means for number of breedings, number of breedings per conception, calving-to-conception intervals (first and second lactation), and AFC between CR (vs MC) groups were not significantly different (Table 6).

Survival plots describing the distribution of calving-to-conception intervals between treatment groups (CR vs MC) in the first and second lactation are presented (Figure 4). No difference in the length of the calving-to-conception interval between groups (CR vs MC) was observed in both first and second lactation periods, respectively.

Multivariable models analysis for breeding performance outcomes

Feeding CR (vs MC) had no significant effect on AFC (i.e. risk of a first calving event, *P* = 0.34), number of breedings per conception in the first (*P* = 0.83) and second (*P* = 0.32) lactations respectively, and calving-to-conception intervals (or pregnancy risk) in the first (*P* = 0.7) and second (*P* = 0.21) lactations, respectively (Table 6).

Descriptive summary for milk yield outcomes

Crude means for milk yield (kg) in first and second lactation periods respectively, and lifetime milk yield up to 54 months of follow-up were not significantly different between CR (vs MC) groups(Table 6).

Multivariable models analysis for milk yield outcomes

Generalized linear modeling indicated that feeding CR (vs MC) significantly decreased first lactation milk yield by 429 kg ($P = 0.02$). In the second lactation, feeding CR (vs MC) increased milk yield by 452 kg but this effect was not significant ($P = 0.18$). However, lifetime milk yield (i.e. up to 54 months of age in this study) of study cows were not significantly different between groups (CR vs MC) (Table 7).

Discussion

The main strength of this study was its original design (i.e. prospective clinical trial) which made it possible to potentially infer a cause-and-effect relationship between type of colostrum fed (CR or MC) at birth and future milk yield, breeding performance, and longevity in the herd, thus providing producers with the needed evidence to compare future productivity for calves enrolled into one of the two colostrum feeding programs.

As previously reported, [142] cows fed CR (vs MC) at birth had experienced a significantly higher risk of FPT during calfhood. As such, the cows fed CR in the present study were expected to perform worse than cows fed MC at birth with respect to lifetime milk yield, breeding performance and longevity outcomes respectively, as lactating adult cows. The rationale being that the high incidence of FPT events experienced by calves fed CR could possibly have compromised future performance of heifers in the CR group once they became lactating cows. The latter was premised upon evidence from earlier studies indicating that heifer calves that experienced FPT events (or fed ≤ 2 L of raw MC at birth) were more likely to produce less milk as lactating adults compared with calves that had satisfactory passive transfer of maternal IgG (or were fed ≥ 4 L of raw MC at birth), and that longevity was compromised in calves experiencing FPT events (or fed ≤ 2 L of raw MC at birth) compared with calves that attained satisfactory serum IgG mass (or were fed ≥ 4 L of raw MC at birth) through ingestion of colostrum.[151, 161]

However despite the significant increase in risk of FPT experienced by cows fed CR during calfhood, there was no significant difference between these groups (CR vs MC) with respect to overall risk of removal from the herd (i.e. longevity) and breeding performance outcomes, respectively. Cows fed CR at birth produced significantly less

actual milk in the first lactation than cows fed MC at birth. This observation was in agreement with findings from earlier studies indicating that calves that experienced a FPT event (vs calves that did not) or consumed less colostrum (≤ 2 L vs ≥ 4 L; assuming an inverse relationship between volume of intake and risk of FPT) at birth were more likely to produce less actual milk and had a decreased mature equivalent milk-and-fat during the first lactation period, respectively. [151, 161] However, the effect of feeding CR (vs MC) on second lactation and overall lifetime milk yield were not significantly different. This observation contradicted reports of an earlier study indicating that calves fed ≥ 4 L of colostrum produced significantly more milk than calves fed ≤ 2 L of colostrum(assuming an inverse relationship between volume of intake and risk of FPT) in the second lactation period. [151]

Since the incidence of subclinical MAP (JD) infection was significantly higher (i.e. 7.6% in CR vs 12% in MC groups) in heifer calves fed MC at birth, [154] an obvious question centers around whether the negative relationship observed in other studies between subclinical JD infection and overall milk yield,[97, 99, 162]culling risks, [162, 163] and breeding performance[97, 164] possibly neutralized the detrimental effects of FPT on the latter outcomes in the CR group, resulting in the failure to observe a significant difference in performance between calves fed CR (vs MC) in the present study. This hypothesis requires further investigation.

Alternatively, the failure to observe an effect of feeding CR (vs MC) in the present study might have resulted from the fact that there really is little to no influence of either the CR or MC feeding programs on longevity, breeding performance, and overall milk yield respectively, although perhaps other management factors unrelated to type of colostrum fed to calves present in study herds may have played a more significant role(s) in influencing performance outcomes, effectively overshadowing any effect of feeding CR (vs MC), if present. In a recent Swedish study, Hultgren and Svensson reported that certain heifer rearing conditions including crowded (> 7 calves) housing of 3-to-7 month old calves in slatted pens (vs litter pens) were negatively associated with length of productive life (i.e. increased risk of removal from the herd after first calving) of Swedish dairy breeds. [165] However all heifers in the present study were subjected to

similar husbandry conditions including housing throughout the study duration making influence on their future performance by such management practices a less likely factor. Moreover possible unmeasured variations in management practices between herds were adjusted for in the statistical models using robust variance estimates. [143, 158, 160, 166]

Though the mechanism(s) by which colostrum intake might influence future lactation performance is not currently understood, it seems reasonable to suggest that feeding MC (or CR) might influence longevity, milk yield, and breeding performance by ensuring adequate transfer of passive immunity in calves upon ingestion followed by a reduction in the risk of neonatal calfhood disease incidence in heifer calves and optimum daily weight gains during the early pre-and post-weaning periods. However, previous observational studies have mostly revealed conflicting findings for the relationship between passive immunity or calfhood disease incidences and subsequent longevity, milk yield, and breeding performance of dairy heifer calves upon becoming lactating adult cows. For example no significant association was observed between calfhood morbidity and first lactation milk yield or long-term survival after calving in calves born on 25 New York Holstein farms.[167, 168] Conversely, calves diagnosed with a respiratory syndrome were significantly less likely to have a first calving event (i.e. translating into a 3-to-6 month increase in AFC) and often required assistance at calving compared with herd mates that did not experience respiratory syndromes.[169-171] In two separate studies, adequate passive transfer of immunity (or ingesting $\geq$ 4 L vs $\leq$ 2 L of high quality MC by calves) was not significantly associated with AFC; although high levels of serum IgG or ingesting $\geq$ 4 L (vs $\leq$ 2 L) of high quality MC by calves were significant predictors for actual milk yield, and mature equivalent milk-and-fat during the first lactation period, respectively. [151, 161] In these same studies it was revealed that $\geq$ 21.5% of calves that experienced a high degree of FPT (FPT = serum IgG $\leq$ 12 mg/ mL within 24 h of birth in this study) died or were culled for low production [161]while calves ingesting $\leq$ 2 L (vs $\geq$ 4 L) of MC were more likely to be culled for low milk production or poor udder health. [151] However, while significant differences in risk of FPT between CR (vs MC) groups were present, the cows in this study did not experience significant differences in calfhood morbidity risks between groups (CR vs MC), [142] making it impossible to verify any

possible role preweaning calfhood morbidity events may have played with respect to influencing future performance of study cows. Contrary to expectations, the increase in risk of FPT experienced by cows fed CR[142] during calfhood had no detrimental effect on overall risk of removal from the herd (i.e. longevity), overall milk yield, and breeding performance outcomes respectively.

Therefore in the face of conflicting findings such as the preceding ones, readers are advised to be cautious while interpreting results of the present study given the lack of corroborative data, since no studies had previously attempted to address similar questions using the study design employed here. Perhaps the response could be different if lacteal-derived (vs plasma-derived) colostrum replacement products were fed in comparison to raw MC. It is also possible that the effect of feeding CR (vs MC) on survival, production, and reproductive efficiency could be different under herd management systems different from those of the herds in the present study. Further investigations are needed with regards to these questions.

Finally, while feeding adequate high quality colostrum remains a key element for neonatal calf health and future productivity, [150, 152, 153, 161] feeding raw MC from cows of unknown(or known) JD status or cows in JD endemic herds is also a significant risk factor for transmission of MAP in young calves.[35, 36, 105, 154] Therefore, feeding CR instead of MC may be one management tool to be used for decreasing the risk of MAP infection in heifer calves at birth as previously demonstrated, [154]while maintaining optimum productivity in the lactating adult cow. One advantage with feeding a CR product is that it allows the flexibility for potentially increasing the IgG mass fed (to approx $\geq$ 200 g/dose) to significantly reduce the high incidence of FPT that was observed with feeding CR in the present study cohorts. [142, 155, 156] However, an obvious limitation to increasing the mass of IgG fed in CR products would be the increased cost to the producer. The long-term benefits and economic cost-benefit of this practice requires a formal evaluation.

Conclusions

The study reported here revealed that there was no difference in the overall risk of death, culling, milk production, and reproductive performance between calves fed CR (vs MC) at birth despite the high FPT rates observed in the CR group.

Acknowledgments

Supported by the Minnesota Rapid Agricultural Response Fund, the American Protein Corporation Incorporated, and the USDA-CREES NRI Competitive Grants Program (No 1716-669-6304). The authors would like to thank the producers for allowing us to use their herds for this study.

Table 4. Unconditional comparison of number of calves fed CR vs MC at birth with respect to risk of death, and culling at various stages during the follow-up period up to 54 months of age.

Variable	CR		MC		*P* value[†]
	No. of events	%	No. of events	%	
Birth to 54 months of follow-up					
Number of calves enrolled	236	47	261	53	—
Death events					
Birth to first calving period	42	18	40	15	0.46
First lactation	7	3	6	2	0.64
Second lactation	8	3	7	3	0.65
Birth to 54 months	58	25	55	21	0.35
Culling events					
Birth to first calving period	14	6	17	7	0.79
First lactation	18	8	25	10	0.44
Second lactation	23	10	30	11	0.53
Birth to 54 months	68	29	81	31	0.59
Death and/or culling events					
Birth to first calving period	56	24	57	22	0.62
First lactation	25	11	31	12	0.65
Second lactation	31	13	37	14	0.74
Birth to 54 months	126	53	136	52	0.78
First calving to 54 months of age					
Number of heifers that calved	165	70	198	76	—
Death events					
First lactation	7	4	6	3	0.54
Second lactation	7	4	7	4	0.73
First calving to 54 months	15	9	15	8	0.60
Culling events					
First lactation	18	11	25	13	0.61
Second lactation	23	14	30	15	0.75
First calving to 54 months	54	33	64	32	0.94
Death and/or culling events					
First lactation	25	15	31	16	0.89
Second lactation	30	18	37	19	0.90
First calving to 54 months	69	42	79	40	0.71

[†]Values were considered significant for $P \leq 0.05$

Table 5. Final proportional hazards models evaluating time-to-removal from the herd for any reason between cows fed CR vs MC at birth, from birth and from date of first calving up to 54 months of age respectively.

Outcome variable	*b*	S.E.*	HR‡	95% CI	*P* value†
Birth to54 months of follow-up					
Death events	0.2	0.14	1.22	0.92-1.62	0.17
Culling events	0.01	0.25	1.01	0.63-1.64	0.95
Death and/or culling events	0.09	0.18	1.1	0.77-1.58	0.61
First calving to 54 months of age					
Death events	0.2	0.27	1.22	0.72-2.04	0.46
Culling events	0.01	0.25	1.01	0.62-1.64	0.98
Death and/or culling events	0.04	0.23	1.05	0.67-1.64	0.85

b = Coefficient. * Standard errors are adjusted for herd effect (clustering).‡ The HR for CR vs MC.† Values were considered significant for $P \leq 0.05$.

Table 6. Unconditional comparison of milk yield and breeding performance indices between cows fed CR vs MC at birth.

Variable	Group		*P* value[†]
	CR	MC	
Number of calves enrolled, n (%)	236 (47)	261(53)	—
No. of first calving events, n (%)	165 (70)	198(76)	0.14[‡*]
AFC (months), Mean ± SD (n)	24.3 ± 1.85 (165)	24.4 ± 1.99 (198)	0.47[§]
No. of breedings			
1st lactation, Mean ± SD (n)	2.84 ± 2.29 (154)	3 ± 2.86 (187)	0.73[*‡]
2nd lactation, Mean ± SD (n)	2.61 ± 2.1 (124)	2.84 ± 2.17 (154)	0.33[*‡]
No. of breedings per conception			
1st lactation, Mean ± SD (n)	2.74 ± 2.2 (141)	2.7 ± 2.2 (174)	0.62[*‡]
2nd lactation, Mean ± SD (n)	2.36 ± 1.69 (110)	2.54 ± 1.83 (128)	0.45[*‡]
Calving-to-conception interval (d)			
1st lactation, Mean ± SD (n)	139 ± 89 (141)	138 ± 103 (174)	0.68[*‡]
2nd lactation, Mean ± SD (n)	118 ± 62 (110)	121 ± 68 (128)	0.98[*‡]
Milk yield (kg)			
1st lactation, Mean ± SD (n)	11,889 ± 4,601 (147)	12,232 ± 4,109 (181)	0.48[§]
2nd lactation, Mean ± SD (n)	11,972 ± 4,698 (127)	11,451 ± 4,587 (162)	0.34[§]
Lifetime, Mean ± SD (n)	22,681 ± 10,166 (159)	22, 944 ± 9,469 (195)	0.8[§]

[†] and [–] *See* Table 4 and 3.2 for key. [‡*] *P* value based on χ^2- test. [*‡] *P* values based Wilcoxon-rank sum test. [§] *P* values based on Student *t*-tests.

Table 7. Final models evaluating breeding performance and milk yield indices of cows fed CR vs MC at birth.

Variable	*b*	S.E.*	95% CI	*P* value†
Likelihood of a first calving event	0.11	0.12	–0.12 to 0.34	0.34
Number of breedings per conception‡				
First lactation	0.02	0.09	–0.15 to 0.18	0.83
Second lactation	–0.07	0.07	–0.22 to 0.07	0.32
Calving-to-conception interval (d)				
First lactation	–0.05	0.12	–0.29 to 0.2	0.7
Second lactation	0.16	0.13	–0.09 to 0.4	0.21
Milk yield (kg)†‡				
First lactation	–429	178	–777 to –80	0.02
Second lactation	452	334	–203 to 1106	0.18
Lifetime up to 54 months of age	–360	529	–1396 to 676	0.5

b = Coefficient.* Standard errors are adjusted for herd effect (clustering).† *See* Table 4 and 3.2 for key.‡ Generalized linear model with a logit link function and a possion outcome family distribution. †‡ Generalized linear model with an identity link function and a Gaussian outcome family distribution. Calving season and AFC were dropped from these models for failure to satisfy a Wald-test $P \leq 0.05$. Lactation length remained a significant ($P < 0.001$) predictor of average milk yield in all models after adjusting for the group variable (CR vs MC).

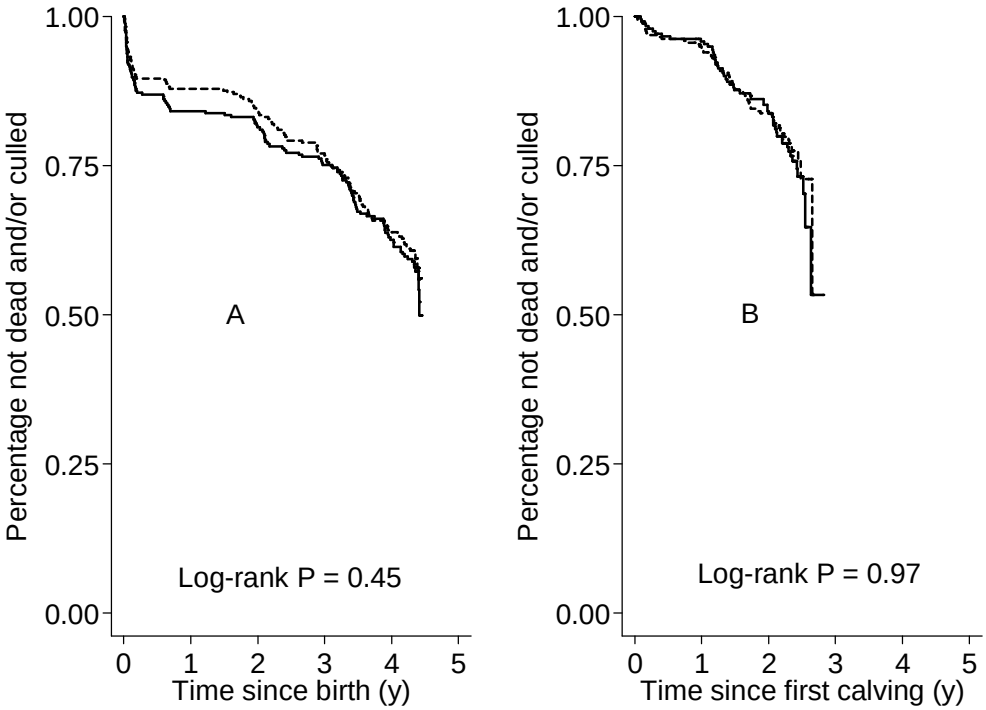

Figure 3. Herd adjusted survival plots for the analysis of time-to-death and/or culling outcome from date of birth (A) and first calving (B) respectively, for cows fed CR (solid lines) or MC (dotted lines) at birth.

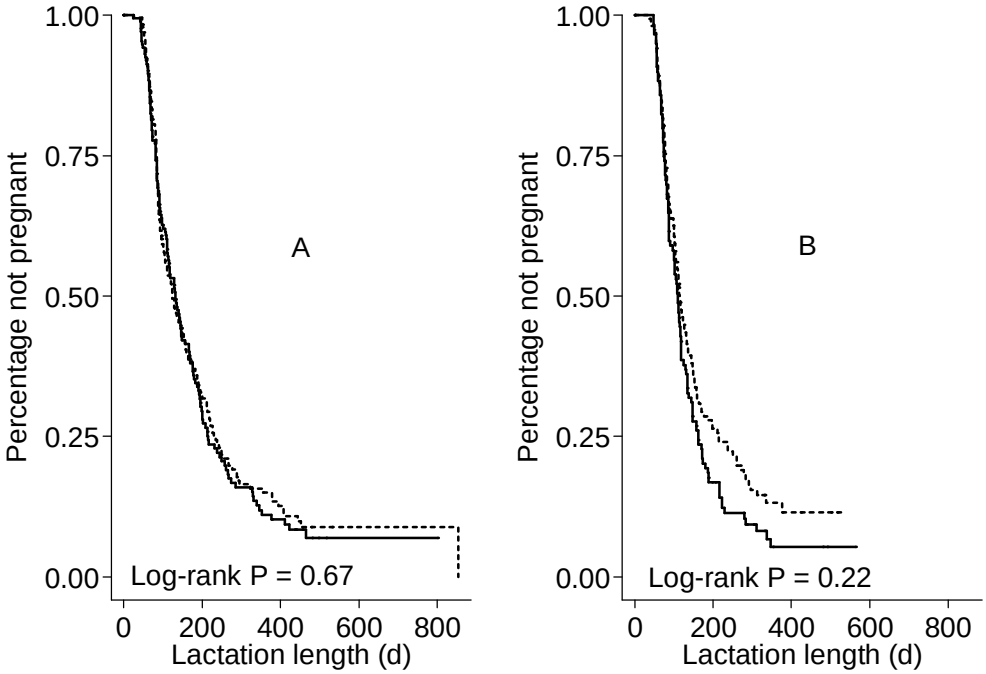

Figure 4. Herd adjusted survival plots for the analysis of time-to-conception outcome in first (A) and second (B) lactation period for cows fed CR (solid lines) or MC (dotted lines) at birth.

Chapter 4: Experimental validation of a nested PCR targeting the genetic element ISMAP02 for detection of MAP in bovine colostrum

Abstract

Colostrum samples experimentally inoculated with MAP (strain K-10) at increasing concentrations between 1×10^{1} and 1×10^{9} cells/mL were tested for recovery of MAP DNA using a modified nested ISMAP*02* target PCR initially developed for detecting MAP DNA in fecal samples.

The following detection limits were achieved for sample replicates inoculated with unsonicated MAP pure stock: 100% between 1×10^{7} and 1×10^{9} cells/mL, 75% between $1 \times10^{3}$and 1×10^{6} cells/mL, and 50% between $1\times10^{1}$and 1×10^{2} cells/mL replicates, respectively. Detection limits achieved for the colostrum sample replicates inoculated with sonicated MAP cells suspension were: 75% for 1×10^{9} cells/mL, 100% between $1 \times 10^{7}$and 1×10^{8} cells/mL, 75% for 1×10^{6} cells/mL, 0 for 1×10^{4} cells/mL, and 25% between $1 \times 10^{1}$and 1×10^{3} cells/mL, respectively. When the known negative colostrum samples were tested, 16/18 (89%) samples were correctly detected as negative for MAP DNA using the current assay.

