TRANSMISSION DYNAMICS OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN CLOSED DAIRY HERDS IN THE US

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TRANSMISSION DYNAMICS OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS IN CLOSED DAIRY HERDS IN THE US*

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In this work we present work focused on within farm dynamics of *Mycobacterium avium* subsp *paratuberculosis* (MAP) infection on commercial US dairies. We focused on using deterministic mathematical models informed by molecular epidemiology and analysis of longitudinal data to evaluate current assumptions of MAP transmission. *In vitro* analysis of bovine-specific MAP strains revealed a difference in survival of bovine-specific MAP strains but no difference in monocyte-derived macrophages from shedding and non-shedding exposed animals. Molecular analysis of strains of MAP in dam-daughter pairs in a seven-year longitudinal dataset showed that vertical transmission plays a small but identifiable role in MAP transmission. In a meta-analysis of experimental infections of cows with MAP, most calves shed MAP within 12 months of infection. Duration of early shedding and time to late shedding were both dependent on age at exposure, while dose delivered played a role in duration of early shedding only. We integrated early shedding into on-farm models and it played an important role in MAP persistence in herds undergoing intervention attempts. Adding age- and dose-dependent early shedding created a forward feedback loop in which MAP can persist in populations which would not support introduction.

BIOGRAPHICAL SKETCH

Rebecca Mitchell is a DVM seeking PhD in the Quality Milk Production Services section of the Department of Population Medicine and Diagnostic Sciences at the Cornell College of Veterinary Medicine. She received an AB *magna cum laude* from Harvard University in 2003. Rebecca began a PhD program at Cornell University in 2003 as a member of the Dual Degree (DVM/PhD) program at Cornell College of Veterinary Medicine. She was supervised by Dr. Ynte Schukken, Dr. Yrjo Grohn, Dr. Hollis Erb, Dr. David Russell and Dr. Martin Weidmann. Rebecca took a one year leave of absence from the veterinary curriculum beginning in the Summer of 2008 to participate in the Fogarty International Clinical Research Scholars program, sponsored by the NIH Fogarty International Center during which she was researching the transmission of drug-resistant HIV-1C in Botswana. In the spring of 2010 she was a CDC Foundation OC Hubert Fellow in Guatemala applying outbreak detection algorithms to longitudinal health datasets. In her Ph.D. research, Rebecca is studying *Mycobacterium avium* subspecies *paratuberculosis* (MAP) transmission on commercial US dairy farms. Her projects involve *in vitro* assessment of host and pathogen heterogeneity, fieldwork and mathematical model development.

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CHAPTER ONE

Introduction

Background

Johne's disease (JD) is a chronic gastroenteritis of ruminants characterized by progressive weight loss in diarrheic adult animals. It is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a slow-growing, acid-fast bacillus. MAP is a member of the same genus as *M. tuberculosis* (MTB), *M. leprae* and many primarily non-pathogenic or opportunistic mycobacterium (e.g. *M. avium*, *M. intracellulare*).

MAP infection causes a chronic progressive inflammation of the gastrointestinal tract in ruminants (Coussens, 2001). Early infection stages of cattle are not necessarily associated with clinical signs (Whitlock and Buergelt, 1996), and infected cattle often remain negative in diagnostic tests until adulthood, even when infection occurred during early calfhood. Eventual clinical signs include decreased milk production, reduced fertility, progressive watery diarrhea and inability to maintain adequate body condition ultimately resulting in early removal from the herd (Whitlock and Buergelt, 1996). The disease is progressive, and there is no viable treatment option.

Pathobiology

Diarrhea and progressive weight loss of clinical MAP infection is brought on by decreased absorption through thickened small intestinal walls. In gross examination of clinically affected animals, MAP lesions are found predominantly in the ileum and to a lesser degree in the jejunum, rarely extending to the large intestine or duodenum (Buergelt, et al., 1978; Dennis, et al., 2008). Intestinal walls exhibit marked interstitial infiltration with inflammatory cells, enteritis and lymphangitis in conjunction with lymphadenitis. Bacteria can be recovered

in many organ systems and in peripheral blood mononuclear cell (PBMC) fractions from clinical animals, indicating systemic dissemination (Clarke, 1997; Antognoli, et al., 2008).

MAP delivered orally is selectively taken up by gut associated lymphoid tissues including tonsils and Peyer's patches. An initial (moderate) cell-mediated immune response which keeps both inflammation and proliferation at a low level gives way to an ineffective humoral immune response producing lesions and clinical disease (Clarke, 1997). The shift in immune response type and subsequent transition from subclinical chronic infections to active disease may be triggered by stressors including negative energy balance (Stabel, et al., 2003) and late gestation (Nielsen, et al., 2002a; Gierke, et al., 2007), but strong correlation to individual animal characteristics or life events is lacking.

Economic Impact

MAP is a costly disease due to both losses to milk production (Gonda, et al., 2007; Smith, et al., 2009; Smith, et al., 2010) and losses due to voluntary and involuntary culling in multiple ruminant species (Stehman, 1996; Bush, et al., 2006; McKenna, et al., 2006). In the 2007 National Animal Health Monitoring Survey, 68% of all United States dairy farms surveyed were MAP-positive, and 95% of farms with greater than 500 animals were MAP-positive (USDA:APHIS:VS, 2008). Overall, MAP infections result in a loss estimated at $97 per animal annually in commercial US dairy herds (Ott, et al., 1999). This loss depends on infection prevalence within a herd, with a $61 annual loss per animal in low prevalence herds and $245 annual loss per animal in high-prevalence herds (Ott, et al., 1999). Milk loss in high shedding animals has more recently been estimated at 4 kg/day as compared to same-farm consistently test-negative animals in a recent study (Smith, et al., 2009).