In conclusion, the analytic sensitivity of the present assay improved with increasing concentration of MAP in the colostrum sample replicates but specificity was compromised possibly due to contamination during sample preparations. Overall the findings presented here suggest that the nested PCR protocol evaluated in this study was useful for the detection of the presence of MAP DNA in experimentally infected bovine colostrum.

Introduction

Mycobacterium avium subsp. *paratuberculosis* infection is characterized by chronic enteritis, progressive diarrhea, weight loss, and eventual death. [28, 62, 172] In cattle, the disease is called paratuberculosis or JD. Susceptibility to infection with MAP is thought to be greatest in calves with most infections occurring within the first month of birth. [23-25] While the most common source of MAP is known to be feces from infected adult cattle,

[24, 30, 31, 33] with most transmissions occurring via the fecal-oral route, [25, 30, 30, 31, 31, 37] MAP has also been detected in milk and colostrum collected from cows subclinically infected with JD[34, 35, 136] suggesting that raw colostrum and waste milk might be one of the earliest sources of MAP by which calves become infected.

While several factors are known to hinder efforts to control and ultimately eliminate JD from MAP infected herds, including the long incubation period of the disease in cattle, [28, 62] the situation is compounded by limited availability of diagnostic assays with sufficient sensitivity to detect MAP infected cattle early in the course of the disease.[50-53, 76, 78, 83, 108-110] Over the past two decades there has been considerable development of new diagnostic tests and refinement of previously existing ones to improve the ability to detect MAP infections.[69] The current commercially available diagnostic tests have been developed and occasionally field tested for direct and indirect detection of MAP in serum, milk, and fecal clinical specimens. [52, 70-75, 78] There is however no validated diagnostic test to date for the direct or indirect detection of MAP in colostrum clinical samples.

Although previous researchers [34, 35] were able to cultivate MAP in milk and colostrum collected from known JD infected cows, bacterial culture of MAP from colostrum samples is considered a tedious and frustrating process with very low success rates due to lack of a suitable sample decontamination protocol (Judith R Stabel, NADC, USDA-ARS, 2008, personal communication). Because of the preceding limitation(s), the bacterial culture of MAP from colostrum samples is not a frequent undertaking across diagnostic and research laboratories and the potential for bacterial culture in MAP diagnostics has not been developed. However, the recent mapping of the complete MAP gene sequence [90, 91] have provided an alternative involving the possible use of unique elements within the MAP genome for development of diagnostic assays. The genetic elements IS900 [88, 89] and ISMAP02 [90, 91] are considered specific to MAP and are detectable in clinical specimens by PCR amplification.[51, 76, 79, 81, 92] Recently, Stabel and Bannantine [81] developed and validated a nested PCR method targeting the multiple copy elements ISMAP02[90, 91] for detection of MAP in fecal samples.

In the present study, we report findings of a validation experiment for the performance of a modified version of the nested ISMAP02 PCR developed by Stabel and Bannantine [81] for detecting MAP in colostrum samples. The objective was to estimate the limits of detection and to determine the ability of the nested ISMAP02 PCR to correctly discriminate between known MAP infected and uninfected colostrum samples prior to a future field sample based assay sensitivity and specificity estimation study.

Materials and Methods

Sample collection

The colostrum samples were collected from a single MAP negative cow in a 45 adult-cow Minnesota dairy herd with a level 4 herd status classification of the Minnesota Voluntary JD herd status program. Prior to collection of the colostrum samples, blood and fecal samples were collected from the donor cow and tested to determine her true MAP infection status as follows: 10 mL of blood was drawn from the coccygeal vein using a 20-gauge, 1-inch needle into a 16 X100-mm blood collection tube [a] and tested for MAP antibodies using a previously described serological method.[52] Additionally, 10 g of feces were collected rectally using disposable plastic rectal examination gloves and tested by a previously described conventional bacteriological fecal culture method for MAP cultivation. [38, 108]

The colostrum sample was collected as follows: 6 h after calving, the teat skin of the donor cow was disinfected using 0.05% povidone iodine detergent, and swabbed with 70% ethyl alcohol-impregnated pledgets prior to actual collection of colostrum samples. The fore stripping colostrum was discarded, and 250 mL of colostrum was collected into a sterile glass tube from all 4 quarters and stored at – 20 °C(– 4 °F). The colostrum samples were then transported to the National Animal Disease Center, USDA-ARS in Ames IA, for further processing and analysis.

[a] BD Vacutainer® Blood Collection Tubes, Franklin Lakes, NJ

Sample preparation

The ability of the nested ISMAP02 PCR to correctly discriminate between known MAP infected and uninfected colostrum samples was evaluated using a predetermined set of experimentally infected colostrum samples while uninfected colostrum samples were used as controls.

The method for preparation of strain K-10 MAP pure cells suspensions used to experimentally inoculate the colostrum samples in this study has been previously described.[81, 173] Briefly, a pure culture of MAP was collected during the log growth phase (Abs $_{540nm}$ = 0.2-0.4). The isolates were pelleted through centrifugation at 7,500 × *g*, washed with phosphate-buffered saline (PBS) (pH = 7.4) twice prior to resuspending in PBS to a concentration of 1×10^9 cells/mL. Half of the preparation was sonicated [b] through short bursts at 35 W for between 10 and 15 sec to break up potentially clustered bacterial particles. This was then followed by a 10-fold (1×10^1 to 1×10^9 cells/mL) serial dilution of both sonicated and unsonicated stocks in PBS. One mL of colostrum samples in quadruplicate were then inoculated with the inocula preparations containing MAP in concentrations that ranged between 1×10^1 and 1×10^9 cells/mL of sample.

To ensure that the inocula contained viable MAP organisms, 1 mL of each concentration (1×10^1 to 1×10^9 cells/mL) of the inocula was plated onto Herrold's Egg York Agar Slants [c] containing Mycobactin J and antibiotics and tubes were loosely capped and incubated in a horizontal position at 37°C for between 1-3 weeks at the Minnesota Veterinary Diagnostic Laboratory. Total bacterial colony counts were read for each dilution at approximately 16 weeks. For each dilution bacterial colony counts was reported as colonies per tube (CPT)/mL of cell suspension (Table 8). At a dilution of 1×10^1 and 1×10^2 cells/mL, the MAP concentration in the cell suspension was below the limit of detection by the bacterial culture tests for cultivation of MAP. This was probably due to the fact that at these dilutions (1×10^1 and 1×10^2 cells/mL), there were only 10 and 100 viable MAP organisms in the inocula, respectively.

[b] Tekmar, Cincinnati, OH
[c] HEYM-BBL; Becton-Dickinson, Franklin Lakes, NJ

In total, 72 (4 samples per dilution level [1×10^1 to 1×10^9 cells/mL]) colostrum samples were spiked with strain K-10 cells suspension. The strain K-10 used to inoculate 36 colostrum samples were sonicated to reduce potential clumping of the bacteria while the remaining 36 colostrum samples were spiked using the unsonicated strain K-10 cells suspension. A total of 18 colostrum samples were used as controls (i.e. no MAP added).

DNA extraction protocol

For extraction of MAP DNA, 1 mL of colostrum was added to a sterile eppendorf tube. Tubes were centrifuged at 15,000 rpm for 5 min and the whey was discarded. To each cell pellet, 1 mg of proteinase K [d] was added and samples were vigorously vortexed to resuspend the pellets. Samples were incubated overnight at 50°C in a shaking water bath. To each sample, 44 µL of TEN buffers [e] (i.e.10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0; 0.1 M NaCl) and 20 µL of 0.4 M NaOH were added and samples were mixed by vortexing. Tubes were then placed in a boiling water bath for 30 min. After cooling, samples were extracted with an equal volume of phenol, chloroform, and isoamyl alcohol [f] (25:24:1) and vortexed, followed by immediate centrifugation at 10,000 rpm for 5 min. The aqueous layer was transferred to a new tube and the DNA precipitated by adding 2.2 volumes of cold 100% ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.2). Samples were mixed gently by inversion and then placed in a -20°C freezer for 1 h. The tubes were centrifuged at 15,000 rpm for 15 min followed by decantation of the supernatants. DNA pellets were air-dried for 2 to 5 min and then pellets were resuspended in 20 µL of sterile water. The DNA was either used immediately or frozen at -20°C to be analyzed later.

The PCR process

Specific primers for the ISMAP02 element were selected for use in a nested PCR format for amplification of DNA isolated from the colostrum samples. The primer

[d] Qiagen, Valencia, CA
[e] Sigma Chemical Co
[f] Amresco, Solon, OH

sequences for the initial amplification included 5'-GCACGGTTTTTCGGATAACGAG-3" (forward primer) and 5'-TCAACTGCGTCACGGTGTCCTG-3" (reverse primer). In the first round, PCR was run using a conventional thermal cycler. [g] The reaction mixture consisted of ultrapure distilled water (DNase and RNase free), GeneAmp 10X PCR buffer II, 2.5 mM $MgCl_2$, 0.25 mM deoxynucleoside triphosphates, 0.3 µM primers, and 2 U AmpliTaq Gold. Negative controls consisted of the reaction mixture alone and the nonspiked colostrum sample collected from the cow previously confirmed to be MAP negative on both the serum ELISA and bacterial fecal culture tests for MAP, respectively. The positive controls consisted of genomic DNA from MAP (strain K-10 isolates) and the colostrum samples spiked with the same MAP strain. Five µL of DNA was added for each sample and samples were run in triplicate in 96-well plates according to the following protocol: 1 cycle at 94°C for 5 min, followed by 20 cycles at 94°C for 45 s, 58°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 7 min.

The second amplification was performed using real-time PCR [h] and included a FAM-labeled probe specific for the ISMAP02 target sequence for the semi-quantitative evaluation of the test samples. Primers nested within the first set included 5'-GCACGGTTTTTCGGATAACGAG-3' (forward primer) and 5'-AACCGACGCCGCCAATACG-3" (reverse primer) for this second amplification. One µL of DNA from the first amplification was added to a reaction mixture consisting of Taqman Universal PCR Mastermix [i], ultrapure distilled water (DNase and RNase free), 0.05 µM primers, and 0.05 µM of the FAM-labeled probe, 5'-/56-FAM/CAACCCGCACGCTG/3BHQ-1/-3'. A standard was constructed by amplifying the ISMAP02 target from MAP strain K-10 genomic DNA and cloned into the TOPO expression vector [j] followed by transformation in *E. coli*. The insert was analyzed for accuracy following plasmid digestion with EcoRI and verification of size on a 4% agarose gel. Further verification of the cloned insert was conducted by sequencing the

[g] Peltier Thermal Cycler, MJ Research, Waltham, MA
[h] 7500 Real-time PCR System, Applied Biosystems, Foster City, CA
[i] TaqMan® Universal PCR Master Mix, Applied Biosystems, Foster City, CA
[j] Invitrogen, Carlsbad, CA

product using the Applied Biosystems 3100 DNA analyzer [k] after labeling the product with Big Dye v 3.1. Primers used for sequencing were M13F, 5' CGTTGTAAAACGACGGCCAGT-3' (forward) and M13R, 5'-CAGGAAACAGCTATGAC-3' (reverse). Optimal concentrations of the plasmid for use as a real-time PCR standard ranged from 1 ng to 100 attg. Real-time PCR conditions for the amplification of test samples and standards were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 25 sec and 60°C for 1 min.

Data analysis

Proportion of samples detected at each dilution (1×10^1 to 1×10^9 cells/mL) was summarized and the assay limits of detection (analytic sensitivity) were estimated. In general the proportions of known MAP infected colostrum samples, correctly detected as positive by the assay was calculated. Similarly, the proportion of control samples (no MAP) correctly detected as negative was also summarized. The 95% confidence intervals (95% CI) for the estimated proportions were calculated using standard statistical software.[l]

Results

The estimated limits of detection or analytic sensitivity for MAP (strain K-10) DNA are presented (Table 9). For the colostrum samples inoculated with unsonicated strain K-10 cell suspensions, the following detection limits were achieved using the current protocol: 100% between 1×10^7 and 1×10^9 cells/mL, 75% between 1×10^3 and 1×10^6 cells/mL, and 50% between 1×10^1 and 1×10^2 cells/mL replicates, respectively. For the colostrum samples inoculated with sonicated strain K-10 cell suspensions, the following detection limits were achieved using the present protocol: 75% for 1×10^9 cells/mL, 100% between 1×10^7 and 1×10^8 cells/mL, 75% for 1×10^6 cells/mL, 0 for 1×10^4 cells/mL, and 25% between 1×10^1 and 1×10^3 cells/mL replicates, respectively.

[k] 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA
[l] Stata® Corp., College Station, TX

The overall proportion of known infected colostrum samples (replicates) based on the unsonicated strain K-10 cell suspensions as the inocula correctly detected as positive for MAP DNA by the current assay was 28/36 or 78% (95% CI, 61 to 90%). However, when the colostrum samples inoculated with sonicated strain K-10 were evaluated, the proportion of colostrum samples (replicates) correctly detected as positive for MAP DNA was 19/36 or 53% (95% CI, 35% to 70%) (Table 10). *Mycobacterium avium* subsp. *paratuberculosis* DNA was correctly detected in 78% (28/36) of colostrum sample replicates inoculated with unnsonicated strain K-10 cell suspension compared with 53% (19/36) of the colostrum sample replicates inoculated with sonicated strain K-10 cell suspension stock. The proportion of control samples (no MAP) correctly detected as negative for MAP DNA using the current protocol was 16/18 or 89% (95% CI, 65% to 99%).

Discussion

The objective of this study was to perform a validation experiment to estimate the analytical sensitivity of a modified nested PCR protocol [81] for detecting MAP (strain K-10) in raw colostrum. Findings of the current study indicated that under laboratory conditions, this nested PCR protocol had an analytical sensitivity for detecting MAP that ranged between $1\times10^{1}$and 1×10^{9} cells/mL in spiked colostrum samples although the analytical sensitivity was compromised at lower concentrations of the inoculum i.e. only one-quarter (25%) and one-half (50%) of the colostrum samples containing between $1\times10^{1}$and 1×10^{2} cells/mL of sonicated and unsonicated strain K-10 suspensions were correctly identified as positive for MAP DNA using the present protocol (Table 9).

The proportion of control samples (no MAP) correctly identified as negative for MAP DNA using the present protocol was 89% (95% CI, 65% to 99%). This finding was contrary to expectations. Stabel and Bannantine[81]had previously evaluated the specificity of ISMAP02 element by analyzing the DNA extracted from other *Mycobacterial* species (e.g. *M. avium* subsp. *avium, M. phlei, M. smegmatis*) and found that only MAP DNA was detectable by PCR using the ISMAP02 primers(i.e. 100% specificity). Therefore the apparent false positive test outcomes observed from the known negative colostrum

samples in the present study might have resulted from laboratory contamination by MAP during the sample preparation process. It is also possible that sample contamination by MAP picked up from the farm environment [42, 117] and present on the teat skin[28] may have occurred during the time of colostrum harvest. However, this source of contamination is unlikely since the single colostrum donor cow was negative for MAP as determined by the bacterial fecal culture and serological ELISA tests for MAP, respectively. Additionally, the farm on which the sampled cow was raised was known to be free of JD with a level 4 herd status classification of the Minnesota Voluntary JD herd status program. Moreover steps were taken to prevent bacterial contamination of colostrum through proper prepping of the udders of the donor cow prior to harvest, and storage of the colostrum in a sanitized sample bottle. These explanations notwithstanding, it should however be noted that while the nested aspect of this assay was designed to improve sensitivity, improvement of sensitivity comes at a price in that the nested design detracts from specificity and increases the likelihood of contamination. It is therefore recommended in practice that a good idea for the present assay within the laboratory would be to run positive and negative controls through the entire process (including the DNA extraction process) to rule out potential contamination. In the present study, negative controls consisting of the reaction mixture alone and the nonspiked colostrum samples (no MAP) and positive controls consisting of genomic DNA from strain K-10 MAP isolates and the colostrum samples spiked with the same MAP strain (K-10) were run as control (positive and negative) to monitor the possibility of contamination.

Though not particularly significant for the present study, sonication through short bursts at 35 W for between 10 and 15 sec to break up potentially clustered MAP cells appeared to compromise analytical sensitivity of the current assay. This observation was contrary to initial expectation since sonication has been shown previously to improve the efficiency of PCR detection of Gram-positive bacteria DNA under laboratory conditions by causing disruption of the cellular walls and subsequent release of DNA.[174] A study by Schleig and others[175] demonstrated that sonication of MAP for 2 min at 100 W did not affect viability of MAP and allowed for maximum CFU counts on HEYM. Though it is unclear why in the present study, sonication compromised analytical sensitivity of the

assay, a possible reason might be that the individual free MAP cells (released following sonication of the pure stock) got trapped and were subsequently lost in the discarded whey following centrifugation. Another possible reason is that the compromised analytic sensitivity might have resulted from the undesirable partial or complete degradation of the released MAP DNA in some samples following sonication. In a study of the effect sonication for the release of DNA from *Bacillus cereus* (model organism for Gram positive bacteria), Fykse and others [174] found that prolonged sonication (for up to 5 min) of cell suspensions resulted in degradation of their DNA. The latter is however unlikely to have occurred in the present study since the sonication for the strain K-10 isolate was undertaken for only 15 s at 35 W for the purpose of breaking up potentially clustered MAP in the pure stock.

Lastly, while the advantage of the present study lay in its laboratory controlled nature, a major limitation was the fact that the MAP (strain K-10) cell suspension concentrations (1×10^1 to 1×10^9 cells/mL) used to spike the colostrum samples prior to analysis, and the frequency of detection at the different concentration levels might not necessarily mimic the levels of MAP shed naturally in colostrum. Though data on the efficacy of this test based on field colostrum samples is currently lacking, work is being undertaken to determine the diagnostic sensitivity of this test on colostrum samples obtained from Minnesota dairy herds naturally infected with JD.

Conclusions

The present findings suggest a potential role of the PCR assay for detecting MAP in colostrum. Though data on the efficacy of this test based on field colostrum samples is lacking, work is currently underway to determine the diagnostic sensitivity of this test on colostrum samples collected from herds naturally infected with JD. As such the adoption of this test for use in routine screening of field colostrum for MAP should not be under taken pending the formal field validation outcomes.

Acknowledgements

The authors would like to extend their appreciation to Margaret Walker for technical assistance.

Table 8. The colony counts associated with MAP concentration for each dilution of the cell suspension used to inoculate colostrum samples.

MAP concentration in cell suspension (cells/mL)	*Mycobacterium avium* subsp *paratuberculosis* strain K-10 cell preparation	
	Sonicated MAP cell suspension counts (CPT/mL)[‡]	Unsonicated MAP cell suspension counts (CPT/mL)[‡]
1×10^{1}[§]	Negative	Negative
1×10^{2}[§]	Negative	Negative
1×10^{3}	1-10	1-10
1×10^{4}	1-10	10-50
1×10^{5}	10-50	10-50
1×10^{6}	> 100	> 100
1×10^{7}	> 100	> 100
1×10^{8}	> 100	> 100
1×10^{9}	> 100	> 100

[§]At a dilution of 1×10^{1} and 1×10^{2} cells/mL, the MAP concentration in the cell suspension was below the limit of detection of the bacterial culture tests for cultivation of MAP. [‡]CPT/mL based on the MVDL count system.

Table 9. The limits of detection at each concentration of incocula for the detection of *Mycobacterium avium* subsp. *paratuberculosis* in colostrum using the nested PCR of the target gene ISMAP02.

	Mycobacterium avium subsp *paratuberculosis* strain K-10 stock preparation					
	Sonicated MAP cell suspension			Unsonicated MAP cell suspension		
Concentration of MAP in cell suspension (cells/mL)	No. of samples test positive/total no. tested	% positive	95% CI, % positive	No. of samples test positive/total no. tested	% positive	95% CI, % positive
1 x 10^1	¼	25	0.63-80.6	2/4	50	6.8-93.2
1 x 10^2	¼	25	0.63-80.6	2/4	50	6.8-93.2
1 x 10^3	¼	25	0.63-80.6	¾	75	19.4-99
1 x 10^4	0/4	0	0	¾	75	19.4-99
1 x 10^5	2/4	50	6.8-93.2	¾	75	19.4-99
1 x 10^6	¾	75	19.4-99	¾	75	19.4-99
1 x 10^7	4/4	100	40-100	4/4	100	40-100
1 x 10^8	4/4	100	40-100	4/4	100	40-100
1 x 10^9	¾	75	19.4-99	4/4	100	40-100
Controlsamples (no MAP)[§]	2/18	11	1.4-35	2/18	11	1.4-35

[§]The proportion of control samples (no MAP) correctly detected as negative for MAP DNA by the current assay was 16/18 or 89%. The 11% positive control samples were false positive results possibly due to laboratory contamination by MAP during sample preparation.

Table 10. The total number of known infected colostrum samples based on the sonicated and unsonicated MAP strain K-10 cell suspensions as the inocula correctly detected as positive for MAP DNA using the a nested PCR of the target element ISMAP02.

MAP cell suspension used as incocula	No. of samples test positive/total no. tested	% positive	95% CI, % positive
Sonicated	19/36	53	35-70
Unsonicated	28/36	78	61-70

[§]The proportion of control samples (no MAP) correctly detected as negative for MAP DNA by the current assay was 16/18 or 89%. The 11% positive control samples were false positive results possibly due to laboratory contamination by MAP during sample preparation.

Chapter 5: Lack of association between ingestion of *Mycobacterium avium* subsp. *paratuberculosis* DNA positive raw colostrum and the risk of infection in Holstein calves

Abstract

The objective of this study was to estimate the relative risk of MAP infection in Holstein calves associated with ingestion of MAP DNA positive (vs MAP DNA negative colostrum) within the first 1 h of birth.

Every calf born was separated from their dam within 30 to 60 minutes of birth and fed raw colostrum originating from their respective dams although in instances where the dam's colostrum was insufficient in quantity, refrigerated colostrum collected from other donors was fed instead. For each calf, samples of the colostrum fed was collected and tested for presence of MAP DNA using a nested PCR of the target gene ISMAP02. The calves were classified into exposed and unexposed categories if fed colostrum that tested positive and negative for MAP DNA, respectively. Each calf was followed to adulthood and tested for MAP infection at 30, 42, and 54 months of age using a serum ELISA and bacterial fecal culture tests, respectively. The relative risk of MAP infection in exposed (vs unexposed) calves was described using the Cox proportional hazards models.

Risk of MAP infection was not different between group (exposed vs unexposed) when the serum ELISA (Hazard ratio = 0.74, $P = 0.65$), bacterial fecal culture (Hazard ratio = 0.92, $P = 0.85$) or both diagnostic tests (i.e. after invoking a parallel test interpretation criteria) (Hazard ratio = 0.82, $P = 0.65$) were used to define MAP infection status of the study cohorts respectively, suggesting the lack of an added risk of MAP infection associated with ingesting raw MAP DNA positive colostrum by Holstein calves.

Introduction

Paratuberculosis (JD) is a chronic enteritis of cattle attributable to infection with MAP. The disease is important to the United States dairy industry because of the economic losses attributable to its high national prevalence (estimated at ≥ 68% in 2007).

[18, 96]In cattle, susceptibility to MAP infection is thought to be greatest in calves within the first 6 months following birth [23-25] and feces of infected adult cattle is thought to be the most important source of MAP [24, 30, 31, 33] with transmission occurring mainly vertically (dam-to-calf) through the fecal-oral route. [25, 30, 30, 31, 31, 37]

Though early and adequate intake of colostrum is considered the single most important factor for preventing FPT(defined here as a calf serum IgG level < 10 g /L between 24 and 48 h after birth), a condition that is thought to compromise neonatal calf health, and overall survival of calves in a herd, [152, 153]researchers have confirmed presence of MAP in colostrum collected from cows that are subclinically infected with MAP[35] suggesting that feeding raw bovine colostrum might be one of the earliest vehicles by which calves may become exposed to MAP.

Recognizing the potential role that colostrum feeding programs might play in the transmission of JD in dairy herds, current JD control programs recommend feeding colostrum from MAP negative donors, pasteurized colostrum, or alternatively feeding commercially available colostrum replacement products,[29, 31, 33, 137-141, 154] despite the cost burden to producers associated with testing cows or heifers to determine MAP status, purchase plus operational costs of an on-farm pasteurizer unit, or purchase of the colostrum replacement products.

While a number of studies have reported a positive association between feeding colostrum and risk of MAP infection at the animal and herd levels [36, 105] respectively, there is very limited evidence [154] to date to suggest that actual feeding of colostrum positive for MAP at birth results in MAP transmission, and subsequent patent JD infection in calves. Similarly, the mechanism(s) by which MAP is excreted in colostrum remains poorly understood. A strict intracellular pathogen, MAP has an affinity for infecting bovine macrophages. [176] They are also known to posses the ability to survive inside macrophages for long periods of time with no detrimental effects on the viability of their cellular hosts. [177] Considering the fact that macrophages constitute the dominant somatic cell type detected in secretions from uninfected mammary glands during the dry and periparturient periods [178, 179] respectively, it is possible that in JD infected cows,

freely circulating MAP infected macrophages end up in the mammary glands with colostrum being the final deposition site. It is therefore possible that calves would ingest macrophages that have been infected and contain MAP resulting in patent MAP infection when fed such colostrum. Alternatively, MAP found in colostrum could result from fecal contamination (e.g. MAP infective feces present on teats) during the harvest, storage, and/or feeding processes, respectively.[172]

The objective of the present study was to estimate the relative risk of MAP infection in calves that ingested MAP DNA positive colostrum compared with calves that ingested MAP DNA negative raw colostrum after birth. The null hypothesis tested in this study was that calves ingesting MAP positive raw colostrum at birth were at no greater risk of MAP infection compared with calves fed MAP negative raw colostrum.

Materials and Methods

The current study was part of a larger study, approved by the Institutional Animal Care and Use Committee of the University of Minnesota (protocol No. 0704A06362), originally designed to compare FPT rates, preweaning health, and risk of MAP infection in calves fed raw bovine colostrum vs a commercially available colostrum replacement product at birth.[142, 154] Detailed colostrum feeding protocols and study design of the original study has been previously described. [142, 154] The sample of interest for evaluating the current objective was the subgroup of heifer calves fed raw colostrum (n = 261) at birth.

Herd selection and description

Herds to be included in the present study were selected based on criteria described in detail elsewhere. [142, 154] Briefly, convenience samples of 12 commercial Holstein herds were recruited to participate in the study, 9 of these were located in south-eastern Minnesota while the remaining 3 herds were located in western Wisconsin. The size of participating herds ranged between 190 and 1,500 cows (median, 594 cows). Fifty-two percent of Minnesota and 54% of Wisconsin dairy herds fall within these categories on

the basis of herd-size, respectively [146]. Mean milk production (rolling herd average) for each farm ranged between 9,773 and 13,636 kg/y (21,500 and 30,000 lb/y), with a median of 11,727 kg/y (25,800 lb/y). Bulk-tank somatic cell count ranged between 180,000 and 280,000 cells/mL (median, 240,000 cells/mL).

All participating herds were infected with JD, with the burden of infection varying among herds. The estimated MAP prevalence for each herd ranged between 0.43% and 64% (median, 8%). [154] Of the 9 farms in Minnesota, 8 were participants in the Minnesota Voluntary JD Control Program. Only 1 of the 3 farms enrolled from Wisconsin reported participating in a similar voluntary JD control program.

Six of the 12 study farms routinely fed colostrum to calves through a bottle while the remaining six herds fed colostrum to calves using a tube. Calves were raised on-site in six farms while the other six farms transported calves at 1-to-3 d old to be raised off-site at the facilities of a professional heifer grower.

Study design

An observational prospective cohort study was conducted to estimate the relative risk of subclinical JD infection in calves fed colostrum positive for MAP DNA (i.e. exposed group) compared with calves fed colostrum negative for MAP DNA (i.e. unexposed group) at birth. Heifer calves born between July and October 2003 were enrolled in the study. Samples and data collection were completed in December 2007.

In the original study, [142, 154] the heifer calves in the colostrum subgroup (n = 261) were separated from their dams within 30 to 60 minutes after birth and were denied the opportunity to suckle their respective dams. Each calf was then fed colostrum originating from their respective dams, although there were instances in which an insufficient quantity of colostrum was available from a dam, which necessitated that refrigerated colostrum from other dams was fed instead.

The colostrum feeding programs were variable between study herds with respect to the quantity fed and frequency of feeding.

Calves in 11 of the 12 participating herds were fed 3.8 L of MC at the first feeding, whereas calves in the other herd were fed 1.9 L of MC during the first feeding. Calves in 5 herds were routinely provided a single meal of MC followed by a commercial milk replacer thereafter. In the other 7 herds, calves were routinely provided a second meal consisting of 1.9 L of MC within 8 to 12 hours after the first colostral feeding. Preweaning diets (up to 56 days of age) for all calves consisted of commercial milk replacer and unrestricted amounts of water and calf starter pellets.

Preweaning sample and data collection

For each enrolled calf, calf managers recorded the identification of the dam, identification of the calf, birth date, number of calves born (singletons or twins), calving score, and interval from birth until feeding of colostrum.

Paired 20 mL samples of the colostrum fed to each calf at the first feeding was collected into sterile sample tubes that were subsequently labeled with the respective identification of the calf and farm identification prior to freezing at –20^{o} C (– 4 ^{o}F) for subsequent analysis to determine contamination with MAP. The intent was to establish whether calves were exposed to MAP through ingestion of raw colsotrum. The colsotrum samples were shipped to the National Animal Disease Center, USDA-ARS in Ames, IA for further processing and analysis.

Post weaning samples and data collection

Calves were weaned at approximately 56 days of age and monitored until adulthood. Calves were tested for MAP infection at approximately 30, 42, and 54 months of age. Data on calves were not officially collected by the investigators between weaning and the first testing event at 30 months of age. However, records of deaths, sale of animals, or culling events during this period were retrieved from commercially available dairy management software[a] installed in participating farms. Alternately, some of the death and culling events were accessed from records maintained manually by the

[a] Dairy Comp 305®, Valley Agricultural Software, Tulare, CA

producers. Despite this effort, some heifers were lost from the study during this period. These heifers were censored at the last known date present within the herd (weaning date). There were no losses to follow-up in the period between the first testing events (30 months of age) until conclusion of the study (54 months of age). Prior to the start of testing of the adult study cows for MAP infection, producers completed a questionnaire describing details of their routine JD management programs. Details of the routine JD management programs for the present study herds have been described elsewhere. [154]

During each sampling event at approximately 30, 42, and 54 months of age, 10 mL of blood was collected from the coccygeal vein of each cow by use of a 20-gauge, 1-inch needle into a 16 X 100-mm blood collection tube. Approximately 10 g of feces was collected manually from the rectum of each cow by use of a disposable plastic examination sleeve and stored in sterile plastic sample containers prior to shipment to the Minnesota Veterinary Diagnostic Laboratory located at the University of Minnesota, College of Veterinary Medicine for processing and testing.

PCR analysis for detection of MAP DNA in colostrum

Mycobacterium avium subsp. *paratuberculosis* DNA was extracted from colostrum samples using a previously described method. [180]

The first round of PCR was run using a conventional thermal cycler. [b] Samples were run in triplicate in 96-well plates according to the following protocol: 1 cycle at 94°C for 5 min, followed by 20 cycles at 94°C for 45 s, 58°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 7 min. The primer sequences for the initial amplification were 5'-GCACGGTTTTTCGGA TAACGAG-3" (forward primer) and 5'-TCAACTGCGTCACGGTGTCCTG-3" (reverse primer).

The second amplification was performed using real-time PCR. [c] Primers nested within the first set were 5'-GCACGGTTTTTCGGATAACGAG-3' (forward primer) and 5'-AACCGACGCCGCCAATACG-3" (reverse primer) for this second amplification.

[b] Peltier Thermal Cycler, MJ Research, Waltham, MA
[c] 7500 Real-time PCR System, Applied Biosystems, Foster City, CA

Negative and positive controls consisted of the reaction mixture alone, and genomic DNA from either strain 19698 or K-10 isolates MAP, respectively.

Serum ELISA for MAP antibodies

Serum derived from blood samples was tested for antibodies against MAP by use of a commercially available serologic ELISA test kit.[d] Seropositive results for MAP were indicated by use of an optical density value. For each sample, values for MAP antibodies were reported as a sample-to-positive ratio(S/P). Samples with an S/P < 0.25 were classified as having negative results, whereas those with an S/P ≥ 0.25 were classified as having positive results for MAP antibodies. In a validation study, [52] sensitivity of this test varied from 15.5% to 88.1%, depending on the clinical stage and amount of MAP fecal shedding of each infected cow.

Bacterial culture for MAP in fecal samples

The method for the cultivation of MAP in feces used in the present study has been described in detail previously. [38, 108] Briefly, sediments derived from the fecal samples were plated onto Herrold's Egg York Agar Slants [e] containing Mycobactin J and antibiotics (0.1 ml of 50 mg amphotericin B) and incubated at 37°C for between 1 to 3 weeks. At approximately 6 weeks post inoculation, the agar slants were examined weekly for growth of MAP. Based on total colony counts per g of fecal sample tested, results were reported as negative (0 colonies/tube) or positive-low shedder (1 to 10 colonies/tube); positive-moderate shedder (10 to 50 colonies/tube); positive-high shedder (51 to 100 colonies/tube); and positive-very high shedder (> 100 colonies/tube).

Disease outcome definitions

The primary outcome for this study was subclinical MAP infection indicated by a positive test result on the serologic ELISA test or bacterial culture test for MAP in feces.

---

[d] HerdChek®, IDEXX Laboratories, Inc, One IDEXX Drive, Westbrook, ME
[e] HEYM-BBL, Becton-Dickinson, Franklin Lakes, NJ

A third outcome variable was derived by applying a parallel diagnostic test interpretation criterion to both test outcomes (i.e. cows were considered infected with MAP when they had positive test results for either the serologic ELISA, bacterial culture of feces, or both diagnostic tests).

Statistical analysis

Proportion of cows with positive test results for MAP at each sampling event (approximately 30, 42, and 54 months of age) and the cumulative proportion positive for MAP (all months combined) was calculated. Differences in the proportion of cows positive for MAP between exposed and unexposed groups were unconditionally tested using the simple χ^2- statistic.

Multivariable survival analysis

Prior to fitting the final survival models, months of observation was calculated from the time of enrollment (calf birth date) to the sampling date when the first positive test result for MAP was established or when the study concluded at approximately 54 months of follow-up monitoring, at which time all surviving cows were censored. Cows that died or were culled for any reason during the 54-month follow-up period were censored at the date of their death or culling. Cows lost during the follow-up monitoring period between weaning and the first testing event (approximately 30 months of age) were assumed to have exited the study at weaning and were therefore censored from the analysis at date of weaning (approximately 56 days after birth).

Proportional hazards models [157] were used to evaluate the association between feeding MAP DNA positive colostrum (vs MAP DNA negative colostrum) and time-to-positive MAP diagnosis (positive serum ELISA, bacterial fecal culture, and serum ELISA and/or fecal culture, respectively). Since cows in the same herd were more likely to be similar (vs cows in other herds) with respect to exposure risks and study outcomes, robust estimates of variance [158]were applied to all models to adjust for the within herd cluster effects.

The proportional hazards model assumption[157] was evaluated in all 3 models by plotting a non-zero slope of scaled Schoenfeld residuals for the exposure group variable against time at risk calculated for each model. A formal statistical test was performed in which non-significant ($P > 0.05$) findings indicated fulfillment of the proportional hazards assumption. The goodness-of-fit of the final models was evaluated by generating plots of cumulative hazard functions against Cox-Snell residuals with a cumulative hazard approximating a 45^{o} line with a zero intercept, suggesting a good fit of the model to the data.[143, 145, 159]

All analyses were undertaken using standard statistical software [f] and two-sided values of $P \leq 0.05$ were considered statistically significant.

Results

Descriptive summary

A total of 261 heifer calves were fed raw colostrum within 1 hour of birth in the original study. [142, 154] Sixty-nine of 205 (34%) calves were fed colostrum that tested positive for MAP DNA (i.e. exposed) vs136 of 205 (66%) calves that were fed colostrum that tested negative for MAP DNA (i.e. unexposed). The exposure category was undefined for 56 of 261 (21%) calves in the study because samples of colostrum fed to these calves were unavailable for testing because the calf care staff did not collect the samples at the time of enrollment. Records for these calves were therefore excluded from this analysis.

Incidence of MAP positive events detected at 30, 42, and 54 months of testing is presented (Table 11). The cumulative proportion of cows with positive results for MAP by use of the ELISA test was 14 of 205 (6.8%), with 10 of 136 (7%) in the unexposed group vs 6 of 69 (5.8%) in the exposed group. Similarly, the cumulative proportion of cows with positive results for MAP on the basis of results of bacterial culture of feces was 24 of 205 (12%), with 16 of 136 (12%) in the unexposed group vs 8 of 69 (12%) in the exposed group. When the positive outcomes for the serologic ELISA and bacterial

[f] Stata® Corp, College Station, TX

culture were combined via a parallel test interpretation criterion, the cumulative proportion of cows with positive test results for MAP was 26 of 205 (13%), with 16 of 136 (13%) in the unexposed group vs 8 of 69 (12%) in the exposed group. No significant differences in unconditional MAP incidence were observed in the exposed (vs unexposed groups; Table 11).

Multivariable survival models

The proportional hazards assumption for the survival models and the assumption of independence between outcome events and censoring were fulfilled for all models fit to the present data.

Feeding MAP DNA positive (vs MAP DNA negative) raw colostrum at birth did not significantly increase the risk of MAP infection in cows as indicated by the ELISA ($P = 0.65$), bacterial culture ($P = 0.85$), and both diagnostic test(parallel interpretation; $P = 0.65$) outcome models, respectively (Table 12).

Discussion

This is the first study undertaken to determine the direct link between raw colostrum feeding programs and transmission of MAP in calves through the actual ingestion of colostrum positive for MAP DNA (vs colostrum negative for MAP DNA) at birth, and testing the exposed calves for subsequent subclinical JD infection over time.

Calves that ingested MAP DNA positive colostrum (i.e. exposed) in the present study were expected to experience a higher risk of MAP infection than the unexposed (i.e. MAP DNA negative colostrum) calves. The latter was premised on the understanding that MAP DNA was a surrogate marker for viable *Mycobacteria* present in the samples of colostrum fed, potentially resulting in MAP infection upon ingestion by calves after birth.

There was however no significant differences in the risk of MAP infection in the group of calves fed MAP DNA positive colostrum in comparison to calves fed MAP

DNA negative colostrum at birth suggesting that there really is little to no added risk of MAP infection associated with ingesting MAP DNA positive colostrum by calves.

Strengths of this study included its longitudinal cohort design while the relatively large number of participating herds (n = 12) ensured inclusion of diverse herd populations. Participating herds were confirmed JD infected herds, providing the ideal herd (animal) populations needed for addressing the present study objective.

Despite the above study strengths, there were also a number of limitations that needed to be considered while interpreting the results presented here. First, samples of colostrum fed to the calves at the second feeding in 7 of the 12 herds that routinely provided a second colostral meal, 8 to 12 h after the first meal, were not tested for presence of MAP DNA yet the latter was potentially an additional and significant source of MAP for the calves in those herds. Secondly, the investigators were unable to confirm dam status for MAP infection (specifically MAP fecal shedding status) prior to, or even after the birth of each enrolled calf. Evidence for possible in utero MAP transmission has been previously reported [43-45] with an estimated 9% of fetuses carried by subclinically infected dams being infected by MAP at any one time through in utero exposure.[46] Johne's disease infected dams are more likely to excrete MAP in colostrum than their MAP negative herd mates as reported in an earlier study. [35] It is therefore possible that some calves in the present study might have been infected congenitally and not through ingestion of MAP DNA positive colostrum. Additionally, calves in this study born to MAP positive dams may have had a disproportionately higher risk of ingesting MAP positive colostrum than calves born to MAP negative dams. If present, the effect of the latter route of transmission and source of error would have been to bias the effect of exposure (i.e. feeding MAP DNA positive colostrum) away from the null, effectively overestimating the association between ingestion of MAP DNA positive colostrum and the risk of transmission of MAP in the calves studied. However, given the lack of knowledge of dam MAP status, it was impossible to verify the role of other potential transmission routes, including in utero MAP transmission, which could have also been associated with a MAP positive status of study cohorts.

The nested PCR assay used to detect MAP in colostrum samples in the present study was shown in a previous experimental validation study, [180] to have an analytic sensitivity that improved with increasing concentration of MAP (range, 100%, between 1×10^7 and 1×10^9 cells/mL; 75%, between 1×10^3 and 1×10^6 cells/mL; and 50%, between 1×10^1 and 1×10^2 cells/mL, respectively) in colostrum, and a specificity that approximated 0.89 (or a false positive rate of approximately 0.11). Given the preceding experimental evidence suggestive of an imperfect sensitivity and specificity of the nested PCR assay, it is likely that the MAP status of some colostrum samples tested in this study were misclassified in both directions (i.e. due to either false positive and negative errors, respectively or both). Effect of this error if present, would have been to effectively drive the relative risk estimates towards the null.

Additionally, while a parallel test interpretation criterion was applied to the serological ELISA and bacterial culture tests, to improve MAP diagnostic sensitivity in the present study, the serologic ELISA and bacterial fecal culture tests used to determine the MAP status of cows in this study are known to have limited and unreliable sensitivity during the early phase of MAP pathology in cattle. [51-53, 108] As such, cows in this study that were shedding below the limit of fecal cultural detection or were in the subclinical phase of the disease might have been misclassified as being MAP negative (i.e. false negative). The consequence of such misclassification of MAP infection status if present would have been to underestimate the incidence of MAP infection resulting in a type-II error while estimating the association of exposure (vs unexposed) and risk of MAP infection in the present study cohorts.