The disease also places an economic burden on farms that test semiannually or annually to identify currently shedding animals (Groenendaal and Wolf, 2008; Pillars, et al., 2009) in an

attempt to eradicate infection. However, due to poor test sensitivity, the majority of infected animals are not successfully identified by fecal and ELISA testing (McKenna, et al., 2005). Consequently, current testing and control strategies are targeting only a small proportion of all infected and potentially infectious animals in a population. Under the assumption of persistent endemicity of infection, an optimal control program would focus on minimizing the economic impact of this important infectious disease on American cattle farms.

Public Health Impact

In addition to being an important economic issue, MAP is potentially a human pathogen. The MAP bacterium continues to be associated with Crohn's disease in humans (Feller, et al., 2007; Behr and Kapur, 2008). In Europe and the United States, humans are primarily exposed to MAP through contaminated dairy products (Streeter, et al., 1995), although there is also a small but non-zero risk of exposure through meat products (Alonso-Hearn, et al., 2009; Mutharia, et al., 2010; Reddacliff, et al., 2010; Whittington, et al., 2010). MAP survives pasteurization in milk better than other common milk-borne pathogens and can be identified from a small proportion of processed milk samples (Sung and Collins, 1998; Eltholth, et al., 2009; National Advisory Committee on Microbiological Criteria for Foods, 2010). Recovery of MAP DNA from raw cheese (Clark Jr., et al., 2006; Stephan, et al., 2007) and viable MAP from both soft and hard cheeses up to 27 weeks into the ripening process (Spahr and Schafroth, 2001; Donaghy, et al., 2004) indicates that raw milk is not the only consumer risk. The presence of MAP in dairy products and the association of MAP with Crohn's disease make MAP a continuing food safety concern in the US and throughout the world.

MAP shedding directly into milk occurs predominantly from cattle that are also shedding high concentrations of MAP in feces (Sweeney, et al., 1992b; Streeter, et al., 1995). Shedding directly into milk has also been reported in infected cows following parturition (Stabel and

Goff, 2004). Fecal shedding into the environment was reported to be an important source of milk contamination with MAP (Weber, et al., 2008). Preventing rapid progression of MAP infections to a state of high shedding through optimizing control programs decreases the prevalence of high-shedding adult animals and therefore the direct and indirect risk of contamination of milk.

MAP prevalence

Less than 5% of animals typically test positive for MAP on each infected farm (USDA, 2005), and high levels of bacterial shedding are noted in only a small proportion of these animals (Whitlock, et al., 2000). However, elimination of MAP from herds has proven exceptionally challenging, with no published reports of successful eradication of MAP from infected dairy farms in the peer-reviewed literature without depopulation of the farm.

Most infected animals are not successfully identified by fecal culture and ELISA testing, as within-herd prevalence estimates are substantially less than estimates based on tissue culture sampling, for which upward of thirty percent of animals are test-positive (McKenna, et al., 2005; Wells, et al., 2009). Consequently, current testing and control strategies are targeting only a small proportion of all infected and potentially infectious animals in a population, resulting in a large reservoir of undiagnosed MAP infected animals. Given this reservoir, current intervention strategies need to be altered to adjust to the expectation of infection control rather than eradication of infection.

Research Needs

It is generally assumed that transmission is predominantly vertical (from dam to daughter) or from adults to young calves and that adult cattle are effectively resistant to MAP infection (Whitlock and Buergelt, 1996; Whittington and Windsor, 2009; Windsor and Whittington,

2010). Recent epidemiological studies indicate that these assumptions need to be challenged (van Roermund, et al., 2007; Weber, et al., 2010). In this body of research, our goal is to improve understanding of MAP endemicity on US dairy farms. We want to combine knowledge gained from mathematical modelling, molecular epidemiology and field studies to determine which areas of transmission that are not yet fully understood may be influential to controlling this endemic infection.

Chapter 2: Model development

There are several simulation models addressing MAP transmission and elimination strategies(Collins and Morgan, 1991; Groenendaal and Galligan, 1999; Beyerbach, et al., 2001). These models use data gathered in previous experimental and observational studies. Factors including rate of contact between animals in a herd, infectiousness of low and high shedding MAP-positive animals, and age at first shedding MAP have necessitated assumptions based on expert opinion where insufficient data were available. Using these models, interruption of cow-to-calf transmission should eliminate successful disease propagation. But in practice, even in herds with excellent hygiene and separation of calves from dams within a brief interval following calving, herds still maintain a low level prevalence of positive animals. This disparity between model predictions and herd infection statuses has led us to re-examine the assumptions concerning routes of transmission.

Recently there has been a focus on quantifying MAP shed by infectious animals. MAP shedding has previously been categorized as low, medium or high, with a maximum of 300 CFU/ culture tube due to limitations of bacterial plating. Recent dilution and plating experiments indicate that shedding levels can encompass six or eight orders of magnitude (Whitlock, et al., 2005). To address exceptionally high level of shedding from a small number of animals, it becomes necessary to either build a model with additional compartments or to

re-evaluate the definition of "high shedding" in a framework similar to previous models.