Closely related to the preceding limitation is the fact that follow-up time to MAP diagnosis or censorship from the study was short relative to the long and variable incubation period of MAP infection in some study cohorts (cows). In the present study, cows were tested for MAP infection status for the last time at approximately 54 months of age. Infection with MAP in cattle is thought to follow a long course with a $\geq$ 24 to72 month's interval from infection (assuming infection occurs in calfhood) and onset of active clinical disease. Therefore given the apparent short follow-up time period in the

current study and the fact that overall 88% (179/205) of calves enrolled were censored from the study, it is possible that several MAP infected calves left the study (censored) for reasons unrelated to their exposure status prior to being tested (between birth and 30 months of age), or returning a positive MAP diagnosis (between 30 and 54 months of follow-up). Moreover the diagnostic tests used for detecting MAP infection in the study cows are known to have unreliable sensitivities during the early phase of MAP pathogenesis in cattle.[51, 52, 108] Consequently, the underlying risk of MAP infection in the cohort of cows studied may have been underestimated. The latter if present, may have resulted in a compromised statistical power to detect a significant JD risk difference between the groups (i.e. exposed vs unexposed).

Notwithstanding the above limitations, findings of the present study suggest lack of an added risk of MAP infection associated with ingesting MAP DNA positive colostrum by calves. However, these findings are in stark contrast with results observed in other studies (albeit with study designs different from that employed in the current study). For example, a recent clinical trial for the efficacy of feeding a commercially available plasma-derived colostrum replacement (CR) product for the prevention of MAP transmission in calves revealed that calves fed raw bovine colostrum collected from their respective dams at birth were approximately twice as likely to be infected by MAP as calves fed the CR product. [154] Other studies have also identified certain colostrum management and feeding practices as being significant risk factors for MAP transmission in JD endemic herds. For example in one such study, herds in which calves were fed colostrum collected from known MAP infected cows were 87 times as likely to be infected with MAP than herds in which such colostrum feeding practices were not the norm.[105] Additionally, a Danish study revealed that calves fed colostrum collected from multiple sources (or cows) were more likely to test positive for MAP in adulthood compared to herd mates fed colostrum collected from their own dams.[36] In an earlier study, Streeter and others reported successfully cultivating MAP from colostrum collected from cows subclinically infected with JD. [35] Collectively therefore, the preceding study findings seem to provide evidence in support of colostrum as a possible

early vehicle by which calves get exposed to MAP in JD endemic herds underscoring the potential role of colostrum feeding programs in the epidemiology of JD in dairy herds. Perhaps the lack of a statistically significant positive association between ingesting MAP DNA positive colostrum and risk of MAP infection observed in the present study was due to the fact that the difference in incidence of MAP infection (ELISA and/or bacterial culture test outcomes) in exposed (vs unexposed) groups was biologically negligible (difference $\leq 1.6\%$) to be statistically significant ($P \leq 0.05$). A *post hoc* power analysis revealed that if the true population difference in incidence of MAP infection in unexposed (vs exposed) groups is $\geq 10.6\%$, then the current sample size (exposed group, $n = 69$ vs. unexposed group, $n = 136$) would have been sufficient to provide the present study with a statistical power $\geq 81\%$ to allow for rejection of the stated null hypothesis at a significance level (α) ≤ 0.05, assuming the incidence of MAP infection in the unexposed group was the same as that estimated from the current data.

However, given the conflicting findings (compared with findings from other studies) and limitations of the present study, readers are advised to be cautious while interpreting the current results. The potential role that other MAP transmission mechanisms (e.g. in-utero and/or fecal-oral transmission mechanisms) may have played in the present study, given the study design, should not be underestimated. Findings of the present study are inconclusive. As such, the present results should not be used as a basis to discount the potential role that raw colostrum feeding programs might play in the transmission of MAP to susceptible calves given the evidence from other studies [35, 36, 105] which suggest that colostrum might be an early source for MAP in JD infected herds. Further research is warranted to establish whether the lack of a relationship between ingesting MAP positive colostrum and risk of JD infection observed in the present study is real. Future studies attempting to address a similar question should determine dam status for MAP fecal shedding (infection) *a priori* with the intent of adjusting for this factor (dam status for MAP) at either the study design phase or during the time of analysis to avoid possible confounding bias due to in-utero transmission of MAP [43-46] or any other possible transmission modes unrelated to ingesting MAP positive colostrum.

Conclusions

In the present study, the risk of JD infection in 205 Holstein calves fed raw colostrum within the first 1 h of birth was reported. Heifer calves fed MAP DNA positive colostrum were at no greater risk of MAP infection in comparison to heifer calves fed MAP DNA negative colostrum. While it might be true that feeding MAP DNA positive colostrum does not necessarily result in added risk of JD infection in the recipient calves, the results of the current study contradict results from other studies. Given the limitations and lack of corroborative data since no studies had previously attempted to address similar questions using the study design employed in the present study, the authors' recommend treating the current results as hypothesis only generating rather than conclusive findings. Future studies that address among other factors the limitations discussed above are encouraged to determine whether the lack of association between feeding MAP positive colostrum and risk of JD infection is real.

Acknowledgements

Supported by the Minnesota Rapid Agricultural Response Fund, the American Protein Corporation Incorporated, and the USDA-CSREES NRI Competitive Grants Program (No. 1716-669-6304).

Table 11. Unconditional comparison of the number of MAP positive events observed in Holstein cows attributable to ingestion of MAP DNA positive colostrum (exposed) vs MAP DNA negative colostrum (unexposed) at birth.

Variable	Unexposed		Exposed		*P* value[†]
	No. positive	%	No. positive	%	
Test outcome at 30 months of age					
ELISA	6	4	1	1.4	0.43
Bacterial culture	6	7	3	4	0.75
Both diagnostic tests[‡]	9	7	3	4	0.75
Test outcome at 42 months of age					
ELISA	2	1.5	1	1.4	1.00
Bacterial culture	2	1.5	3	4	0.34
Both diagnostic tests	3	2.2	3	4	0.41
Test outcome at 54 months of age					
ELISA	2	1.5	2	2.9	0.60
Bacterial culture	5	3.7	2	2.9	1.00
Both diagnostic tests	6	4	2	2.9	1.00
Test outcome at all age					
ELISA	10	7	4	6	0.78
Bacterial culture	16	12	8	12	0.97[§]
Both diagnostic tests	18	13.2	8	11.6	0.74[§]

[†] *P* values were based on Fisher's exact test with values ≤ 0.05 considered significant. [‡] Cows were considered infected with MAP when they had positive test results for the serologic ELISA, bacterial fecal culture, or both tests (i.e. parallel interpretation criterion). [§] *P* values were based on Pearson χ^2-test statistic with values ≤ 0.05 considered significant.

Table 12. Final proportional hazards models describing the association between ingesting MAP DNA positive colostrum (vs MAP DNA negative colostrum) and time-to-MAP positive diagnosis (hazard).

Test	Group	*b*	S.E.*	Hazard ratio	95% CI	*P* value†
ELISA						
	Exposed	–0.31	0.68	0.74	0.2-2.77	0.65
	Unexposed	—	—	Baseline	—	—
Bacterial culture						
	Exposed	–0.09	0.45	0.92	0.38-2.22	0.85
	Unexposed	—	—	Baseline	—	—
Both diagnostic tests‡						
	Exposed	–0.2	0.45	0.82	0.34-1.95	0.65
	Unexposed	—	—	Baseline	—	—

b = Coefficient. — = Not applicable. *Standard errors are adjusted for herd effect (i.e. clustering). See Table 11 for remainder of Key.

Chapter 6: *Mycobacterium avium* subsp. *paratuberculosis* DNA detection in colostrum and teat skin associated with *Mycobacterial* fecal excretion: Implications for prevention of transmission in Holstein calves

Abstract

The objective of this study was to describe the relationship between fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) around calving time and detection of MAP in colostrum samples and on teat skin surfaces, respectively.

Fecal samples collected within 48 to 72 h prior to actual calving, colostrum (composite sample from all 4 quarters of the udder) and teat swab samples collected immediately after calving from 112 cows in 4 JD endemic herds, were tested for MAP using the conventional bacterial culture method (fecal samples) and a nested PCR targeting the genetic element ISMAP*02* (colostrum and teat swabs samples). Logistic regression modeling was used to evaluate the relationship between MAP fecal shedding status (positive/negative) and MAP detection in colostrum samples or teat swabs. Population attributable fractions (PAF) describing the proportion of colostrum and teat swab samples positive for MAP attributable to fecal shedding status of the donor cows were calculated from the odds ratios (OR) derived from the logistic regression models.

The OR for detection of MAP in colostrum samples or teat swabs in fecal culture positive (vs fecal culture negative) cows were 2.02 (95% CI: 1.32, 3.10; $P < 0.001$) and 1.87 (95% CI: 1.18, 2.97; $P < 0.008$), respectively. These findings suggested that in practice, withholding colostrum collected from MAP fecal culture positive cows might reduce the risk of exposing calves to MAP through ingestion of colostrum by 18.2% (PAF) and that limiting the chances of natural nursing by calves might reduce the risk of exposing calves to MAP present on teats of MAP fecal shedding dams by approximately 19.5% (PAF). However, it was also found that while the latter interventions might achieve some reduction in risk of exposure to MAP, they by no means eliminate the risk of exposing calves to MAP through ingestion of MAP present in colostrum or on bovine teats in JD endemic herds, given that a greater proportion of MAP detected in the

colostrum samples and teat swabs had sources unrelated to the fecal shedding status of the donors. These findings underscored the need for strict adherence to practices that limit contact of calves with adult cows from the time of birth and to practices that promote hygienic colostrum handling to avoid possible contamination during harvest, storage or feeding.

Introduction

Paratuberculosis (JD) is an economically costly chronic and contagious infection of cattle and other ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). [33, 37, 93, 96] Johne's disease is endemic in the United States (US) with a reported national prevalence ≥ 68 %.[18] Infection with MAP is difficult to control because of lack of an approved drug for treating the condition,[125] the long incubation period of infections,[28] and lack of diagnostic tests with satisfactory sensitivity for early detection of the infection in cattle.[50-53, 76, 78, 83, 108-110] Susceptibility to MAP infection is thought to be greatest in calves with most calves acquiring infections at ≤ 6 months of age.[23, 25, 31] Transmission occurs mainly via the fecal-oral route.[25, 30, 31, 33, 37] Though the most important source of MAP for within-herd transmission is considered to be feces (manure) from infected adult cattle, [24, 30, 31, 33, 40] MAP has also been detected in colostrum and milk collected from MAP fecal culture positive cows.[34, 35, 136] Feeding raw colostrum to calves might therefore be one of the earliest means by which calves become exposed to MAP. In a recent study, calves fed raw bovine colostrum had a significantly higher risk of infection with MAP vs calves fed a plasma-derived commercial colostrum replacement product. [154] Similarly, in one Danish study, cows fed colostrum collected from multiple cows or sources were more likely to test positive for MAP vs herd mates fed colostrum collected from their own dams at birth. [36] Additionally, herds in which calves were fed colostrum collected from known MAP infected cows were 87 times as likely to be infected with MAP as herds in which such colostrum feeding practices were not the norm.[105]

The scientific literature relating fecal shedding of MAP to possible contamination of colostrum with MAP is scarce. In one Ohio study, colostrum samples collected from 36 subclinically infected cows that were shedding MAP in their feces were cultured for isolation of MAP.[35] Overall, MAP was present in the colostrum samples of 22% (8 of 36) of the MAP fecal culture positive cows. Thirty-one percent (11 of 36) of these were classified as heavy fecal shedders (> 3,000 colonies/tube) vs 70% (25 of 36) that were classified as light fecal shedders (< 3,000 colonies /tube) of MAP. The colostrum samples collected from 36% (4 of 11) of the heavy shedders were positive for MAP vs 16% (4 of 25) of the colostrum samples collected from the light shedding cows. However, the failure to utilize controls (i.e. cows negative for MAP on the bacterial fecal culture test) in this early study rendered it impossible to quantify the relationship between a positive MAP fecal shedding status, and risk of recovering MAP in colostrum. Furthermore, to the author's knowledge, there is no evidence to date relating fecal shedding of MAP and contamination of bovine teats and udders with feces, soils, and/or other environmental contaminants containing MAP respectively, although fecal contamination of teat surfaces is thought to facilitate transmission of MAP in calves that might be permitted to suckle naturally. [172] In a recent observational study, the risk of MAP infection was twice as high among calves that suckled their dams or were fostered by alien cows vs calves fed milk replacer. [36]

The objective of the present study was to describe the relationship between fecal shedding of MAP around calving time and detection of MAP in colostrum samples and teat skin surfaces, respectively.

Materials and Methods

Herd selection

Cows in the present study were enrolled from a convenience sample of 4 commercial Holstein dairy herds located in southeast Minnesota. The original herd selection criteria have been described in detail elsewhere. [181] Briefly, requisites for herd selection into the study included evidence for the endemic presence of JD in the herd and

use of group maternity pens. The evidence for presence of JD in the herds was initially ascertained based on a history of culling due to JD during the year prior to the start of this study and past serological test records for MAP, although herd screening testing to determine true herd infection status was later performed. The requirement for utilizing group maternity pens was due to the fact that short- and long-term goals of the study were to evaluate the efficacy of single cow calving pens (vs group maternity pens) for the prevention of neonatal calf diseases, [181] and to evaluate the effect of single cow calving pens (vs group maternity pens) for prevention of transmission of MAP in Holstein calves, respectively. Additional criteria for herd selection included (1) routine testing for milk production through the Minnesota Dairy Herd Improvement Association (DHIA),(2) willingness to comply with study protocols, and (3) proximity (≤ 160 km radius) to the College of Veterinary Medicine, University of Minnesota.

Number of cows in participating herds ranged between 265 and 580 cows (median, 405 cows). In-terms of herd-size, 3.7% and ≤ 1% of Minnesota dairy herds had between 200 and 499, and ≥ 500 milking cows respectively, in 2004. [182] Annual herd milk production (i.e. rolling herd average) ranged between 9,752 and 13,607 kg/y (median, 10,381 kg/y). Bulk-tank somatic cell counts ranged between 180,000 and 240,000 cells/mL (median, 230,000 cells/mL). Number of cows enrolled in the original study ranged between 50 and 229 cows (median, 110 cows). The estimated within-herd prevalence for MAP infection as determined through bacterial culture of feces for MAP, ranged between 4% and 14% (median, 8%) (Table 13).

Study design and animal selection

A cross sectional study was conducted to evaluate the objective of the present study. The original study, of which the current study is part, was approved by the Institutional Animal Use and Care Committee at the University of Minnesota (Protocol Number 0406A1181).

Feces, colostrum, and teat swab samples were collected (*see* details below) from all cows (n = 499) in the 4 participating herds that calved between January and December,

2005 for the recovery and detection of MAP, respectively. However, while all collected fecal samples (*n* = 499) were tested for MAP (via fecal culture, *see* details below), not all collected colostrum and teat swab samples were tested to confirm presence of MAP for budgetary reasons. As such, colostrum and teat swab samples tested for MAP were selected from the stored samples collected during the enrollment period as follows.

The colostrum and teat swab sample pairs collected from fecal culture positive cows (i.e. *n* = 38 or 7.6% of 499 cows tested) were all tested for presence of MAP, respectively. For each MAP positive cow in the study herds, approximately 2 MAP fecal culture negative cows were randomly selected from the list of fecal culture negative cows in the same herd as a comparison group (i.e. controls). The colostrum and teat swab samples collected from the controls (i.e. *n* = 74 or 15% of 499 cows tested) were all tested for presence of MAP, respectively. Number of cows sampled per herd ranged between 7 and 83 cows (median, 11 cows) (Table 13).

Sample collection

For each cow that was about to calve, approximately 10 g of feces was collected manually by the herdsmen from the rectum using a disposable plastic examination sleeve within 48 to 72 h prior to actual calving. Fecal samples were stored in a sterile plastic sample container and labeled with the respective cow identification prior to refrigeration at ≤ 8 °C (46 °F). Newborn calves were separated from their dams within 1 h of being born prior to having the opportunity to nurse.

Sterile gauze swabs (impregnated with a solution of 4.5 g/L sodium chloride) were prepared in advance of an impending teat swabbing exercise. Teat skin surfaces of the front and rear teats were swabbed using the sterile gauze preparations prior to being washed by the herdsmen. The teat swabs were labeled with the respective cow identification and placed in an opaque air tight plastic specimen tube containing 5mL of 4.5 g/L sodium chloride solution and kept in storage at – 20 °C (– 4 °F). The teat surfaces were then disinfected using 0.05% povidone iodine commercial teat dip and cleansed with 70% ethyl alcohol-impregnated pledgets prior to collection of colostrum samples.

The fore stripping colostrum was discarded, and composite colostrum (50 mL) samples were collected into a sterile plastic tube from all 4 quarters of the udder, labeled with the respective cow identification, and stored at – 20 °C (– 4 °F).

For the collection of colostrum samples and obtaining the teat swabs, latex gloves were worn and fresh pairs of gloves were used each time a new cow was sampled. All samples (fecal samples, colostrum samples, and teat swabs) were picked up on a biweekly farm visiting schedule by the investigator and transported on ice to the University of Minnesota. Fecal samples were tested for MAP at the Minnesota Veterinary Diagnostic Laboratory (MVDL). Colostrum and teat swab samples were shipped to the National Animal Disease Center, USDA-ARS in Ames, IA for further processing and analysis.

Cultivation of MAP from fecal samples

The method for the cultivation of MAP in feces used in the present study has been described in detail previously. [38, 108] Briefly, sediments derived from the fecal samples were plated onto Herrold's Egg York Agar Slants [a] containing Mycobactin J and antibiotics (0.1 ml of 50 mg amphotericin B) and incubated at 37°C for between 1 to 3 weeks. At approximately 6 weeks post inoculation, the agar slants were examined weekly for growth of MAP. Based on total colony counts per g of fecal sample tested, results were reported as negative (0 colonies/tube) or positive-low shedder (1 to 10 colonies/tube); positive-moderate shedder (10 to 50 colonies/tube); positive-high shedder (51 to 100 colonies/tube); and positive-very high shedder (> 100 colonies/tube).

PCR analysis for detection of MAP DNA in colostrum and teat swab samples

Mycobacterium avium subsp. *paratuberculosis* DNA was extracted from colostrum samples using a previously described method [180] and from the teat swabs, using a commercially available kit. [b]

[a] HEYM-BBL, Becton-Dickinson, Franklin Lakes, NJ
[b] ZR Fecal DNA Kit®, Zymo Research Corp., Orange, CA

The first round of PCR was run using a conventional thermal cycler. [c] Samples were run in triplicate in 96-well plates according to the following protocol: 1 cycle at 94°C for 5 min, followed by 20 cycles at 94°C for 45 s, 58°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 7 min. The primer sequences for the initial amplification were 5'-GCACGGTTTTTCGGA TAACGAG-3" (forward primer) and 5'-TCAACTGCGTCACGGTGTCCTG-3" (reverse primer).

The second amplification was performed using real-time PCR. [d] Primers nested within the first set were 5'-GCACGGTTTTTCGGATAACGAG-3' (forward primer) and 5'-AACCGACGCCGCCAATACG-3" (reverse primer) for this second amplification.

Negative and positive controls consisted of the reaction mixture alone, and genomic DNA from MAP (strain K-10 isolates), respectively.

Statistical analysis

The distribution of cow parity (1^{st} vs $\geq 2^{nd}$ lactation), number of colostrum samples and teat swabs positive for MAP (yes/no), and MAP fecal shedding categories (1 = low shedder, 1 to 10 colonies/tube; 2 = moderate shedder, 10 to 50 colonies/tube; 3 = high shedder, 50-100 colonies/tube; 4 = very high shedder, > 100 colonies/tube; and 0 = negative, 0 colonies/tube) between fecal culture positive and negative groups of cows was summarized.