Although early models serve as the first and most widely recognized transmission models developed for paratuberculosis in a dairy setting, they have implicit limitations. For example, the assumption that halving exposure (such as assumed by Beyerbach *et al.* 2001) will halve the disease incidence is not necessarily the case, since transmission dynamics are necessarily non-linear and are determined by susceptibility as well as infection (Edmunds, et al., 1999). Infection dynamics often follow and are modeled allowing exponential growth and recovery (Diekmann and Heesterbeek, 2000). Mathematical modeling allows the flexibility required to incorporate our biological understanding of infection processes and the effectiveness of control programs (Anderson and May, 1999). The previously developed models reflect our current knowledge of the biology of MAP transmission, with limited input as to infectious status of calves and age at which animals are no longer susceptible to infection.

In **Chapter 2** we seek to understand how our assumptions about MAP shedding patterns and contact rates can influence prevalence. We build a series of mathematical models representing increasing system complexity and evaluate output from the same initial conditions in the multiple increasingly complex models. This chapter uses parameters based on assumptions of infectious progression from previously published work. Herd turnover assumptions are informed by the National Animal Health Monitoring Survey in 2002. We hypothesize that the contribution of high-shedding adults and herd contact structure are important to maintaining endemic infection within farms.

Chapter 3: Heterogeneity

Pathogen

MAP strains exhibit virulence differences at a host-species level (Saxegaard, 1990; Motiwala, et al., 2003; O'Brien, et al., 2006). Although there have not been specific proteins identified which explain species specificity, recent sequencing of the MAP genome (Li, et al., 2005) and microarray analysis have illustrated genome level differences between sheep and cattle strains at a genome level (Marsh, et al., 2006). At the time of this study, there were very limited data available on virulence differences between bovine-specific MAP strains. This was partially due to strain typing techniques which, until recently, did not adequately characterize diversity of host-specific strains. Recent addition of multi-locus short sequence repeat (MLSSR) strain typing techniques to the arsenal of typing tools allows better differentiation among bovine-specific isolates (Motiwala, et al., 2003; Amonsin, et al., 2004; Harris, et al., 2005).

Host

Host susceptibility to mycobacteria could be related to host genetics, nutritional status or other stresses on the host (parturition, environmental change, etc.). There are several host genes (Bellamy, 2003; Alcais, et al., 2005; Baghdadi, et al., 2006) and additional chromosomal loci identified via LOD score which show promise for explaining heterogeneity in genetic susceptibility to tuberculosis (Bellamy, 2003). Both MTB and MAP invasion and replication have been linked to available iron concentration (Lepper, et al., 1988; Johnson-Ifearulundu and Kaneene, 1999; Wagner, et al., 2005).

In **Chapter 3** our goal is to evaluate the contribution of diversity of host susceptibility and

pathogen strain on MAP survival. This work evaluated if MAP strains with different host specificities would survive better in monocyte-derived macrophages (MDMs) from cows that had been previously exposed to MAP than MAP strains that were bovine-specific. Additionally, we examined whether MDMs from known-infectious animals had greater MAP killing capacity in *vitro* than MDMs from non-shedding animals from the same farm environment.

In **Chapter 4** we compare quantitation methodologies for MAP survival in macrophages. Previous work has measured *M avium* survival using fluorescent staining and visual quantitation, but this system is tedious and imprecise due to differential distribution of cells and bacteria on a coverslip. As only a small portion of the coverslip is processed, this variability can greatly influence findings. We developed a qPCR system to quantify both host cells and bacteria. The objective of this methods paper was to evaluate the repeatability and reliability of each assay in an experimental setting which involves multiple MAP strains.

Chapter 5: Vertical transmission

It is generally assumed that most MAP infections take place at an early age. It is assumed that the vertical transmission route (dam-to-daughter) is an important source of infection maintenance in farms (Benedictus, et al., 2008). However, recent studies on genetic susceptibility potentially challenge the importance of the vertical infection route and suggest that infection of both dam and daughter is attributable to increased genetic susceptibility and resulting from horizontal transmission (Koets, et al., 2000; Nielsen, et al., 2002b; Mortensen, et al., 2004; Gonda, et al., 2006). Data obtained from the Regional Dairy Quality Management Alliance (RDQMA) longitudinal field study will allow the distinction of these infection routes. Based on the within-herd prevalence of MAP-infected animals and number of dam-daughter MAP-positive pairs in the dataset, we calculate the number of expected

positive dam-daughter pairs due to horizontal transmission. If there were no association between dam and daughter infections (all horizontal infections), then we would expect a high proportion of strain-mismatched pairs of dam-daughter infections. Genetic studies have shown heritability of MAP infection in the RDQMA herds, so these animals may be in a highly susceptible class, rather than infected only as a result of a high level of contact with positive dams (Settles, et al., 2009; Zanella, et al., 2010). If all the transmission is vertical, we would expect many dam-daughter pairs with matching strain types.

In **Chapter 5,** we evaluate the contribution of dam infection to daughter infection by studying dam-daughter pairs in which both animals are MAP-positive by fecal culture. We investigate strains of MAP that were carried by other potentially infectious animals on the farm environment and study the probability of vertical transmission given other simultaneous sources of MAP. We hypothesize that most MAP positive dam-daughter pairs represent vertical transmission.

Chapter 6: Experimental infections

Successful experimental infection challenge trials using calves include intravenous (Rankin, 1958; Rankin, 1961), oral (Gilmour, et al., 1965; Larsen, et al., 1977; Sweeney, et al., 1992a), subcutaneous (Larsen, et al., 1977) and intra-tonsilar routes (Waters, et al., 2003). The predominant transmission route on dairy farms is assumed to be via consumption of feed or water contaminated with infected feces (Clarke, 1997), although Corner proposes aerosol transmission (Corner, et al., 2004). Infected milk and colostrum from heavy shedding animals are thought to be an important but non-dominant transmission route (Sweeney, 1996). There can be *in utero* transmission to fetuses in high-shedding animals (Whittington and Windsor, 2009). Semen of fecal-culture-positive bulls can carry MAP (Larsen, et al., 1981; Khol, et al., 2010) as can transplanted embryos from clinical cows (Bielanski, et al., 2006), but these are

not considered major infection routes.

In **Chapter 6**, we present a meta-analysis of experimental infections of cattle with MAP. The goal of this work was to understand shedding patterns of infected animals. . Using time-to-event analysis techniques, we evaluate impact of dosing (delivery method, multiple or single dose, CFUs) on likelihood of shedding in young animals and progression of infection. Specifically we hypothesized that high doses of MAP and infections at a young age are associated with increased probability of shedding within twelve months of exposure and increase time in shedding categories.

Chapter 7: Backward bifurcation

In Chapter 2, we developed a series of mathematical models of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) transmission on commercial US dairy farms, building upon and modifying the assumptions in previous work to best reflect the pathobiology of disease transmission. It was clear from all models that high-shedding animals could have a significant impact on prevalence of MAP-infected animals in a herd, yet more complex models illustrated that these animals were not alone responsible for disease persistence. The maintenance of MAP in a low-prevalence herd which removed high-shedding adult animals upon detection appeared to be caused by other (potentially multiple) factors. Persistence of infection with a relative low level of prevalence was more easily predicted in models that incorporated calf-to-calf transmission and dam-to-daughter transmission from latent and low-shedding dams.

Based on our retrospective analysis of published datasets in Chapter 6, it appears that the likelihood and duration of transient shedding of MAP depends on both age at exposure and dose given. Animals which are exposed at a younger age or given a higher dose are more likely to have a transient-shedding phase, and will stay in this phase longer when they do

enter it. A trend where animals will become more infectious with increased infection prevalence could allow herds to sustain MAP even when contact between animals is reduced to a level which would not support introduction. This phenomenon is termed a "backward bifurcation".

For a backward bifurcation to occur, infectious dynamics require a forward feedback loop. Increased infection prevalence within a herd leads to increased transmission from each individual, and overcomes the decreased number of susceptible animals in a population relative to a naive population. In epidemiologic terms, the effective reproduction ratio exceeds the basic reproduction ratio. The basic reproduction rate (R_0) is defined as the number of animals that would be infected by the introduction of one infected animal into a population of susceptibles. The effective reproduction ratio (R_e) is the number of animals that are infected by each infected animal when the population is not completely susceptible. Generally, as more animals become infected, R_e becomes progressively smaller than R_0 until prevalence stabilizes in an endemically infected population. However, it is possible to have R_e exceed R_0, so that as prevalence increases, each infected animal can cause more secondary infections than if introduced in a completely susceptible population. In this case, if one infected animal were introduced to the population, prevalence of infection would return to zero. If many infected animals were introduced (more than the unstable equilibrium), then infection would be maintained in the herd at the same R_0. In this scenario, the stable equilibrium prevalence would not be near zero for an R_0 marginally greater than one, and an unstable equilibrium would bifurcate in the backward direction.

In **Chapter 7**, we explore the potential contribution of an age- and dose-related infectious progression to within-herd transmission dynamics. There has not yet been a data-based (vs. theoretical) paper published on veterinary pathogens which illustrates backward bifurcation in both age- and dose-dependent fashion. Backward bifurcations are one method by which we

can explain sustained disease at a low prevalence or where aggressive intervention strategies are hypothesized to bring R_0 <1, but MAP persists. We use deterministic mathematical models to look at farms undergoing active interventions. We hypothesize that the combined influence of high shedding animals and high contact between shedding young animals and susceptible young animals result in the establishment of a bistable equilibrium.

Chapter 8: Discussion and future directions

In **Chapter 8** we synthesize the most important findings from this collection of work and concurrent progress in the field. Based on the outcome of this work, we propose future research hypotheses.

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CHAPTER TWO

Simulation modeling to evaluate the persistence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) on commercial dairy farms in the United States[◊]

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Abstract

We developed a series of deterministic mathematical models of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) transmission on commercial US dairies. Our models build upon and modify models and assumptions in previous work to better reflect the pathobiology of the disease. Parameter values were obtained from literature for animal turnover in US dairy herds and rates of transition between disease states. The models developed were used to test three hypotheses. (1) Infectious transmission following intervention is relatively insensitive to the presence of high-shedding animals. (2) Vertical and pseudovertical transmission increases prevalence of disease but is insufficient to explain persistence following intervention. (3) Transiently shedding young animals might aid persistence. Our simulations indicated that multiple levels of contagiousness among infected adult animals in combination with vertical transmission and MAP shedding in infected young animals explained the maintenance of low-prevalence infections in herds. High relative contagiousness of high-shedding adult animals resulted in these animals serving as the predominant contributor to transmission. This caused elimination of infection in herds using the test-and-cull intervention tested in these simulations. Addition of vertical transmission caused persistence of infection in a moderately complicated model. In the most complex model that allowed age-based contacts, calf-to-calf transmission was required for persistence.

1. Introduction

Johne's disease is caused by Mycobacterium avium subsp. paratuberculosis (MAP), a slow-growing bacterial pathogen of ruminants. Herd-level prevalence of MAP infection on US dairy farms was estimated to be at least 22% generally, and at least 40% for herds of over 300 animals (Wells and Wagner, 2000). The high prevalence of infected herds makes MAP an infection of interest from animal welfare and economic perspectives, as well as from a human health perspective due to the potential link between MAP and human Crohn's disease. Crohn's is an intestinal disorder of humans that several authors have hypothesized to be of mycobacterial origin (Shulaw and Larew-Naugle, 2003; Naser et al., 2004).