Multivariable analysis

The number of colostrum samples and teat swabs positive for MAP (yes/no) was submitted to two separate logistic regression models as dependent variables. Initially, the MAP fecal shedding categories (1 = low shedder, 1 to 10 colonies/tube; 2 = moderate shedder, 10 to 50 colonies/tube; 3 = high shedder, 50-100 colonies/tube; 4 = very high shedder, > 100 colonies/tube; and 0 = negative, 0 colonies/tube) and lactation number of the cow (1^{st} vs $\geq 2^{nd}$ lactation) were included as independent variables in each fitted

[c] Peltier Thermal Cycler, MJ Research, Waltham, MA
[d] 7500 Real-time PCR System, Applied Biosystems, Foster City, CA

model. However, after grouping the MAP fecal shedding categories into a dichotomous variable, the MAP fecal shedding status (positive/negative) and lactation number of the cow (1^{st} vs $\geq 2^{nd}$ lactation) were included in the final logistic models as independent variables. Since cows in the same herd were more likely to be similar (vs cows in other herds) with respect to study outcomes, robust estimates of variance [143, 160, 166] were used to adjust for the within-herd cluster effects. Because statistical significance ($P \leq 0.05$) was absent for the lactation number variable in all of the fitted models, this variable was excluded from all final models. The odds ratios (OR) expressing the strength of the relationship between MAP fecal shedding status (positive/negative) and fecal shedding categories (1 = low shedder, 1 to 10 colonies/tube; 2 = moderate shedder, 10 to 50 colonies/tube; 3 = high shedder, 50-100 colonies/tube; 4 = very high shedder, > 100 colonies/tube; and 0 = negative, 0 colonies/tube) respectively, and risk of detecting MAP present in colostrum samples and teat swabs, respectively were calculated from each model. Model fit to the data was tested using the Hosmer-Lemshow goodness-of-fit test for all models. [166]

All analyses were implemented using standard statistical software [e] and values of $P \leq 0.05$ were considered significant.

Estimation of the population attributable fraction (PAF)

To estimate the proportion of colostrum samples and teat swabs positive for MAP due to the fact that the cows donating these samples were MAP fecal culture positive, the PAF were estimated as follows:

$$PAF = pd\ (OR - 1/OR)$$

where *pd* represented the overall proportion of colostrum samples or teat swabs testing positive for MAP and that were collected from MAP fecal culture positive cows, and *OR* was the adjusted odds ratio for the relationship between MAP fecal shedding status (positive/negative) or shedding category (low, moderate, high, and very high) and risk of detecting MAP in colostrum samples and teat swabs, respectively. [183]

[e] Stata® Corp., College Station, TX

Results

Descriptive summary

Overall 22% (112 of 499) of the cows enrolled in the original study were available for analysis. Thirty-four percent (38 of 112) of these were fecal culture positive for MAP while 66% (74 of 112) were fecal culture negative. Low, 1 to 10 colonies/tube; moderate, 10 to 50 colonies/tube; and very high, > 100 colonies/tube shedding categories constituted 66% (25 of 38), 13% (5 of 38), and 21% (8 of 38) of the total number of MAP fecal culture positive cows, respectively. There were no cows in the high shedding category. Nineteen percent (21 of 112) of the teat swab samples collected from 13% (5 of 38) fecal culture positive cows vs 22%(16 of 74) fecal culture negative cows respectively were missing. Similarly, 7% (8 of 112) of the colostrum samples collected from 13% (5 of 38) fecal culture positive cows vs 4 %(3 of 74) fecal culture negative cows respectively were missing. Records with missing data were excluded from all subsequent analysis. Sixty-nine percent (72 of 104) of all colostrum samples tested were positive for MAP, 79% (26 of 33) of these were collected from MAP fecal culture positive cows vs 65% (46 of 71) collected from MAP fecal culture negative cows, respectively. Sixty percent (55 of 91) of all teat swab samples tested were positive for MAP, 70% (23 of 33) of these were collected from MAP fecal culture positive cows vs 55% (32 of 58) collected from MAP fecal culture negative cows, respectively (Table 14).

Multivariable logistic regression

The goodness-of-fit analyses for the final models suggested that the models fit the data reasonably well. Logistic regression modeling indicated that overall OR for the association between MAP fecal shedding status (positive/negative) and detection of MAP in colostrum was 2.02 (95% CI: 1.32, 3.10; $P < 0.001$). Similarly, the OR for the association between MAP fecal shedding status (positive/negative) and detection of MAP in teat swabs was 1.87 (95% CI: 1.18, 2.97; $P < 0.008$) (Table 15). The OR for the association between low, 1 to10 colonies/tube; moderate, 10 to 50 colonies/tube; and very high, > 100 colonies/tube MAP fecal shedding categories and risk of detecting MAP in

colostrum were 1.74 (95% CI: 0.79, 3.81; *P* = 0.17), 2.17(95% CI: 0.56, 8.38; *P* = 0.26), and 3.26 (95% CI: 0.65, 16.5; *P* = 0.15), respectively. Similarly, the estimated OR for the association between low, moderate, and very high MAP fecal shedding categories and risk of detecting MAP in teat swabs were 1.63 (95% CI: 1.07,2.46; *P* = 0.02), 1.63 (95% CI: 1.00,2.64; *P* = 0.05), and 4.06 (95% CI: 0.07,19.0; *P* = 0.07), respectively (Table 16). In both cases, there was an increasing trend in the risk of MAP detection in colostrum samples and teat swabs the higher the MAP fecal shedding category, although these estimates were not statistically significant for the MAP positive colostrum outcome.

Estimates for population attributable fraction

The PAF estimates associated with each MAP fecal shedding category plus all MAP shedding categories combined (i.e. MAP fecal shedding status defined as positive or negative) are presented (Table 17). Overall, the proportion of colostrum samples and teat swabs positive for MAP attributable to the MAP fecal culture positive (vs negative) status of the donor cows in the population studied was 18.2% and 19.5% respectively, based on OR estimates(Table 15) from the final logistic regression models.

Discussion

Mycobacterium avium subsp. *paratuberculosis* has previously been detected through the bacterial culture of MAP in colostrum of 22% (8 of 36) of MAP fecal culture positive cows. [35] However, it was impossible to quantify the magnitude of the association between a positive MAP fecal shedding status and risk of detecting MAP in colostrum since this early study did not include MAP fecal culture negative cows as a control group. The present study was undertaken to quantify the impact of MAP fecal shedding on the risk of detecting MAP in colostrum and teat swabs of cows inhabiting JD endemic herds.

Though a major strength of this study was the fact that the selected herds were heavily infected with JD (i.e. prevalence range, 4% to 14%; median 8%) providing the ideal group of animals for the evaluation of the current study objectives, there were also a number of limitations. For example, the cross sectional observational design of this study

made it impossible to infer cause-and-effect relationships using the present data. The nested PCR assay used to detect MAP in colostrum samples and teat swabs in the present study was shown in a previous experimental validation study,[180] to have an analytic sensitivity that improved with increasing concentration of MAP (range, 100%, between 1×10^7 and 1×10^9 cells/mL; 75%, between 1×10^3 and 1×10^6 cells/mL; and 50%, between 1×10^1 and 1×10^2 cells/mL, respectively) in colostrum, and a specificity that approximated 0.89 (or a false positive rate of approximately 0.11). Therefore, given the preceding experimental evidence suggestive of an imperfect sensitivity and specificity of the nested PCR assay, it is likely that the MAP status of some colostrum samples and teat swabs tested in this study were misclassified in both directions (i.e. due to either false positive and negative errors, respectively or both). Similarly, the bacterial fecal culture test used to determine the MAP fecal shedding status of cows in this study is known to have limited and unreliable sensitivity during the early phase of MAP pathology in cattle. [51-53, 108] As such, cows in this study that were shedding below the limit of fecal cultural detection might have been misclassified as being MAP negative, particularly those in their first lactation that made up approximately 38% of the total cows sampled. Such misclassification errors (due to the imperfection of the tests used in this study) if present may have biased the association between the positive MAP fecal shedding status and risk of detecting MAP in colostrum samples and teat swabs towards the null, effectively lowering the magnitude of the estimated ORs.

In spite of the above limitations, there was still a significant association detected between positive MAP fecal shedding status of the cow and risk of detection of MAP in colostrum samples and teat swabs, respectively (Table 15). There was also evidence of an increasing trend in the strength of the association between risks of detecting MAP in colostrum or teat swabs from the low MAP fecal shedding category to the very high category, although the MAP fecal shedding category estimates of associations were not statistically significant ($P > 0.05$) when the MAP positive colostrum outcome variable was considered (Table 16). These findings were not surprising since the authors had expected an increase in the risk of detecting MAP in colostrum and teat swabs the greater

the quantity of MAP present per g of fecal sample tested. The lack of statistically significant associations observed between MAP fecal shedding status categories and risk of detecting MAP in colostrum might have been due to a compromised statistical power resulting from a relatively small number of cows within each MAP fecal shedding category resulting from such categorization of data (Table 16). Nonetheless there was an overall significant positive association between positive MAP fecal shedding status of the cow (positive/negative) and risk of detection of MAP in colostrum (Table 15).

While mechanisms by which MAP may contaminate colostrum are still poorly understood, two theories have been advanced as potential explanations for the latter phenomenon. First, it is thought that MAP infected feces present on the teat skin might play a significant role in contamination of colostrum during the collection or harvesting process. [172] There was however no evidence in support of the latter theory in the present study since no significant association (i.e. correlation) was observed between MAP DNA positive colostrum status and MAP DNA positive teat swab status, respectively. Perhaps the lack of a significant association between MAP DNA positive colostrum status and MAP DNA positive teat swabs may have been a result of the level of hygiene (i.e. sterile collection of colostrum) observed during the collection of colostrum samples in this study. The second mechanism is thought to be through the systemic or hematogenous dissemination of *Mycobacteria* in an infected host into the mammary gland. [35] In this mechanism (i.e. hematogenous mechanism), freely circulating MAP infected macrophages are thought to end up in the mammary glands with colostrum being their final site of deposition. This follows the observation that macrophages constitute the dominant somatic cell type detected in secretions from uninfected mammary glands during the dry and periparturient periods respectively.[178, 179] While in an infected host, MAP is a strict intracellular pathogen with an affinity for infecting macrophages, and the ability to survive inside macrophages for long periods of time with no detrimental effects on the viability of their cellular hosts.[176, 177]

The presence of MAP on teat skin may result from fecal contamination of the cow's teat directly from MAP-infected manure excreted by the same cow (or another

cow), [172]although it is possible that the environment of JD infected herds might also serve as an important source of MAP from which infected feces is picked up by the udders and teats when cows lie in bedding containing MAP infected manure, or floors on which MAP infected manure has been allowed to accumulate. In a previous study of distribution of MAP in the environment of Minnesota herds, $\geq$ 78% of fecal sample pools collected from surroundings of JD endemic herds were positive for MAP, with fecal pools collected from cow alleys (77%), manure storage areas (68%), maternity pens (21%), and sick cow pens (18%) testing positive for MAP, respectively.[42] This finding underscored the ubiquitousness of MAP in the surroundings of JD infected herds and the possible role of the environment in MAP transmission.

Interestingly the proportion of MAP positive colostrum samples and teat swabs potentially attributed to positive MAP fecal shedding status of the donor cows (i.e. PAF), possibly through the mechanisms described above, were only 18% and 20%, respectively (Table 17). This finding suggests that approximately 80% of MAP detected in the colostrum samples and from teat swabs had sources unrelated to the MAP fecal shedding status of the donor cows, respectively. Since JD was endemic in the study herds with an estimated within-herd prevalence of MAP fecal shedders ranging between 4% and 14% (median, 8%), it is possible that the environments of participating herds may have played a major role as reservoirs for MAP, providing a source for MAP that eventually contaminated the colostrum samples and teat surfaces. In absolute terms, 65% of the colostrum samples and 55% of the teat swabs derived from MAP fecal culture negative cows in the present study had MAP detected in them (Table 14).

In spite of the apparently greater potential role played by the MAP-laden environment in contamination of colostrum and teat surfaces of cows in JD endemic herds, the PAF estimates obtained in the present study have practical implications for within-herd JD control efforts. For example, the PAF estimates indicated that by avoiding the feeding of colostrum from known fecal shedders of MAP within a JD infected herd, the risk of exposing calves to MAP (assuming the MAP DNA present in colostrum was indicative of a viable infectious *Mycobacteria*) within 24 to 48 h of birth through

ingestion of colostrum could be diminished by approximately 18%. Similarly, by avoiding the possibility of natural nursing of cows shedding MAP in their feces through prompt separation of calves from their dams within 30 minutes of birth, the risk of exposing calves through ingestion of MAP present (assuming the MAP DNA present on the teats was indicative of a viable infectious *Mycobacteria*) on bovine teats could be diminished by approximately 20% (Table 16). However, important to note is that while the preceding interventions might achieve some reduction in risk (by approximately 18% and 20% risk of MAP exposure reduction, respectively) of exposure, they by no means could completely eliminate the risk for MAP exposure to calves through ingestion of MAP present in colostrum or on bovine teats in JD endemic herds, since a larger percentage (≥ 81%) of the MAP (again assuming the MAP DNA present in these samples were indicative of a viable infectious *Mycobacteria*) detected in colostrum samples and teat swabs in the present study had sources unrelated to the MAP fecal shedding status of cows from which the samples were collected. As such, implementing other strategies that might assist with preventing MAP contamination (from other sources such as the environment in JD endemic herds) of colostrum and teats to reduce the risk of calves' exposure to MAP is highly encouraged. For example, dairy producers might limit contamination of colostrum by manure cycle pathogens including MAP by adequately prepping udders prior to harvesting colostrum and storing or handling colostrum using sanitized equipment. Details on how to prevent potential bacterial contamination of colostrum during the harvest, storage, and/or feeding processes have been discussed elsewhere. [153, 184] A recent report indicated that herds in which calves were fed colostrum collected from known MAP infected cows were 87 times as likely to be infected with MAP as herds in which such colostrum feeding practices are not the norm.[105] As such, further effort could also be directed towards identifying individual cows that are shedding MAP in feces within-herds through regular bacterial fecal cultural screening for possible removal from the herd to reduce the pool of animals that may be contributing directly to increased environmental loads of MAP and/or donors of MAP contaminated colostrum. Permitting natural nursing or fostering of calves by alien cows within a herd is a

significant predictor for future MAP positive test outcomes in the affected calves.[36] As such, newborn calves should not be allowed to nurse their dams or other alien cows (i.e. cows different from their own dams) whenever possible through prompt separation from their dams within 30 minutes of birth. Implementing measures that promote proper manure waste management should be undertaken additional to using individual maternity pens (vs group maternity pens) that are cleaned and freshly bedded between successive calvings. A study is currently in progress at the University of Minnesota to evaluate the efficacy of the latter approach in preventing MAP transmission in calves. [181]

Lastly, the herds in this study were not a random selection from the entire population of dairy herds in Minnesota. In-terms of herd size, the selected herds were medium (100 to 499 cows) to large (≥ 500 cows) in size. According to the National Agricultural Statistics Service (NASS), approximately 3.7% and ≤ 1% of the total Minnesota dairy herds belonged to these herd size categories in 2004, respectively.[182] As such, caution should be exercised when attempting to extrapolate the current results to smaller herds (> 100 cows) or herds with different management systems.

Conclusions

Findings of the present study underscored the need for strict adherence to practices that limit contact of calves to adult cattle shedding MAP in their feces at birth in addition to employing strategies that limit potential environmental contamination of colostrum during the process of harvesting, storage, and feeding, as part of a wider JD control effort. Overall the OR of detecting MAP in colostrum samples and teat swabs of cows that were shedding MAP in their feces (vs cows that were not shedding MAP in feces) was 2.02 ($P < 0.001$) and 1.87 ($P < 0.008$) respectively, suggesting an increased risk of contamination associated with MAP fecal shedding. The present study results also indicated that by withholding colostrum collected from MAP fecal culture positive cows the risk of exposing calves to MAP through ingestion of colostrum could be reduced by approximately 18%. Similarly, limiting the chances of natural nursing by calves could potentially reduce the risk of exposing calves to MAP present on teat surfaces of MAP

fecal shedding dams by approximately 20%. However, these study results suggest that the preceding interventions alone will be insufficient in eliminating the risk of calves' exposure to MAP through colostrum, or ingestion of MAP present on bovine teats in JD endemic herds due to the ubiquitous presence of MAP in the environment surrounding JD infected herds, the latter serving as a significant source of MAP from which colostrum and dams' teats might be contaminated during the harvesting, storage, and feeding processes, or when the cows lie down, respectively. Therefore in attempting to control JD in affected herds' efforts should be made to indentify for possible removal from the herds, all cows actively shedding MAP in their feces to reduce the pool of animals that may be contributing directly to increased loads of MAP in the environment. Other areas to be emphasized include proper manure waste management and environmental hygiene including hygiene within the calving pens additional to the calf and adult cow housing areas, respectively.

Acknowledgments

This study was funded through the USDA-CSREES NRI Competitive Grants Program (#1716-669-6295). We would also like to thank the Minnesota Board of Animal Health for providing contacts of the dairy herds, and the producers whose herds were used in this study, for their cooperation.

Table 13. The general characteristics of four Minnesota dairy herds enrolled in 2005 for a study of the association between positive MAP fecal shedding status and risk of detecting MAP in colostrum and teat swabs collected from Holstein cows around calving time.

	Herd 1	Herd 2	Herd 3	Herd 4
Herd size (*n*)	580	530	280	265
Number of cows enrolled* (*n*)	229	157	63	50
Number of cows analyzed (*n*)	83	12	7	10
Rolling herd average milk production (*kg/y*)	9,752	13,607	10,432	10,330
Average somatic cell count estimates (*per ml*)	230,000	180,000	230,000	240,000
MAP prevalence estimates† (*%*)	14	4	8	8

*Number of cows enrolled in original study; findings for the short-term objectives have been described elsewhere.[181]†Estimated within-herd apparent prevalence of MAP fecal shedding cattle.

Table 14. The comparison of number of colostrum samples and teat swabs in which MAP was detected, and the distribution of other variables in a study of the association between positive MAP fecal shedding status and risk of detecting MAP in colostrum and teat swabs collected from Holstein cows around calving time in four Minnesota dairy farms in 2005.

Variable	Fecal culture positive	
	Yes	No
Number of cows (*n*)	38	74
Lactation		
1, *n* (%)	15 (48)	28(38)
≥ 2, *n* (%)	16 (52)	45(62)
MAP fecal shedding category[†]		
Low, *n* (%)	25 (66)	0
Moderate, *n* (%)	5(13)	0
High, *n* (%)	0	0
Very high, *n* (%)	8(21)	0
Negative, *n* (%)	0	74 (100)
MAP detected in colostrum[‡]		
Yes, *n* (%)	26(79)	46(65)
No, *n* (%)	7(21)	25(35)
MAP detected in teat swabs[*]		
Yes, *n* (%)	23(70)	32(55)
No, *n* (%)	10(30)	26(45)

[†]Low shedder, 1 to 10 colonies/tube; moderate shedder, 10 to 50 colonies/tube; high shedder, 50-100 colonies/tube; very high shedder, > 100 colonies/tube; and negative, 0 colonies/tube. [‡] Due to exclusion of missing records from analysis, denominator used in calculation of proportion of colostrum samples positive for MAP was 33 fecal culture positive vs 71 fecal negative cows. [*]For the teat skin outcome, denominator used in calculation of proportion of teat swab samples positive for MAP was 33 fecal culture positive vs 58 fecal negative cows respectively, due to exclusion of missing data from the analysis.

Table 15. Final logistic-regression models estimating the association between MAP fecal shedding status and risk of detecting MAP in colostrum and teat swabs collected from Holstein cows around calving time for fecal culture positive cows.

Outcome variable	Fecal culture positive	OR	S.E ‡	95% CI,OR		*P* value
				Lower	Upper	
MAP detected in colostrum						
	Yes	2.02	0.44	1.32	3.10	< 0.001
	No	Baseline	—	—	—	—
MAP detected in teat swabs						
	Yes	1.87	0.44	1.18	2.97	0.008
	No	Baseline	—	—	—	—

‡ Standard errors adjusting for cluster effect of herd. — = Not applicable.

Table 16. Logistic regression models representing the association between MAP fecal shedding category, and the risk of detecting MAP in colostrum and teat swabs around calving time for fecal culture positive cows.

Outcome variable	MAP fecal shedding category[†]	OR	S.E[‡]	95% CI, OR		*P* value
				Lower	Upper	
MAP detection in colostrum						
	Low	1.74	0.70	0.79	3.81	0.17
	Moderate	2.17	1.50	0.56	8.38	0.26
	High	—	—	—	—	—
	Very high	3.26	2.70	0.65	16.5	0.15
	Negative	Baseline	—	—	—	—
MAP detection in teat swabs						
	Low	1.63	0.34	1.07	2.46	0.02
	Moderate	1.63	0.40	1.00	2.64	0.05
	High	—	—	—	—	—
	Very high	4.06	3.19	0.87	19.0	0.07
	Negative	Baseline	—	—	—	—

See Table 14 and 6.3 for key.

Table 17. Population attributable fraction (PAF) estimates for the risk of MAP detection in colostrum and teat swabs attributable to a positive MAP fecal shedding status around calving time.