Less than 10% of animals typically test positive for MAP on each infected farm (van Schaik et al., 2003; NAHMS, 2005), and high shedding of the organism (>100 cfu/g of feces) is observed in less than 20% of animals (Whitlock et al., 2000; Crossley et al., 2005). However, elimination of MAP from herds has proven challenging, with no reports in the peer-reviewed literature of successful long-term eradication of MAP from dairy farms without depopulation of all animals.

Several previous models addressed not only how MAP was spread on a farm, but also tested intervention strategies (Collins and Morgan, 1992; Groenendaal and Galligan, 1999; Beyerbach et al., 2001). The transmission of MAP in these models focused on infection of young calves via *in utero* infection, MAP in colostrum or milk, or exposure to infected feces. All models assigned shedding status to infected adult animals and based incident infections on the number of infectious adult animals present. Collins and Morgan (1992) developed the first dynamic deterministic model of MAP transmission that tracked the prevalence of four categories of animals. In their model, young animals remained susceptible up to age 1 year, when uninfected animals entered a resistant category and infected animals became latent. In this model 'latent' implied non-infectious, subclinical animals. Calves from infected dams

had the same risk of infection as all other calves. At 2 years old, latent animals entered an infectious compartment for the remaining time in the herd.

Groenendaal and Galligan's (1999) stochastic model of MAP transmission introduced three adult-shedding categories (low, high and clinical). Farm-level risk factors established a base level of exposure as a result of environmental contamination. Their model tracked infection via identifiable point-source exposures. There was an exponential decay in susceptibility of animals up to 1 year of age and animals became fully resistant to infection at 1 year old. Infected animals were assigned a future entry date for the high-shedding category at time of infection based on age at infection. Infected animals entered the low-shedding compartment two lactations previous to their assigned high-shedding compartment entry date. The probability of a successful infectious contact increased as infected animals moved from low-shedding to high-shedding and then to clinical status.

Beyerbach et al. (2001) constructed a deterministic model of herd-level prevalence. A relatively straightforward set of prevalence equations was used to model infection prevalence over time. The two main factors evaluated in the model are the farm hygiene level and the culling discipline of test-positive animals. The model requires relatively little input and subsequently allows only limited simulation. Although these models served as the first and most widely recognized transmission models developed for paratuberculosis in a dairy setting, they have implicit limitations. For example, the assumption that halving exposure (such as assumed by Beyerbach et al., 2001) will halve the disease incidence is naive, because transmission dynamics are necessarily non-linear and are determined by susceptibility as well as infection (Edmunds et al., 1999). Infection dynamics often follow, and are modeled allowing, exponential growth and recovery (Diekmann and Heesterbeek, 2000).

The previously described models reflect our current knowledge of the biology of MAP transmission, with limited input as to the infectious status of calves and age that animals are no longer susceptible to infection. Recently there has been a focus on quantifying MAP shed by infected animals (Whitlock et al., 2005). Although MAP shedding has previously been categorized as low, medium or high due to limitations of bacterial plating, shedding levels encompass a greater range than has previously been assumed (Whitlock et al., 2005). To address an exceptionally high level of shedding from a small number of animals, it becomes necessary to either build a model with additional compartments or to re-evaluate the definition of ‘‘high shedding’’ in a framework similar to previous models.

Methods of detection for MAP have low sensitivity among animals early in the progress of infection. Animals which are fecal-culture-negative might be shedding MAP. There is no way to distinguish between animals that are infected but not yet shedding MAP (true latents) and those that are infected and shedding below the threshold of detection by fecal culture (test-negative animals). In this paper these two populations are both defined as latent. They are assumed to be non-infectious except to their own calves.

Dam-to-daughter transmission from high-shedding animals has been addressed in previous models (Groenendaal and Galligan, 1999) by increasing the risk of infection at birth. A proportion of fetuses from high-shedding dams are culture-positive *in utero* (Sweeney et al., 1992). In a 20-year longitudinal dataset from one Pennsylvania farm analyzed by Benedictus et al. (2008), the risk of becoming MAP-positive as adults was not significantly different among calves born to latent dams and currently shedding dams. Calves born to latent dams did have increased risk of becoming MAP-positive as adults relative to calves from dams which never cultured positive in their lifetimes and were therefore considered uninfected. The risk of vertical or pseudo-vertical transmission from latent dams has not been addressed in previous models.

Calf-to-calf transmission of MAP also has not been addressed in any previous models. Calves shed MAP after experimental infection (Rankin, 1961b); however, until recently this transmission has not been considered as a source of MAP transmission on the farm (Weber et al., 2005; Bolton et al., 2005; van Roermund et al., 2007). Although shedding is detected, it might be infrequent or at a low level (Rankin, 1961a; van Roermund et al., 2007). The objective of this paper was to develop a series of mathematical models of MAP transmission building upon and modifying the assumptions in previous work to reflect our improved knowledge of the pathobiology of infection transmission. This series of models was used to explore sensitivity of transmission dynamics to three possibilities: presence of a highshedding compartment that reflects a much greater difference in shedding levels than previously assumed; all infected adults producing infected calves (not just restricting this transmission to high-shedding adults); and young animals shedding MAP rather than being latent. The future goal will be to use this model to test control strategies for eliminating clinical paratuberculosis and MAP infection from the herd.

2. Materials and methods

We developed three models that represent herd dynamics and disease process at an increasingly detailed level.

2.1. Base population and assumptions

The three series of differential equations presented in the appendices model all female animals on a fictional, 100-cow, commercial US dairy farm. Time steps of 1-month duration were used to allow incorporation of short-duration disease-status compartments. Outputs are presented as from a 100-cow dairy farm; however, because these models are frequency dependent rather than density-dependent, any herd size used would produce the same

proportion of infected animals. The selection of herd-size-independent dynamics follows from research indicating that the influence of herd size on MAP-infection prevalence might be quite small (Collins et al., 1994; NAHMS, 1997; van Schaik et al., 2003), or might increase with log rather than linear herd-size changes (Ott et al., 1999). A frequency-dependent rate of contact allows limited sensitivity to herd-size changes while a population-size dependent rate of contact (as in conventional state-transition models) results in rapid increase of MAP prevalence with an increase in herd size. In this series of models, rate of contact between animals on all farm sizes is constant due to an assumption of size-independent management that maintains constant density of animals. This assumption could be relaxed in future studies to allow herd size to influence prevalence.

Herd turnover values were taken from the National Animal Health Monitoring System dairy survey (NAHMS, 2003) to reflect averages from the US national herd. In simpler models, animal turnover was age independent. In the most complex model, age-dependent exit reflected average turnover at the age categories ‘‘calves’’ (μ_1), ‘‘heifers’’ (μ_2) and ‘‘cows’’ (μ_3) as defined in Table 2.1. In all models, calves entered the herd as replacements at the same rate as animals exited to maintain a constant population size. Excess female calves and all male calves were not included in the models. It was assumed that this was a closed herd with sufficient replacements being produced. The models could accommodate external replacements in open herds, but this would require an additional set of assumptions concerning herds of origin that have not been addressed in this paper. Calves born to infected dams entered the population at the same rate as calves born to uninfected dams unless there was active intervention. Population turnover was not affected by the presence of MAP, because increased rate of exit due to MAP resulted in decreased exit due to other causes.

We divided the distribution of shedding animals into two populations. The low-shedding population included all animals shedding MAP at levels <300 colony forming units

(cfu) per fecal culture tube (van Schaik et al., 2005). The high-shedding population was comprised of all animals with >300 cfu/g. It is expected that the population of animals shedding MAP in excess of the maximum quantifiable level is comprised of cows shedding orders of magnitude above this level (Whitlock et al., 2005). Although in this model the two categories are treated as distinct populations, it is understood that there might be a continuum of shedding levels. It was initially assumed that if young animals were shedding MAP, it was at a low level and therefore they were not contributing to infection. Because disease progression was uniform and deterministic, animals progressed through the same disease stages regardless of age at infection.

In models that evaluated calf-to-calf transmission and its potential impact on disease prevalence, calves could enter the population as transient shedders infected at or before birth. Calves were susceptible to MAP until 1 year old when they became completely resistant. The 1-year age cut-off for susceptibility followed assumptions of both the Collins & Morgan and Groenendaal & Galligan models.

Table 2.1. Definition of symbols used in compartmental models. Values used in figures and sources of data are provided.

Symbol	Definition	Value	Reference
α	death rate from clinical disease among Y_2 animals	1/year	4
α_1	α x (Y2/Y from model B)	calculated	
β_{1C}	transmission parameter from calves, model C	rate of contact x infectiousness=$10\pi\beta_{3C}$	1
β_{12B}	transmission parameter from transients, model B	$\pi\beta_{3B}$	1
β_{2C}	transmission parameter from heifers, model C	rate of contact x infectiousness=$\pi\beta_{3C}$	1
β_3**	transmission parameter from cows	calculated	
	Model A: β_{3A}	$\beta_{3A}=\beta_{in}(Y_1+Y_2)/(Y_1+\varepsilon Y_2)$	
	Model B: β_{3B}	$\beta_{3B}=\beta_{in}(Tr+Y_1+Y_2)/(\pi Tr+Y_1+\varepsilon Y_2)$	
	Model C: β_{3C} π x $RC_{1,3}$,	$\beta_{3C}=\beta_{in}(Tr_1+Tr_2+Y_1+Y_2)/(10\pi Tr_1+\pi Tr_2+Y_1+\varepsilon Y_2)$	
β_{in}	input value of transmission parameter β	2	2
δ	additional cull rate of Y during intervention (model A)	0.089/year	1
δ_1	additional cull rate of Y_1 animals during intervention	0.25/year	1
δ_2	additional cull rate of Y_2 animals during intervention	1/year	1
ε	ratio of infectiousness of contacts Y_2:Y_1	1, 10, 100	1
ϕ	rate of exit from transient stage (Tr/Tr_2)		7
	Model B	1/year	
	Model C	2/year	
γ_n	proportion calves infected at or before birth		6
	γ_H from H/H_3 dams	0.15	
	γ_{Y1} from Y_1 dams	0.15	
	γ_{Y2} from Y_2 dams	0.17	
γ*	rate of infected calves entering herd	calculated	
	Model B without intervention	$\gamma=(\gamma_H H/N+\gamma_{Y1}Y_1/N+\gamma_{Y2}Y_2/N)\mu$	
	Model B with intervention	$\gamma=(\gamma_H H/(N-Y_2)+\gamma_{Y1}Y_1/(N-Y_2))\mu$	
	Model C without intervention	$\gamma=(\gamma_H H_3/N_3+\gamma_{Y1}Y_1/N_3+\gamma_{Y2}Y_2/N_3)\mu$	
	Model C with intervention	$\gamma=(\gamma_H H_3/(N_3-Y_2)+\gamma_{Y1}Y_1/(N_3-Y_2))\mu$	
$\lambda_{A/B/C}$	model specific force of infection	calculated	