Outcome variable	MAP detected in colostrum		MAP detected in teat swabs	
	OR	% PAF	OR	%PAF
MAP fecal shedding category[†]				
Low	1.74	9.45	1.63	11.2
Moderate	2.17	3	1.63	1.41
High	—	—	—	—
Very high	3.26	5.78	4.06	6.85
Negative	Baseline	—	Baseline	—
Fecal culture positive				
Yes	2.02	18.2	1.87	19.5
No	Baseline	—	Baseline	—

See Table 14 and 6.3 for key

Chapter 7: Lack of evidence for detectable fecal excretion of *Mycobacterium avium* subsp. *paratuberculosis* in calves born in a high Johne's disease prevalence herd

Abstract

The objective of this study was to detect the presence of calves excreting *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in their feces following natural exposure. A total of 60 heifer calves born in one Minnesota commercial Holstein dairy herd were enrolled into the study between July and December, 2005. Between 1-to-3 d of age, herd personnel collected approximately 10 g of fecal samples followed by monthly visits to the farm at which time 10 g of fecal samples were again collected by the study investigator from each calf at approximately 30, 60 and 90 d of age, respectively. *Mycobacterium avium* subsp. *paratuberculosis* was not recovered from any of the fecal samples tested via the bacterial culture method, suggesting that the calves in the present study did not excrete detectable levels of MAP in feces.

Introduction

Paratuberculosis or JD is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection. Though several animal species are known to be infected, paratuberculosis is predominantly a disease of ruminants.[27, 28] Calves are considered particularly vulnerable to MAP infections early in life (≤ 6 months of age) [23, 28, 172, 185] with an age-related resistance to infection potentially developing thereafter.[25] Calves are thought to acquire infections mainly through oral ingestion of MAP[25, 30, 31, 37] with manure excreted by infected adult cattle being the most important source[24, 30, 31, 33] although the practice of feeding raw colostrum and permitting natural nursing have been shown to be significant predictors of MAP transmission in calves. [36, 105, 154]

The model for JD control at the herd-level is premised upon prevention of the vertical transmission of MAP from dam-to-calf through calf segregation from adult cattle and avoiding exposure of calves to adult cattle manure, raw colostrum, and milk.[27, 30, 31]

Though shown in simulation modeling studies to be effective in reducing prevalence of MAP at the farm-level,[122] a limitation for the preceding JD control model lies in its critical assumption that calves acquire MAP infections mainly through the vertical (i.e. dam-to-calf) route, hence the emphasis placed on the dam-calf environment. However, while it is generally assumed that MAP infected calves are not infectious until ≥ 2 years of age[186] and that segregating youngstock from adult cattle is sufficient in breaking the MAP transmission cycles in JD infected herds, [27, 29-31] recent findings from experimental studies have suggested that calves and young stock (< 2 years of age) are capable of excreting detectable quantities of MAP in their feces. [24, 40, 41] While fecal excretion of MAP by calves may have minimal contribution towards overall burden of MAP in the environment, [187] MAP fecal shedding by calves may be significant in maintaining infection in calves housed in groups. This is premised upon the observation that group housing of calves is a significant predictor of herds infected with JD. [21, 38, 54] Recent experimental studies have demonstrated that MAP can be successfully transmitted horizontally (i.e. calf-to-calf),[24, 40, 41] underscoring the potential significance of MAP infected calves in the maintenance of JD within-herds, even if interventions targeting dam-to-calf transmission routes were to be successful in preventing MAP transmission within herds.

Limited studies have been undertaken to evaluate fecal excretion of MAP by calves naturally exposed to infection. [48, 48, 185, 187] The goal of this study was to describe patterns of fecal excretion of MAP by calves following natural exposure. The objective was to detect presence of calves excreting MAP in their feces following natural exposure.

Materials and Methods

Study design

A longitudinal, prospective, 90-d long study involving calves born in a single commercial dairy herd located in southeast Minnesota was conducted to evaluate the objective of the present study. The original study, [181] of which the current study is part,

was approved by the Institutional Animal Use and Care Committee at the University of Minnesota (Protocol No. 0406A1181).

Herd selection criteria and description

Cows in the present study were enrolled from a commercial Holstein dairy herd located in southeast Minnesota in 2005. The original herd selection criteria have been described in detail elsewhere [181] and included evidence for the endemic presence of JD in the herd and use of group maternity pens. The requirement for utilizing group maternity pens was due to the fact that short- and long-term goals of the study were to evaluate the efficacy of single cow calving pens (vs group maternity pens) for the prevention of neonatal calf diseases, [181] and for prevention of transmission of MAP in Holstein calves, respectively. Other criteria for herd selection included routine testing for milk production through the Minnesota Dairy Herd Improvement Association (DHIA), willingness to comply with study protocols, and proximity (≤ 160 km radius) to the College of Veterinary Medicine, University of Minnesota.

Number of cattle in the selected herd was 580 cows in 2005. Milk production (rolling herd average) for this farm was 9,752 kg/y while the bulk-tank somatic cell count was 230,000 cells/mL. The estimated within-herd apparent prevalence for MAP infection determined on the basis of bacterial culture of fecal samples collected from individual cows was 14% in 2005.

Calf management in the selected herd

Pregnant heifers and cows were vaccinated at approximately 3 months prior to the expected calving date using a multiple antigen vaccine [a] containing inactivated rotaviruses, coronaviruses, a bacterin-toxoid from *Clostridium perfringens* types C and D, and a free cell extract of *E. coli*, to provide protection to unborn calves against scours. All calves born in this herd were raised on the farm property and housed in calf hutches until weaning (60 d of age). Following weaning, all calve were transferred into loose

[a] Gardian, Schering-Plough Animal Health Corp, Omaha, NE

houses with bedded pack. The calf preweaning diets (up to 60 d of age) consisted of commercial milk replacer and unrestricted amounts of water and calf starter pellet.

Sample collection

Fecal samples were collected from a total of 60 dam-daughter pairs as follows: For each cow that was about to calve, approximately 10 g of feces was collected manually by the herdsmen from the rectum using a disposable plastic examination sleeve within 48 to 72 h prior to actual calving. Newborn calves were separated from their dams within 1 h of being born prior to having the opportunity to nurse. Between 1-to-3 d of birth, herd personnel collected approximately 10 g of fecal samples via the rectum using individual latex gloves and a lubricant that consisted of sterile water. This was followed by monthly visits to the farm at which time 10 g of fecal samples were again collected by study investigators from each calf at approximately 30, 60 and 90 d of age, respectively. Fecal samples were stored in sterile plastic sample containers and labeled with the identification of the dam and calf respectively prior to storage at ≤ 8 °C (46 °F). All samples were picked up on a weekly schedule by study investigators and transported on ice to the Minnesota Veterinary Diagnostic Laboratory for analysis.

Laboratory analysis

The method for the cultivation of MAP in feces used in the present study has been described in detail previously. [38, 108] Briefly, sediments derived from the fecal samples were plated onto Herrold's Egg York Agar Slants [b]containing Mycobactin J and antibiotics (0.1 ml of 50 mg amphotericin B) and incubated at 37°C for between 1 to 3 weeks. At approximately 6 weeks post inoculation, the agar slants were examined weekly for growth of MAP. Based on total colony counts per g of fecal sample tested, results were reported as negative (0 colonies/tube) or positive-low shedder (1 to 10 colonies/tube); positive-moderate shedder (10 to 50 colonies/tube); positive-high shedder (51 to 100 colonies/tube); and positive-very high shedder (> 100 colonies/tube).

[b] HEYM-BBL, Becton-Dickinson, Franklin Lakes, NJ

Data analysis

Data was summarized using contingency tables.

Results

Fecal samples were collected from a total of 60 calves between July and December, 2005. Sixty-two percent (37 of 60) of these calves were born in individual calving pens that were cleaned between successive calvings while 38 % (23 of 60) of the calves were born in multiple calving pens. Of the 60 dams tested prior to calving, 8% (5 of 60) was bacterial culture positive for MAP. Overall 240 fecal samples collected from calves at 1-to- 2, 30, 60, and 90-d of age were cultured for MAP. None (0 of 240) of these samples tested positive on the bacterial culture test.

Discussion

The goal of the present study was to describe patterns of fecal excretion of MAP by calves following natural exposure to MAP during the first 90-d of testing. Though the main strength of this study was its longitudinal prospective sampling design spanning 90-d since the birth of each study calf, there were also limitations that needed consideration while interpreting the current data. For example, detection of MAP infection in calves remains a daunting task generally due to the long incubation period of the infection following exposure and the lack of validated assays specific for detection of infection in calves. [187] While it is possible to establish exposure to MAP by measuring the cell mediated immune response profiles using the bovine γ interferon assay, [187] the inability to collect blood samples from study calves within 1-to-9 d of birth rendered it impossible to perform this test. Moreover sensitivity and specificity of the bovine γ interferon have not been fully validated for use in calves. [187] Lastly, the objective of this study was evaluated using data collected from a single Minnesota dairy herd. Thus, the finding reported is not representative of the entire population of dairy herds in Minnesota.

The present study failed to detect evidence of fecal excretion of MAP by calves born in a high JD prevalence (i.e., 14%) herd. This finding contradicts preliminary results

from an earlier study which revealed that 8 % (1 of 12) of calves, 0-to-90 d old, under natural exposure conditions excreted detectable levels of MAP in feces. [185] Other than the fact that the detection of MAP infection in calves remains a daunting task generally, [187] for the reasons discussed in the preceding paragraph, the failure to detect fecal excretion of MAP by calves under natural farm exposure conditions may have resulted from the following reasons:

First, it is possible that despite the high prevalence of JD infection in the study herd, the calves were not sufficiently exposed to on-farm sources of MAP to result in infection or detectable levels of MAP in feces using the bacterial culture method. In the present study, natural exposure of calves to MAP was verified through fecal cultural testing of their respective dams 24-to-48 h prior to the calves' birth. This was premised upon evidence from earlier dam-daughter pair studies which suggested that positive MAP infection status of dams was a significant predictor of exposure to- and-subsequent infection of daughters by MAP. [61, 64, 185] However, the daughters (i.e. calves) born to 8% (5 of 60) of the cows that tested positive for MAP via fecal culture in the present study were consistently negative for MAP over the sampling duration (up to 90 d of age). Also, some management factors in place on the study farm may have compromised the extent of exposure of calves to MAP infections resulting in their inability to be exposed and therefore excrete detectable levels of MAP in feces. For example, calves in the present study were separated from their dams within 1 h of birth and housed in individual calf hutches during the preweaning period (up to 60 d old) and later transferred into group housing pens. Though all calves in the present study were fed 3.8 L of raw colostrum collected from their respective dams within 24 h of birth, these calves were not permitted the opportunity to be naturally nursed by their dams. In a previous study, calves that were naturally nursed by their dams or fostered by alien cows were twice as likely to be infected by MAP as herd mates fed milk replacer. [36]Additionally, while there is an increased risk of excreting MAP in colostrum associated with a positive MAP fecal excretion status of donor cows, [35] only 8% (5 of 60) of the cows in the present study were fecal culture positive for MAP. Therefore the possibility of calf exposure through

ingestion of raw colostrum containing MAP might have been minimal overall, although it is also possible that exposure could have been high but the method employed for detecting fecal excretion might have been less sensitive. Thus, some of the above factors may have compromised exposure and subsequent fecal excretion of MAP by study calves.

Lastly, it is possible that calves in the present study were exposed to MAP but that exposure to MAP in calfhood does not necessarily result in fecal excretion of MAP. Sporadic fecal excretion of MAP has been previously reported in calves under both natural farm exposure conditions [185] and in experimental studies.[24, 40, 187] It is therefore possible that the failure to detect MAP in feces of calves in this study may have arisen due to low or intermittent excretion of MAP by calves and the inability of the fecal sampling interval employed in the present study to capture samples containing MAP.

Notwithstanding the above explanations, findings of the present study suggest lack of evidence for detectable levels of MAP potentially excreted in feces collected from calves under natural farm exposure conditions. While it is possible that calves of this age (0-to-90 d) do not excrete MAP in their feces, this is contradictory to findings from earlier [24, 40, 185, 187] reports which suggest otherwise. Thus, in the face of conflicting findings such as the preceding ones, readers should be cautious while interpreting results of the present study as these results are inconclusive. Further investigations are warranted with respect to understanding MAP fecal excretion in calves and the implication for infection control and JD management. A study involving multiple and diverse herds that includes as an outcome of interest, subclinical MAP infection (to be determined through testing when the apparently exposed calves are $\geq$ 2 years of age) additional to potential fecal MAP excretion by such calves might assist in substantiating the evidence for presence of calves potentially excreting MAP in their feces following natural exposure in JD infected herds, and the potential role of such calves in calf-to-calf transmission of MAP.

General conclusions

The general goal of the studies presented in this thesis was to describe the efficacy of plasma-derived colostrum replacer (CR) feeding programs in preventing MAP transmission and the role of raw bovine maternal colostrum (MC) in the epidemiology of JD. Evidence for calf excretion of MAP following natural exposure was also evaluated.

In chapter 2, the relative risk of JD in calves fed CR (vs MC) was estimated. [154] Calves fed the CR product were significantly less likely to be infected by MAP compared with calves fed MC at birth implying that feeding MC is an early source of MAP for calves as previously suggested. [35, 36, 105, 153] The findings also implied that CR products are an effective tool for reducing the risk of transmission of MAP in calves that could be acquired from oral ingestion of MC after birth.[154] While it is thought that CR products might be useful for controlling other pathogens potentially transmissible to calves through ingestion of MC (e.g. *Escherichia coli*, *Salmonella* spp, *Mycoplasma* spp, and bovine leukemia virus), the efficacy of CR (vs MC) with respect to preventing the transmission of such pathogens has not been evaluated. Future research focusing on understanding the role that CR (vs MC) feeding programs might play in the control and management of these pathogens at the farm level is warranted. Additionally while findings of the present study (chapter 2) [154] provide evidence of the efficacy of plasma-derived CR (vs MC) products in preventing MAP transmission in calves, the efficacy of other colostrum replacement products (i.e. lacteal-derivatives) in preventing transmission of MAP in calves has not been validated despite its growing use on dairy farms. Future research focusing on determining the efficacies of plasma-derived vs lacteal-derived CR products vs raw MC feeding programs for the prevention of transmission of colostral pathogens in general is warranted.

Chapter 3 was a description of the effect of CR (vs MC) calf feeding programs on subsequent survival in the herd (longevity), milk yield, and breeding performance in the lactating adult cow during the first 54 months of life. Cows fed CR at birth were no different than cows fed MC with respect to overall risk of death, culling, death and/or culling (from birth to 54 months of follow-up and from first calving to 54 months of age,

respectively), lifetime milk yield, and breeding performance. However while no differences were observed between the two groups (CR vs MC) of cows with respect to adult performance and longevity outcomes and the fact that feeding adequate high quality colostrum remains a key element for calf health and future performance[150, 152, 153, 161] respectively, feeding raw MC from cows of unknown(or known) JD status or cows in JD endemic herds is also a significant risk factor for transmission of MAP in young calves.[35, 36, 105, 154] Therefore, feeding CR instead of MC may be one management tool to be used for decreasing the risk of MAP infection in heifer calves at birth as previously demonstrated (chapter 2),[154] while maintaining optimum productivity in the lactating adult cow. One advantage with feeding a CR product is that it allows the flexibility for potentially increasing the IgG mass fed (to approx ≥ 200 g/dose) to significantly reduce the high incidence of FPT that was observed in the group of cows fed CR at birth. [142, 155, 156] It is important to recognize that an obvious limitation to increasing the mass of IgG in CR would also increase cost of feeding an IgG fortified dose of the product. The long-term benefits and economic cost-benefit of this feeding management program requires a formal evaluation. The effect of plasma-derived vs lacteal-derived CR vs raw MC feeding programs on adult cow performance and longevity requires further evaluation to substantiate the observation made in the present study (chapter 3).

Detection of MAP present in colostrum and milk continues to be difficult using currently available tests. In chapter 4, the limits of detection and the ability of the nested ISMAP02 PCR to correctly discriminate between known MAP infected and uninfected colostrum samples were experimentally determined. The experimental validation of the nested ISMAP02 PCR assay was necessary for the interpretation of the data generated in chapters 5 and 6. The analytic sensitivity of the nested ISMAP02 PCR assay improved with increasing concentration of MAP in the colostrum sample replicates but specificity was compromised possibly due to contamination during sample preparations. Overall the findings suggested that the nested PCR protocol evaluated in this study was useful for the detection of the presence of MAP DNA in experimentally infected bovine colostrum. However, data on the efficacy of this test based on field colostrum samples continues to

be lacking. As such the adoption of this test for use in routine screening of field colostrum for MAP prior to feeding should not be under taken pending the completion of a formal field validation study involving perhaps testing colostrum samples collected from cows in a set of certified MAP negative herds for estimation of specificity, and colostrum collected from cows in a set of known MAP infected herds for estimation of sensitivity with the unit of analysis in each case being the colostrum sample donor cows. Further evaluations of tests for diagnosing MAP infections using milk and colostrum clinical specimens are also warranted.

The relative risk of MAP infection in calves that ingested MAP DNA positive colostrum (exposed) compared with calves that ingested MAP DNA negative (unexposed) raw colostrum after birth was estimated (chapter 5). The risk of MAP infection was not different between group (exposed vs unexposed) when the serum ELISA, bacterial fecal culture or both diagnostic tests were used as the basis for determining MAP infection status respectively suggesting the lack of an added risk of MAP infection associated with ingesting raw colostrum by Holstein calves. The preceding findings were in stark contrast to previous reports [35, 36, 105, 154] that seem to provide evidence in support of colostrum as a possible early vehicle by which calves get exposed to MAP in JD endemic herds underscoring the potential role of colostrum feeding programs in the epidemiology of JD in dairy herds. Given the limitations (discussed in chapter 5) and lack of corroborative data (since no studies had previously attempted to address similar questions using the study design employed in the present study), the results in chapter 5 need be treated as hypothesis only generating rather than conclusive findings. Future studies that address among other factors the limitations discussed (see chapter 5) are encouraged to determine whether the lack of association between feeding MAP positive colostrum and risk of JD infection is real.

The relationship between fecal shedding of MAP around calving time and detection of MAP in colostrum samples and on teat skin surfaces respectively was described (chapter 6). Overall, positive (vs negative) fecal excretion status was significantly associated with an increased risk of detecting MAP in colostrum and on teat

skin surfaces, respectively. The percentage of MAP transmission risk that could be eliminated if exposure through feeding colostrum from infected cows excreting MAP in their feces were to be avoided while nothing else changed was estimated at 18%. Similarly, the percentage of MAP transmission risk that could be eliminated if exposure through permitting calves to be nursed by infected cows excreting MAP in their feces were to be avoided while nothing else changed was estimated at 20%. Interestingly however, while the latter interventions might achieve some reduction in risk of exposure to MAP, they by no means eliminate the risk of exposing calves to MAP through ingestion of MAP present in colostrum or on bovine teats in JD endemic herds, given that a greater proportion of MAP detected in the colostrum samples and teat swabs had sources unrelated to the fecal shedding status of the donors with MAP present in the environment perhaps playing a greater role ($\geq$ 80%)in contaminating colostrum and teat skin. Though environmental management including manure waste management aimed at controlling the spread of MAP within and between dairy farms is commonly recommended, [27, 29-31, 33, 57] the significance of MAP present in the environment in the transmission of the disease remains unknown. As such studies designed to specifically understand the role of the environment in the survival and transmission dynamics of MAP should be pursued. It would also be much beneficial to substantiate using randomized controlled field based clinical trial studies, the efficacy of withholding colostrum and preventing natural nursing of MAP infected adults for preventing transmission of MAP in calves since the cross sectional design employed in chapter 6 does not demonstrate efficacies of the said interventions.

The objective of chapter 7 was to detect presence of calves excreting MAP in their feces following natural exposure. *Mycobacterium avium* subsp. *paratuberculosis* was not recovered from the fecal samples tested suggesting that the calves in the present study did not excrete detectable levels of MAP in feces. Although it is possible that calves of this age (0-to-90 d) do not excrete MAP in their feces, this observation was contradictory to findings from earlier [24, 40, 185, 187] reports which suggest otherwise. Therefore in the face of conflicting findings such as that in chapter 7, cautious

interpretation of the data is in order since the results presented are inconclusive. Further investigations are warranted with respect to understanding MAP fecal excretion in calves and the implication for infection control and JD management.

References

1. Thorel MF, Krichevsky M, Levy-Frebault VV. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. *nov*., *Mycobacterium avium* subsp. *paratuberculosis* subsp. *nov*., and *Mycobacterium avium* subsp. *silvaticum* subsp. *nov*. *Int J Syst Bacteriol* 1990; 40(3):254-260.

2. Wray C. Survival and Spread of Pathogenic Bacteria of Veterinary Importance within the Environment. *Vet Bull* 1975;45(8):543-550.

3. Larsen AB, Merkal RS, Vardaman TH. Survival time of *Mycobacterium paratuberculosis*. *Am J Vet Res* 1956; 17(64):549-551.