μ	whole herd introduction rate	0.223/year	3
μ_1	death rate from calves	0.111/year	3
μ_2	death rate from heifers	0.007/year	3
μ_3	death rate from cows	$\mu_{3inv}+\mu_{3v}$	3
μ_{3v}	voluntary cull rate from cows	$\mu_{3vol\ 1}-[\delta_1 Y_1/N_3+(\delta_2+\alpha)Y_2/N_3]$	5
$\mu_{3vol\ 1}$	without adjustment for disease related exit	0.285/year	3
μ_{3inv}	involuntary cull rate from cows	0.048/year	3
μ_d	average death rate (models A and B)		3
	base value without adjustment for disease related exit	0.223/year	
	Model A	$\mu_d=\mu-(\alpha+\delta)Y/N$	
	Model B	$\mu_d=\mu-[\delta_1 Y_1/N+(\delta_2+\alpha)Y_2/N]$	
π	ratio of infectiousness of contacts Tr:Y_1 or Tr_1 & Tr_2:Y_1	0, 0.008, 0.05, 0.08	1
ρ_i	rate of aging	1/year	5
σ	rate of exit from latent compartment (H or H_2)		4
	Model B	0.667/year	
	Model C	1/year	
t	duration of a time step (years)	1/12	1
υ	rate of exit from low shedding compartment (Y_1)	0.33/year	4
w	rate of contact between different age classes (model C)		1

1:user defined 2:estimated by model sensitivity 3:(USDA, 2005) 4: (Whitlock et al., 2000;van Schaik et al., 2003a) 5: calculated 6: (Whitlock et al., 2005b) 7: (Rankin, 1961a) *Culling in the adult herd (μ_3) is maintained as a constant by decreasing voluntary culls when there are MAP-related culls or deaths. This calculation reflects turnover in a commercial herd, where increased culling for MAP can result in reduced culling for other voluntary causes. This stable herd turnover maintains constant input of susceptible calves. ** Any values in italics are determined based on model inputs rather than calculated stepwise based on model outputs. These ratios are based on steady-state assumptions of rate of exit from each category, and therefore do not change over time.

2.2. Infection transmission models

There were three progressively more complicated state-transition model frameworks that modeled MAP transmission (models A, B and C). These models used series of differential equations to define progression through infection states (see Appendices A.1, A.2 and A.3, respectively). Model A placed all infected animals in one category in a herd with age independent turnover. Models B and C allowed more specific disease dynamics and age dependent dynamics, respectively. All parameters of the models are defined in Table 2.1. Because model C allowed us to study all phenomena of interest, we defined our initial infectious transmission parameter (i.e. β_{in}) on plausible output prevalence in model C. In the US the mean 'true prevalence' among adult animals on MAP-infected commercial farms is between 5 and 10% (van Schaik et al., 2003; NAHMS, 2005). Maximum prevalence within a herd can be over 50% test-positive. 'True prevalence' is calculated to account for the low-shedding animals that are not detected in a cross-sectional survey. This value does not include any latent animals or MAP-positive calves or heifers. A mean 'true prevalence' of between 5 and 10% could account for less than half the total infected animals when including latents.

The infectious transmission parameter (β) was modified so that the number of infectious 'units' among infectious adult animals (Y_i) was the same for all models across all simulation series. Scaling of infectious transmission parameter across simulations allowed output from all three models to be compared simultaneously with a reasonable amount of infectious animals. If β were not adjusted, then for each model with additional infectious compartments or increased infectiousness of high shedders, disease prevalence quickly increased to an unreasonable level. β calculations used an infectious animal in model A as the baseline ($\beta_A = \beta_{in}$). For each series, the β values as calculated by the formula shown in Table 2.1 are written in the figure caption.

Each model output was evaluated as to whether it reflected a stable endemic situation with current, relatively modest, intervention schemes as defined in Section 2.3.1. Very high prevalence and continuously increasing prevalence when extended to a 100-year time-frame, as well as very low prevalence with negative slopes leading to eventual elimination were deemed to be non-consistent with observed data on within-herd prevalence. In this series of models that represented average farms, plausible output was defined as a prevalence of infected animals of less than 30% for simulations with no intervention, reflecting a maximum prevalence of shedding adults (the population measured by 'true prevalence') of approximately 10%. For runs with an intervention imposed, output prevalence should have a low-positive or zero slope at t = 300 (20 years following initiation of an intervention strategy), indicating movement towards a stable threshold of infection.

The first, most basic, model A (Appendix A.1, Fig. 2.1) used three states with one infectious stage. Births of calves that are retained as replacement animals are assumed to be constant and sufficient to equal deaths and culling, and only half of the calves born are eligible to enter the replacement population (μ). Baseline turnover (μ_d) is adjusted depending on both disease-related death (α_1) and intervention-related culling (δ) to maintain constant overall rate of exit.

Model B (Fig. 2.2, Appendix A.2) maintains a homogenous population of cattle (no age-dependent death, homogeneous mixing) but introduces more disease states. This model has an additional flow of animals entering the system (γ) accounting for calves infected at or before birth. Input of calves from higher- or lower-risk replacement herds could be introduced to account for assumptions of open herd status. Animals on commercial US dairy farms are generally not mixing homogeneously; instead animals are routinely housed by age group that

will be addressed further in model C. Homogeneous mixing across all disease states requires that the transient-shedding group has the same contact rate with susceptible animals (X_1) as do other infectious compartments (Y_1 and Y_2).