4. Jorgensen JB. Survival of *Mycobacterium paratuberculosis* in slurry. *Nord Vet Med* 1977; 29(6):267-270.

5. Hendrick S, Duffield T, Leslie K, Lissemore K, Archambault M, Kelton D. The prevalence of milk and serum antibodies to *Mycobacterium avium* subspecies *paratuberculosis* in dairy herds in Ontario. *Can Vet J* 2005; 46(12):1126-1129.

6. Salem M, Zeid AA, Hassan D, El-Sayed A, Zschoeck M. Studies on Johne's disease in Egyptian cattle. *J Vet Med B Infect Dis Vet Public Health* 2005;52(3):134-137.

7. Dreier S, Khol JL, Stein B, Fuchs K, Gutler S, Baumgartner W. Serological, bacteriological and molecular biological survey of paratuberculosis (Johne's disease) in Austrian cattle. *J Vet Med B Infect Dis Vet Public Health* 2006; 53(10):477-481.

8. Lee KW, Jung BY, Moon OK, Yang DK, Lee SH, Kim JY, Kweon CH. Seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Korean black goats (Capra hircus aegagrus). *J Vet Med Sci* 2006; 68(12):1379-1381.

9. Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW. Johne's disease in Canada Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. *Can Vet J* 2006; 47(9):874-882.

10. VanLeeuwen JA, Tiwari A, Plaizier JC, Whiting TL. Seroprevalences of antibodies against bovine leukemia virus, bovine viral diarrhea virus, *Mycobacterium avium* subspecies *paratuberculosis*, and *Neospora caninum* in beef and dairy cattle in Manitoba. *Can Vet J* 2006; 47(8):783-786.

11. Dieguez FJ, Arnaiz I, Sanjuan ML, Vilar MJ, Lopez M, Yus E. Prevalence of serum antibodies to *Mycobacterium avium* subsp. *paratuberculosis* in cattle in Galicia (northwest Spain). *Prev Vet Med* 2007; 82(3-4):321-326.

12. Kobayashi S, Tsutsui T, Yamamoto T, Nishiguchi A. Epidemiologic indicators associated with within-farm spread of Johne's disease in dairy farms in Japan. *J Vet Med Sci* 2007; 69(12):1255-1258.

13. Ristow P, Marassi CD, Rodrigues AB, Oelemann WM, Rocha F, Santos AS, Carvalho EC, Carvalho CB, Ferreira R, Fonseca LS, Lilenbaum W. Diagnosis of paratuberculosis in a dairy herd native to Brazil. *Vet J* 2007; 174(2):432-434.

14. Haghkhah M, Ansari-Lari M, Novin-Baheran AM, Bahramy A. Herd-level prevalence of *Mycobacterium avium* subspecies *paratuberculosis* by bulk-tank milk PCR in Fars province (southern Iran) dairy herds. *Prev Vet Med* 2008; 86(1-2):8-13.

15. Shahmoradi AH, Arefpajohi R, Tadayon K, Mosavari N. Paratuberculosis in Holstein-Friesian cattle farms in Central Iran. *Trop Anim Health Prod* 2008; 40(3):169-173.

16. Singh SV, Singh AV, Singh R, Sharma S, Shukla N, Misra S, Singh PK, Sohal JS, Kumar H, Patil PK, Misra P, Sandhu KS. Sero-prevalence of bovine Johne's disease in buffaloes and cattle population of North India using indigenous ELISA kit based on native *Mycobacterium avium* subspecies *paratuberculosis* 'Bison type' genotype of goat origin. *Comp Immunol Microbiol Infect Dis* 2008; 31(5):419-433.

17. Nielsen SS, Toft N. A review of prevalences of paratuberculosis in farmed animals in Europe. *Prev Vet Med* 2009; 88(1):1-14.

18. National Animal Health Monitoring System. Johne’s disease on U.S. Dairies, 1991–2007. Available at: http://nahms.aphis.usda.gov/dairy/index.htm#dairy2007. Accessed March, 11, 2009.

19. Adaska JM, Anderson RJ. Seroprevalence of Johne's-disease infection in dairy cattle in California, USA. *Prev Vet Med* 2003; 60(3):255-261.

20. Thorne JG, Hardin LE. Estimated prevalence of paratuberculosis in Missouri, USA cattle. *Prev Vet Med* 1997; 31(1-2):51-57.

21. Obasanjo IO, Grohn Y, Mohammed HO. Farm factors associated with the presence of *Mycobacterium paratuberculosis* infection in dairy herds on the New York State Paratuberculosis Control Program. *Prev Vet Med* 1997; 32(3-4):243-251.

22. Hirst HL, Garry FB, Morley PS, Salman MD, Dinsmore RP, Wagner BA, McSweeney KD, Goodell GM. Seroprevalence of *Mycobacterium avium* subsp *paratuberculosis* infection among dairy cows in Colorado and herd-level risk factors for seropositivity. *J Am Vet Med Assoc* 2004; 225(1):97-101.

23. Larsen AB, Merkal RS, Cutlip RC. Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis*. *Am J Vet Res* 1975; 36(3):255-257.

24. van Roermund HJ, Bakker D, Willemsen PT, de Jong MC. Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: calves can transmit the infection to other calves. *Vet Microbiol* 2007; 122(3-4):270-279.

25. Windsor PA, Whittington RJ. Evidence for age susceptibility of cattle to Johne's disease. *Vet J* 2009;In Press, Corrected Proof.

26. Larsen AB, Merkal RS, Cutlip RC. Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis*. *Am J Vet Res* 1975; 36(3):255-257.

27. Julian RJ. A short review and some observations on Johne's disease with recommendations for control. *Can Vet J* 1975; 16(2):33-43.

28. Whitlock RH, Hutchinson LT, Glickman LT, Meinersmann R, Rossiter C, Bruce J, Merkal R, Dick J. Paratuberculosis (Johnes's disease) update. *The Bovine Pract* 1986; 21:24-30.

29. Rossiter CA, Burhans WS. Farm-specific approach to paratuberculosis (Johne's disease) control. *Vet Clin North Am Food Anim Pract* 1996; 12(2):383-415.

30. Caldow GL. The prospects for herd level control of paratuberculosis in cattle: a consultant's view, in *Proceedings. 8th International Colloquium on Paratuberculosis.* 2005; 165-173.

31. Wells SJ. The prospects for herd level control of paratuberculosis- A scientists view, in *Proceedings. 8th International Colloquium on Paratuberculosis.* 2005; 174-181.

32. Jubb TF, Galvin JW. Effect of a test and control program for bovine Johne's disease in Victorian dairy herds 1992 - 2002. *Aust Vet J* 2004; 82(4):228-232.

33. Johnson-Ifearulundu YJ, Kaneene JB. Epidemiology and economic impact of subclinical Johne's disease: a review. *Veterinary Bulletin* 1997; 67(6):437-447.

34. Sweeney RW, Whitlock RH, Rosenberger AE. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *J Clin Microbiol* 1992; 30(1):166-171.

35. Streeter RN, Hoffsis GF, Bech-Nielsen S, Shulaw WP, Rings DM. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. *Am J Vet Res* 1995; 56(10):1322-1324.

36. Nielsen SS, Bjerre H, Toft N. Colostrum and milk as risk factors for infection with *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle. *J Dairy Sci* 2008; 91(12):4610-4615.

37. Sweeney RW. Transmission of Paratuberculosis. *Vet Clin North Am Food Anim Pract* 1996; 12(2):305-312.

38. Wells SJ, Wagner BA. 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 that herd and use of preventive measures. *J Am Vet Med Assoc* 2000; 216(9):1450-1457.

39. Raizman EA, Wells SJ, Godden SM, Bey RF, Oakes MJ, Bentley DC, Olsen KE. The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. *J Dairy Sci* 2004; 87(9):2959-2966.

40. van Roermund HJW, de Jong MCM. Horizontal transmission experiment of paratuberculosis, in *Proceedings. 8th International Colloquium on Paratuberculosis.* 2005; 672-678.

41. Weber MF, Kogut J, de Bree J, van Schaik G. Evidence of *Mycobacterium avium* subsp. *paratuberculosis* shedding in young cattle, in *Proceedings. 8th International Colloquium on Paratuberculosis.* 2005; 679-689.

42. Raizman EA, Wells SJ, Godden SM, Bey RF, Oakes MJ, Bentley DC, Olsen KE. The distribution of *Mycobacterium avium* ssp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. *J Dairy Sci* 2004; 87(9):2959-2966.

43. McQueen DS, Russell EG. Culture of *Mycobacterium paratuberculosis* from bovine foetuses. *Aust Vet J* 1979; 55(4):203-204.

44. Seitz SE, Heider LE, Heuston WD, Bech-Nielsen S, Rings DM, Spangler L. Bovine fetal infection with *Mycobacterium paratuberculosis*. *J Am Vet Med Assoc* 1989; 194(10):1423-1426.

45. Sweeney RW, Whitlock RH, Rosenberger AE. *Mycobacterium paratuberculosis* isolated from fetuses of infected cows not manifesting signs of the disease. *Am J Vet Res* 1992; 53(4):477-480.

46. Whittington RJ, Windsor PA. In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: a critical review and meta-analysis. *Vet J* 2009;179(1):60-69.

47. Larsen AB, Stalheim OH, Hughes DE, Appell LH, Richards WD, Himes EM. *Mycobacterium paratuberculosis* in the semen and genital organs of a semen-donor bull. *J Am Vet Med Assoc* 1981; 179(2):169-171.

48. Ayele WY, Bartos M, Svastova P, Pavlik I. Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in organs of naturally infected bull-calves and breeding bulls. *Vet Microbiol* 2004; 103(3-4):209-217.

49. Tiwari A, Vanleeuwen JA, Dohoo IR, Keefe GP, Haddad JP, Scott HM, Whiting T. Risk factors associated with *Mycobacterium avium* subspecies *paratuberculosis* seropositivity in Canadian dairy cows and herds. *Prev Vet Med* 2009; 88(1):32-41.

50. Sockett DC, Conrad TA, Thomas CB, Collins MT. Evaluation of four serological tests for bovine paratuberculosis. *J Clin Microbiol* 1992; 30(5):1134-1139.

51. Collins MT. Diagnosis of paratuberculosis. *Vet Clin North Am Food Anim Pract* 1996; 12(2):357-371.

52. Dargatz DA, Byrum BA, Barber LK, Sweeney RW, Whitlock RH, Shulaw WP, Jacobsen RH, Stabel JR. Evaluation of a commercial ELISA for diagnosis of paratuberculosis in cattle. *J Am Vet Med Assoc* 2001; 218(7):1163-1166.

53. Nielsen SS, Toft N. Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. *Vet Microbiol* 2008; 129(3-4):217-235.

54. Collins MT, Sockett DC, Goodger WJ, Conrad TA, Thomas CB, Carr DJ. Herd prevalence and geographic distribution of, and risk factors for, bovine paratuberculosis in Wisconsin. *J Am Vet Med Assoc* 1994;204(4):636-641.

55. Goodger WJ, Collins MT, Nordlund KV, Eisele C, Pelletier J, Thomas CB, Sockett DC. Epidemiologic study of on-farm management practices associated with prevalence of *Mycobacterium paratuberculosis* infections in dairy cattle. *J Am Vet Med Assoc* 1996; 208(11):1877-1881.

56. Cetinkaya B, Erdogan HM, Morgan KL. Relationships between the presence of Johne's disease and farm and management factors in dairy cattle in England. *Prev Vet Med* 1997; 32(3-4):253-266.

57. Johnson-Ifearulundu YJ, Kaneene JB. Management-related risk factors for *M. paratuberculosis* infection in Michigan, USA, dairy herds. *Prev Vet Med* 1998; 37(1-4):41-54.

58. Johnson-Ifearulundu Y, Kaneene JB. Distribution and environmental risk factors for paratuberculosis in dairy cattle herds in Michigan. *Am J Vet Res* 1999; 60(5):589-596.

59. Muskens J, Elbers AR, van Weering HJ, Noordhuizen JP. Herd management practices associated with paratuberculosis seroprevalence in Dutch dairy herds. *J Vet Med B Infect Dis Vet Public Health* 2003; 50(8):372-377.

60. Jakobsen MB, Alban L, Nielsen SS. A cross-sectional study of paratuberculosis in 1155 Danish dairy cows. *Prev Vet Med* 2000; 46(1):15-27.

61. Woodbine KA, Schukken YH, Green LE, Ramirez-Villaescusa A, Mason S, Moore SJ, Bilbao C, Swann N, Medley GF. Seroprevalence and epidemiological characteristics of *Mycobacterium avium* subsp. *paratuberculosis* on 114 cattle farms in south west England. *Prev Vet Med* 2009; In Press, Corrected Proof.

62. Whitlock RH, Buergelt C. Preclinical and Clinical Manifestation of Paratuberculosis (Including Pathology). *Vet Clin North Am Food Anim Pract* 1996; 12(2):345-356.

63. Nielsen SS, Enevoldsen C, Grohn YT. The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation. *Prev Vet Med* 2002; 54(1):1-10.

64. Aly SS, Thurmond MC. Evaluation of *Mycobacterium avium* subsp *paratuberculosis* infection of dairy cows attributable to infection status of the dam. *J Am Vet Med Assoc* 2005; 227(3):450-454.

65. Whittington RJ, Marshall DJ, Nicholls PJ, Marsh IB, Reddacliff LA. Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. *Appl Environ Microbiol* 2004; 70(5):2989-3004.

66. Reviriego FJ, Moreno MA, Dominguez L. Soil type as a putative risk factor of ovine and caprine paratuberculosis seropositivity in Spain. *Prev Vet Med* 2000;43(1):43-51.

67. Ward MP, Perez AM. Association between soil type and paratuberculosis in cattle herds. *Am J Vet Res* 2004; 65(1):10-14.

68. Dhand NK, Eppleston J, Whittington RJ, Toribio JLML. Association of farm soil characteristics with ovine Johne's disease in Australia. *Prev Vet Med* 2009; 89(1-2):110-120.

69. Collins MT, Gardner IA, Garry FB, Roussel AJ, Wells SJ. Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. *J Am Vet Med Assoc* 2006; 229(12):1912-1919.

70. Whitlock RH, Wells SJ, Sweeney RW, Van Tiem J. ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. *Vet Microbiol* 2000; 77(3-4):387-398.

71. Wells SJ, Whitlock RH, Lindeman CJ, Fyock T. Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis*. *Am J Vet Res* 2002; 63(8):1207-1211.

72. Wells SJ, Whitlock RH, Wagner BA, Collins J, Garry F, Hirst H, Lawrence J, Saville WJ, Naugle AL. Sensitivity of test strategies used in the Voluntary Johne's Disease Herd Status Program for detection of *Mycobacterium paratuberculosis* infection in dairy cattle herds. *J Am Vet Med Assoc* 2002; 220(7):1053-1057.

73. Wells SJ, Godden SM, Lindeman CJ, Collins JE. Evaluation of bacteriologic culture of individual and pooled fecal samples for detection of *Mycobacterium paratuberculosis* in dairy cattle herds. *J Am Vet Med Assoc* 2003; 223(7):1022-1025.

74. Tavornpanich S, Gardner IA, Anderson RJ, Shin S, Whitlock RH, Fyock T, Adaska JM, Walker RL, Hietala SK. Evaluation of microbial culture of pooled fecal samples for detection of *Mycobacterium avium* subsp *paratuberculosis* in large dairy herds. *Am J Vet Res* 2004; 65(8):1061-1070.

75. Wells SJ, Collins MT, Faaberg KS, Wees C, Tavornpanich S, Petrini KR, Collins JE, Cernicchiaro N, Whitlock RH. Evaluation of a rapid fecal PCR test for detection of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. *Clin Vaccine Immunol* 2006; 13(10):1125-1130.

76. Sockett DC, Carr DJ, Collins MT. Evaluation of conventional and radiometric fecal culture and a commercial DNA probe for diagnosis of *Mycobacterium paratuberculosis* infections in cattle. *Can J Vet Res* 1992; 56(2):148-153.

77. McKenna SL, Keefe GP, Barkema HW, Sockett DC. Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. *Vet Microbiol* 2005; 110(1-2):105-111.

78. Hendrick SH, Duffield TE, Kelton DE, Leslie KE, Lissemore KD, Archambault M. Evaluation of enzyme-linked immunosorbent assays performed on milk and serum samples for detection of paratuberculosis in lactating dairy cows. *J Am Vet Med Assoc* 2005; 226(3):424-428.

79. Whipple DL, Kapke PA, Andersen PR. Comparison of a commercial DNA probe test and three cultivation procedures for detection of *Mycobacterium paratuberculosis* in bovine feces. *J Vet Diagn Invest* 1992; 4(1):23-27.

80. Thoen CO, Haagsma J. Molecular techniques in the diagnosis and control of paratuberculosis in cattle. *J Am Vet Med Assoc* 1996; 209(4):734-737.

81. Stabel JR, Bannantine JP. Development of a nested PCR method targeting a unique multicopy element, ISMap02, for detection of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples. *J Clin Microbiol* 2005; 43(9):4744-4750.

82. Clark DL, Koziczkowski JJ, Radcliff RP, Carlson RA, Ellingson JL. Detection of *Mycobacterium avium* subspecies *paratuberculosis*: comparing fecal culture versus

serum enzyme-linked immunosorbent assay and direct fecal polymerase chain reaction. *J Dairy Sci* 2008; 91(7):2620-2627.

83. Nielsen SS, Grønbæk C, Agger JF, Houe H. Maximum-likelihood estimation of sensitivity and specificity of ELISAs and faecal culture for diagnosis of paratuberculosis. *Prev Vet Med* 2002; 53(3):191-204.

84. Sweeney RW, Whitlock RH, Hamir AN, Rosenberger AE, Herr SA. Isolation of *Mycobacterium paratuberculosis* after oral inoculation in uninfected cattle. *Am J Vet Res* 1992; 53(8):1312-1314.

85. Sweeney RW, Whitlock RH, Buckley CL, Spencer P, Rosenberger AE, Hutchinson LJ. Diagnosis of paratuberculosis in dairy cattle, using enzyme-linked immunosorbent assay for detection of antibodies against *Mycobacterium paratuberculosis* in milk. *Am J Vet Res* 1994; 55(7):905-909.

86. Sweeney RW, Whitlock RH, Buckley CL, Spencer PA. Evaluation of a commercial enzyme-linked immunosorbent assay for the diagnosis of paratuberculosis in dairy cattle. *J Vet Diagn Invest* 1995; 7(4):488-493.

87. Osterstock JB, Fosgate GT, Norby B, Manning EJ, Collins MT, Roussel AJ. Contribution of environmental mycobacteria to false-positive serum ELISA results for paratuberculosis. *J Am Vet Med Assoc* 2007; 230(6):896-901.

88. McFadden JJ, Butcher PD, Chiodini R, Hermon-Taylor J. Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. *J Clin Microbiol* 1987; 25(5):796-801.

89. Green EP, Tizard ML, Moss MT, Thompson J, Winterbourne DJ, McFadden JJ, Hermon-Taylor J. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Res* 1989; 17(22):9063-9073.

90. Paustian ML, Amonsin A, Kapur V, Bannantine JP. Characterization of novel coding sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*: implications for diagnosis of Johne's Disease. *J Clin Microbiol* 2004;42(6):2675-2681.

91. Li L, Bannantine JP, Zhang Q, Amonsin A, May BJ, Alt D, Banerji N, Kanjilal S, Kapur V. The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proc Natl Acad Sci USA* 2005; 102(35):12344-12349.

92. Vary PH, Andersen PR, Green E, Hermon-Taylor J, McFadden JJ. Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. *J Clin Microbiol* 1990; 28(5):933-937.

93. Benedictus G, Dijkhuizen AA, Stelwagen J. Economic losses due to paratuberculosis in dairy cattle. *Vet Rec* 1987; 121(7):142-146.

94. Kormendy B, Kopal T, Balint T, Szilagyi M, Beki L. Economic losses caused by paratuberculosis in a dairy herd: case report. *Acta Vet Hung* 1989; 37(1-2):45-53.

95. Johnson-Ifearulundu Y, Kaneene JB, Lloyd JW. Herd-level economic analysis of the impact of paratuberculosis on dairy herds. *J Am Vet Med Assoc* 1999; 214(6):822-825.

96. Ott SL, Wells SJ, Wagner BA. Herd-level economic losses associated with Johne's disease on US dairy operations. *Prev Vet Med* 1999; 40(3-4):179-192.

97. Raizman EA, Fetrow J, Wells SJ, Godden SM, Oakes MJ, Vazquez G. The association between *Mycobacterium avium* subsp. *paratuberculosis* fecal shedding or clinical Johne's disease and lactation performance on two Minnesota, USA dairy farms. *Prev Vet Med* 2007; 78(3-4):179-195.

98. Raizman EA, Wells SJ, Godden SM, Fetrow J, Oakes JM. 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* 2007; 80(2-3):166-178.

99. Gonda MG, Chang YM, Shook GE, Collins MT, Kirkpatrick BW. Effect of *Mycobacterium paratuberculosis* infection on production, reproduction, and health traits in US Holsteins. *Prev Vet Med* 2007; 80(2-3):103-119.

100. Groenendaal H, Wolf CA. Farm-level economic analysis of the US National Johne's Disease Demonstration Herd Project. *J Am Vet Med Assoc* 2008; 233(12):1852-1858.

101. Johnson-Ifearulundu YJ, Kaneene JB. Relationship between soil type and *Mycobacterium paratuberculosis*. *J Am Vet Med Assoc* 1997; 210(12):1735-1740.

102. Ifearulundu YJ, Kaneene JB. Distribution and Environmental Risk Factors for Paratuberculosis in Dairy Cattle Herds in Michigan. *Am J Vet Res* 1999; 60(5):589-596.

103. Chi J, VanLeeuwen JA, Weersink A, Keefe GP. Management factors related to seroprevalences to bovine viral-diarrhoea virus, bovine-leukosis virus, *Mycobacterium avium* subspecies *paratuberculosis*, and Neospora caninum in dairy herds in the Canadian Maritimes. *Prev Vet Med* 2002; 55(1):57-68.

104. Fredriksen B, Djonne B, Sigurdardottir O, Tharaldsen J, Nyberg O, Jarp J. Factors affecting the herd level of antibodies against *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle. *Vet Rec* 2004; 154(17):522-526.

105. Dieguez FJ, Arnaiz I, Sanjuan ML, Vilar MJ, Yus E. Management practices associated with *Mycobacterium avium* subspecies *paratuberculosis* infection and the effects of the infection on dairy herds. *Vet Rec* 2008; 162(19):614-617.

106. Wells SJ, Hartmann WL, Anderson PL. Evaluation of progress made by dairy and beef herds enrolled in the Minnesota Johne's Disease Control Program. *J Am Vet Med Assoc* 2008; 233(12):1920-1926.

107. Ferrouillet C, Wells SJ, Hartmann WL, Godden SM, Carrier J. Decrease of Johne's disease prevalence and incidence in six Minnesota, USA, dairy cattle herds on a long-term management program. *Prev Vet Med* 2009; 88(2):128-137.

108. Whitlock RH, Wells SJ, Sweeney RW, Van Tiem J. ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. *Vet Microbiol* 2000; 77(3-4):387-398.

109. Nielsen SS, Grohn YT, Enevoldsen C. Variation of the milk antibody response to paratuberculosis in naturally infected dairy cows. *J Dairy Sci* 2002; 85(11):2795-2802.

110. Nielsen SS. Transitions in diagnostic tests used for detection of *Mycobacterium avium* subsp. *paratuberculosis* infections in cattle. *Vet Microbiol* 2008; 132(3-4):274-282.

111. Godfroid J, Boelaert F, Heier A, Clavareau C, Wellemans V, Desmecht M, Roels S, Walravens K. First evidence of Johne's disease in farmed red deer (*Cervus elaphus*) in Belgium. *Vet Microbiol* 2000; 77(3-4):283-290.

112. Daniels MJ, Ball N, Hutchings MR, Greig A. The Grazing Response of Cattle to Pasture Contaminated with Rabbit Faeces and the Implications for the Transmission of Paratuberculosis. *Vet J* 2001; 161(3):306-313.

113. Greig A., Beard P., Daniels M. J., Henderson D., Hutchings M. R., Stevenson K. 84. The potential role of wildlife in the epidemiology of paratuberculosis in domestic animals. *Res Vet Sci* 2002; 72(Supplement 1):30-30.

114. Hutchings M. R., Daniels M. J., Henderson D., Hueig A. 83. Wildlife to ruminant transmission routes for *M.a.paratuberculosis*. *Res Vet Sci* 2002; 72(Supplement 1):30-30.

115. Daniels MJ, Lees JD, Hutchings MR, Greig A. The Ranging Behaviour and Habitat Use of Rabbits on Farmland and Their Potential Role in the Epidemiology of Paratuberculosis. *The Veterinary Journal* 2003; 165(3):248-257.

116. Judge J, Greig A, Kyriazakis I, Hutchings MR. Ingestion of faeces by grazing herbivores--risk of inter-species disease transmission. *Agric , Ecosyst Environ* 2005; 107(2-3):267-274.

117. Raizman EA, Wells SJ, Jordan PA, DelGiudice GD, Bey RR. *Mycobacterium avium* subsp. *paratuberculosis* from free-ranging deer and rabbits surrounding Minnesota dairy herds. *Can J Vet Res* 2005; 69(1):32-38.

118. Judge J, Greig A, Kyriazakis I, Hutchings MR. Risk of inter-species *Mycobacterium avium* subspecies *paratuberculosis* transmission from rabbits to livestock via the fecal-oral route, in *Proceedings. 8th International Colloquium on Paratuberculosis.* 2005;632-637.

119. Lovel R, Levi M, Francis J. Studies on the survival of Johne's Bacilli. *J Comp Pathol* 1944; 54:120-129.

120. Jorgensen JB. Survival of *Mycobacterium paratuberculosis* in slurry. *Nord Vet Med* 1977; 29(6):267-270.

121. Kopecky KE. Distribution of paratuberculosis in Wisconsin, by soil regions. *J Am Vet Med Assoc* 1977; 170(3):320-324.

122. Groenendaal H, Nielsen M, Jalvingh AW, Horst SH, Galligan DT, Hesselink JW. A simulation of Johne's disease control. *Prev Vet Med* 2002; 54(3):225-245.

123. Groenendaal H, Galligan DT. Economic consequences of control programs for paratuberculosis in midsize dairy farms in the United States. *J Am Vet Med Assoc* 2003; 223(12):1757-1763.

124. Groenendaal H, Nielsen M, Hesselink JW. Development of the Dutch Johne's disease control program supported by a simulation model. *Prev Vet Med* 2003; 60(1):69-90.

125. St Jean G. Treatment of clinical paratuberculosis in cattle. *Vet Clin North Am Food Anim Pract* 1996; 12(2):417-430.

126. Brumbaugh GW, Edwards JF, Roussel AJ, Thomson TD. Effect of monensin sodium on histological lesions of naturally occurring bovine paratuberculosis. *J Comp Pathol* 2000; 123(1):22-28.

127. Whitlock RH, Sweeney RW, Fyock TL, McAdams S, Gardner IA, McClary DG. Johne's disease: The effect of feeding Monensin to reduce the bio-burden of *Mycobacterium avium* subspecies *paratuberculosis* in neo-natal calves, in *Proceedings. 8th International Colloquium on Paratuberculosis.* 2005; 270.

128. Hendrick SH, Duffield TF, Leslie KE, Lissemore KD, Archambault M, Bagg R, Dick P, Kelton DF. Monensin might protect Ontario, Canada dairy cows from paratuberculosis milk-ELISA positivity. *Prev Vet Med* 2006; 76(3-4):237-248.

129. Hendrick SH, Kelton DF, Leslie KE, Lissemore KD, Archambault M, Bagg R, Dick P, Duffield TF. Efficacy of monensin sodium for the reduction of fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in infected dairy cattle. *Prev Vet Med* 2006; 75(3-4):206-220.

130. Hendrick SH, Duffield TF, Leslie KE, Lissemore KD, Archambault M, Bagg R, Dick P, Kelton DF. Monensin might protect Ontario, Canada dairy cows from paratuberculosis milk-ELISA positivity. *Prev Vet Med* 2006; 76(3-4):237-248.

131. Hendrick SH, Kelton DF, Leslie KE, Lissemore KD, Archambault M, Bagg R, Dick P, Duffield TF. Efficacy of monensin sodium for the reduction of fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in infected dairy cattle. *Prev Vet Med* 2006; 75(3-4):206-220.

132. Lu Z, Mitchell RM, Smith RL, Van Kessel JS, Chapagain PP, Schukken YH, Grohn YT. The importance of culling in Johne's disease control. *J Theor Biol* 2008; 254(1):135-146.

133. Munoz M, Garcia Marin JF, Garcia-Pariente C, Reyes LE, Verna A, Moreno O, Fuertes M, Doce J, Puentes E, Garrido J, Perez V. Efficacy of a killed vaccine (SILIRUM) in calves challenged with MAP, in *Proceedings. 8th International Colloquium on Paratuberculosis.* 2005; 208-217.

134. Garcia-Pariente C, Perez V, Geijo MV, Moreno O, Munoz M, Fuertes M, Puentes E, Doce J, Ferreras MC, Garcia Marin JF. Efficacy of a killed vaccine against paratuberculosis (SILIRUM) in cattle. A field study, in *Proceedings. 8th International Colloquium on Paratuberculosis.* 2005; 260.

135. Sweeney RW, Whitlock RH, Bowersock TL, Cleary DL, Meinert TR, Habecker PL, Pruitt GW. Effect of subcutaneous administration of a killed *Mycobacterium avium* subsp *paratuberculosis* vaccine on colonization of tissues following oral exposure to the organism in calves. *Am J Vet Res* 2009; 70(4):493-497.

136. Giese SB, Ahrens P. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. *Vet Microbiol* 2000; 77(3-4):291-297.

137. Quigley JD, Strohbehn RE, Kost CJ, O'Brien MM. Formulation of colostrum supplements, colostrum replacers and acquisition of passive immunity in neonatal calves. *J Dairy Sci* 2001; 84(9):2059-2065.

138. Quigley JD, Kost CJ, Wolfe TA. Absorption of IgG in calves fed colostrum replacer products derived from bovine
serum. In *Proceedings. 7th International Colloquium on Paratuberculosis.* 2002; 93.

139. Godden SM, Smith S, Feirtag JM, Green LR, Wells SJ, Fetrow JP. Effect of on-farm commercial batch pasteurization of colostrum on colostrum and serum immunoglobulin concentrations in dairy calves. *J Dairy Sci* 2003; 86(4):1503-1512.

140. Godden S, McMartin S, Feirtag J, Stabel J, Bey R, Goyal S, Metzger L, Fetrow J, Wells S, Chester-Jones H. Heat-treatment of bovine colostrum II: Effects of heating duration on pathogen viability and immunoglobulin G. *J Dairy Sci* 2006; 89:3476-3483.

141. Johnson J, Godden S, Molitor T, Ames T, Hagman D. The effect of feeding heat treated colostrum on passive transfer of immune and nutritional parameters in dairy calves. *J Dairy Sci* 2007; 90:5189-5198.

142. Swan H, Godden S, Bey R, Wells S, Fetrow J, Chester-Jones H. Passive transfer of immunoglobulin G and preweaning health in Holstein calves fed a commercial colostrum replacer. *J Dairy Sci* 2007; 90(8):3857-3866.

143. Dohoo I, Martin W, Stryhn H. *Veterinary Epidemiologic Research.* Prince Edward Island: AVC, 2003.

144. Gutierrez GR. Parametric frailty and shared frailty survival models. *The Stata J* 2002; 2(1):22-44.

145. Cleves AC, Gould WW, Gutierrez RG, eds. *An Introduction to Survival Analysis Using Stata.* Revised edition ed. Stata Corporation 4905 Lakeway Drive, College Station, Texas 77845: Stata press, 2004.

146. NASS. Quick Stats[database online] Washington, DC: National Agricultural Statistics Services,2008. Available at: www.nass.usda. gov/Data_and_Statistics/Quick_Stats/. Accessed Sep/6, 2008.

147. Schukken YH, Grohn YT, McDermott B, McDermott JJ. Analysis of correlated discrete observations: background, examples and solutions. *Prev Vet Med* 2003; 59(4):223-240.

148. McDermott JJ, Schukken YH, Shoukri MM. Study design and analytic methods for data collected from clusters of animals. *Prev Vet Med* 1994; 18(3):175-191.

149. McMartin S, Godden S, Metzger L, Feirtag J, Bey R, Stabel J, Goyal S, Fetrow J, Wells S, Chester-Jones H. Heat treatment of bovine colostrum. I: effects of temperature on viscosity and immunoglobulin G level. *J Dairy Sci* 2006; 89(6):2110-2118.

150. Robison JD, Stott GH, DeNise SK. Effects of passive immunity on growth and survival in the dairy heifer. *J Dairy Sci* 1988; 71(5):1283-1287.

151. Faber SN, Faber NE, McCauley TC, Ax RL. Case study: Effects of colostrum ingestion on lactational performance. *Professional animal scientist* 2005;1(5):420-425.

152. Davis CL, Drackley JK. *The Development, Nutrition, and Management of the Young Calf.* Ames, Iowa: Iowa State University Press, 1998.

153. Godden S. Colostrum Management for Dairy Calves. *Veterinary Clinics of North America: Food Animal Practice* 2008; 24(1):19-39.

154. Pithua P, Godden SM, Wells SJ, Oakes MJ. Efficacy of feeding plasma-derived commercial colostrum replacer for the prevention of transmission of *Mycobacterium avium* subsp *paratuberculosis* in Holstein calves. *J Am Vet Med Assoc* 2009; 234(9):1167-1176.

155. Foster DM, Smith GW, Sanner TR, Busso GV. Serum IgG and total protein concentrations in dairy calves fed two colostrum replacement products. *J Am Vet Med Assoc* 2006; 229(8):1282-1285.

156. Godden SM, Haines DM, Hagman D. Improving passive transfer of immunoglobulins in calves. I: dose effect of feeding a commercial colostrum replacer. *J Dairy Sci* 2009; 92(4):1750-1757.

157. Cox DR. Regression Models and Life-Tables. *Journal of the Royal Statistical Society. Series B (Methodological)* 1972; 34(2):187-220.

158. Lin DY, Wei LJ. The Robust Inference for the Cox Proportional Hazards Model. *Journal of the American Statistical Association* 1989; 84(408):1074-1078.

159. Hosmer DW, Lameshow S, May S. *Applied survival analysis: regression modeling of time-to-event data.* 2nd ed. New Jersey: Wiley and Sons, 2008.

160. Kirkwood BR, Stern JAC. *Essential Medical Statistics.* 2nd ed. Massachusetts: Blackwell Science, 2003.

161. DeNise SK, Robison JD, Stott GH, Armstrong DV. Effects of Passive Immunity on Subsequent Production in Dairy Heifers. *J Dairy Sci* 1989; 72(2):552-554.

162. Lombard JE, Garry FB, McCluskey BJ, Wagner BA. Risk of removal and effects on milk production associated with paratuberculosis status in dairy cows. *J Am Vet Med Assoc* 2005; 227(12):1975-1981.

163. Raizman EA, Wells SJ, Godden SM, Fetrow J, Oakes JM. 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* 2007; 80(2-3):166-178.

164. Johnson-Ifearulundu YJ, Kaneene JB, Sprecher DJ, Gardiner JC, Lloyd JW. The effects of subclinical *Mycobacterium paratuberculosis* infection on days open in Michigan, USA, dairy cows. *Prev Vet Med* 2000; 46(3):171-181.

165. Hultgren J, Svensson C. Heifer rearing conditions affect length of productive life in Swedish dairy cows. *Prev Vet Med* 2009; In Press, Corrected Proof.

166. Hosmer DW, Lameshow S. *Applied logistic regression.* 2nd ed. New York: Wiley and Sons, 2000.

167. Warnick LD, Erb HN, White ME. Lack of Association between Calf Morbidity and Subsequent First Lactation Milk Production in 25 New York Holstein Herds. *J Dairy Sci* 1995; 78(12):2819-2830.

168. Warnick LD, Erb HN, White ME. The relationship of calfhood morbidity with survival after calving in 25 New York Holstein herds. *Prev Vet Med* 1997; 31(3-4):263-273.

169. Correa MT, Curtis CR, Erb HN, White ME. Effect of calflhood morbidity on age at first calving in New York Holstein herds. *Prev Vet Med* 1988; 6(4):253-262.

170. Erb HN. The long-term effects of calfhood diseases. *Proceedings. 56th meeting of the Cornell Nutrition Conference for Feed Manufacturers* 1994:143-146.

171. Erb HN, Warnick LD, White ME. Long-term effects of calfhood diseases. *In Proceedings. New Zealand Veterinary Association and Australian Veterinary Association Pan Pacific Conference.* 1996289-301.

172. Merkal RS. Paratuberculosis: prevalence, diagnosis, prevention, and treatment. in *Proceedings. 17th Annual Convention-American Association of Bovine Practitioners.* 1985; 64-66.

173. Stabel JR, Palmer MV, Harris B, Plattner B, Hostetter J, Robbe-Austerman S. Pathogenesis of *Mycobacterium avium* subsp. *paratuberculosis* in neonatal calves after oral or intraperitoneal experimental infection. *Vet Microbiol* 2009; In Press, Corrected Proof.

174. Fykse EM, Olsen JS, Skogan G. Application of sonication to release DNA from Bacillus cereus for quantitative detection by real-time PCR. *J Microbiol Methods* 2003; 55(1):1-10.

175. Schleig PM, Buergelt CD, Davis JK, Williams E, Monif GR, Davidson MK. Attachment of *Mycobacterium avium* subspecies *paratuberculosis* to bovine intestinal organ cultures: method development and strain differences. *Vet Microbiol* 2005; 108(3-4):271-279.

176. Coussens PM. *Mycobacterium paratuberculosis* and the bovine immune system. *Anim Health Res Rev* 2001; 2(2):141-161.

177. Zurbrick BG, Czuprynski CJ. Ingestion and intracellular growth of *Mycobacterium paratuberculosis* within bovine blood monocytes and monocyte-derived macrophages. *Infect Immun* 1987; 55(7):1588-1593.

178. McDonald JS, Anderson AJ. Total and differential somatic cell counts in secretions from noninfected bovine mammary glands: the early nonlactating period. *Am J Vet Res* 1981; 42(8):1360-1365.

179. McDonald JS, Anderson AJ. Total and differential somatic cell counts in secretions from noninfected bovine mammary glands: the peripartum period. *Am J Vet Res* 1981; 42(8):1366-1368.

180. Experimental validation of a nested PCR targeting the genetic element ISMAP02 for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine colostrum. Unpublished.

181. Pithua P, Wells SJ, Godden SM, Raizman EA. Clinical trial on type of calving pen and the risk of disease in Holstein calves during the first 90 d of life. *Prev Vet Med* 2009; 89:8-15.

182. National Agricultural Statistics Services. Farms, Land in Farms, and Livestock Operations: 2003-2004. Available at: http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1259. Accessed February/22, 2009.

183. Rockhill B, Newman B, Weinberg C. Use and misuse of population attributable fractions. *Am J Public Health* 1998; 88(1):15-19.

184. Stewart S, Godden S, Bey R, Rapnicki P, Fetrow J, Farnsworth R, Scanlon M, Arnold Y, Clow L, Mueller K, Ferrouillet C. Preventing Bacterial Contamination and Proliferation During the Harvest, Storage, and Feeding of Fresh Bovine Colostrum. *J Dairy Sci* 2005; 88(7):2571-2578.

185. Bolton MW, Grooms DL, Kaneene JB. Fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in calves: implication for infection control and management, in *Proceedings. 8th International Colloquium on Paratuberculosis.* 2005; 596-600.

186. Chiodini RJ, Van Kruiningen HJ, Merkal RS. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Vet* 1984; 74(3):218-262.

187. McDonald WL, Ridge SE, Hope AF, Condron RJ. Evaluation of diagnostic tests for Johne's disease in young cattle. *Aust Vet J* 1999; 77(2):113-119.

AVMA American Veterinary Medical Association

Diane A Fagen,
Librarian
1931 N. Meacham Rd.
Schaumburg, IL
60173-4360

phone 847.925.8070
fax 847.925.9329
dfagen@avma.org
www.avma.org

This email is to request a letter authorizing the use of a material published in the May 01, 2009 issue of the *Journal of the American Veterinary Medical Association* as a chapter in my PhD thesis. The following specifications apply:

Patrick Pithua
College of Veterinary Medicine, University of Minnesota
Dept of Veterinary Population Medicine, University of Minnesota,
136 Andrew Boss Laboratory,
St. Paul, MN 55108

Material to republish:
Publication: *Journal of the American Veterinary Medical Association*
Title: Efficacy of feeding plasma-derived commercial colostrum replacer for the prevention of transmission of *Mycobacterium avium* subsp *paratuberculosis* in Holstein calves
Page No: 1167-1176
Figures: 1 and 2
Tables: 1, 2 and 3
Other content: Abstract
Please see attached copy

Intended use:
PhD Thesis title: Efficacy of interventions role of colostrum in the transmission of *Mycobacterium avium* subsp *paratuberculosis* **Author:** Patrick Pithua
Publisher: UMI® Dissertation publishing: **www.il.proquest.com** **Publication year:** 2009
Language: English **Form of reproduction:** Print and electronic

Permission granted provided the *Journal of the American Veterinary Medical Association* is clearly credited and the material is used for the stated purpose only. Permission includes authorization to Proquest to reproduce and distribute single copies upon demand for scholarly use.

Diane A. Fagen
Diane A Fagen
Permissions Coordinator

8 May 2009
Date

Dear Dr. Pithua:

Congratulations on your accomplishment. We look forward to future contributions to our journals.

Best regards,

Diane A Fagen
Diane