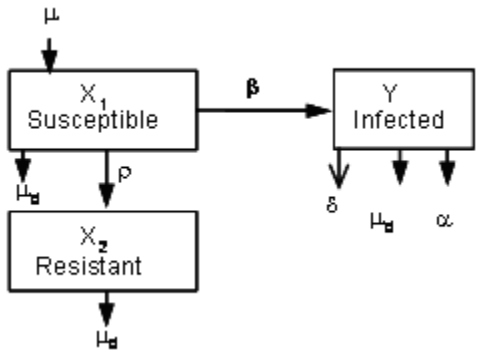


Fig. 2.1 Model A is a basic state-transition model including age-based resistance. Animals move from susceptible (X_1) status to either infectious (Y) at a rate λ_A or resistant (X_2) at a rate ρ. Transitions between states are represented as arrows. Animals enter the susceptible compartment at a rate μ and exit all compartments at a rate μ_d. There is additional exit from the infectious compartment at a rate a1 due to disease-related deaths and d due to additional culling during intervention. Values of transmission rates are defined in Table 2.1.

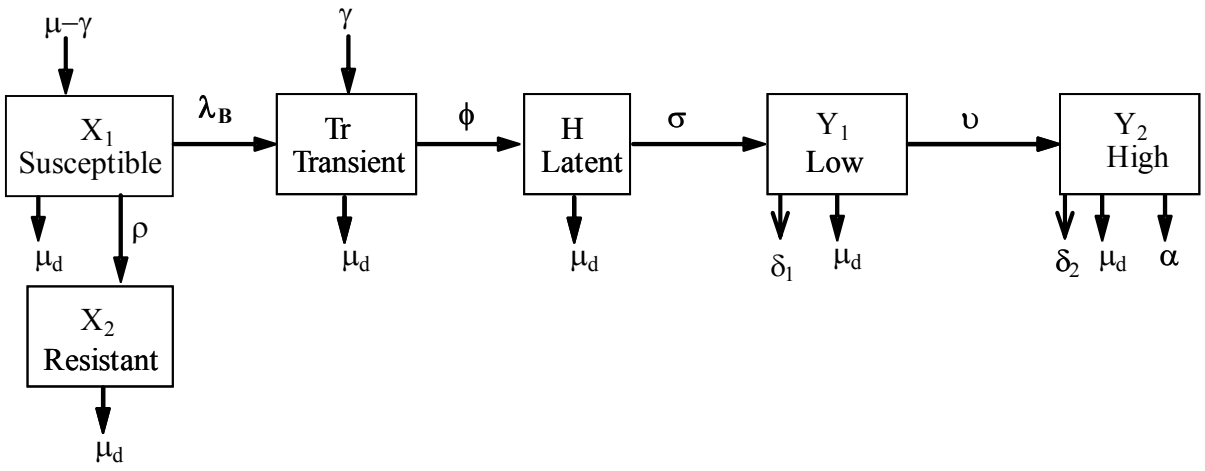


Fig. 2.2. Model B is an intermediate state-transition model, including low-shedding (Y_1) and high-shedding (Y_2) states. Transmission rates between states are represented as arrows and defined in Table 2.1. Animals move from susceptible (X_1) status to either transient (Tr) at a rate λ_B or resistant (X_2) at a rate ρ. Transients move to the latent category at a rate ϕ, then to low shedding (Y_1) at a rate σ and high shedding (Y_2) at a rate ν. Animals enter the susceptible compartment at a rate μ–γ and the transient compartment at rate γ. There is exit from all compartments at rate μ_d and additional disease-related exit at rate α from the high-shedding compartment. Intervention-related exit is from both low-shedding and high-shedding compartments at rates δ_1 and δ_2, respectively.

Model C (Fig. 2.3, Appendix A.3) introduces age-class divisions based on age-dependent exit rates. ‘‘Calves’’ are here defined as animals less than 1 year old, ‘‘heifers’’ as animals 1–2 years old, and ‘‘cows’’ as animals greater than or equal to 2 years old, regardless of reproductive status. Several compartments with otherwise-identical characteristics are split based on age of animals (Tr_1 and Tr_2, H_2 and H_3, X_2 and X_3). This division limits progression through disease states to a more biologically plausible minimum duration and also allows age-dependent contact rates. A modified WAIFW (Who Acquires Infection From Whom) matrix (Anderson and May, 1999) represents contact rates between animals in different age classes (Fig. 2.4). The matrix describes rate of contact ($RC_{n;n'}$) between age classes rather than likelihood of successful transmission (β) because disease progression is dependent on age at exposure. It is reasonable to assume a much greater rate of intra-class contact than inter-class contact on a commercial dairy farm based on barn and pen layouts; rates of inter-class and intra-class contact were selected to illustrate the effect of contact limitation between classes without completely eliminating inter-class contact.

2.3. Simulation studies

2.3.1. Simulation background

State-transition models were constructed in Modelmaker Version 4.0 software (Cherwell Scientific Ltd., Oxford, UK). Equations were solved numerically via the Runge-Kutta integration method (Bray and Lay, 1994). Data gathered from simulations were transferred to Microsoft Excel (2003) for graph development. Simulations were run to determine model output with a range of input parameters as defined in Table 2.1 to test sensitivity of the model to our assumptions. Simulations followed an average herd following the introduction of MAP graphed

at time zero. In this series of models, population introduced into the model was 99 young animals and 1 adult.

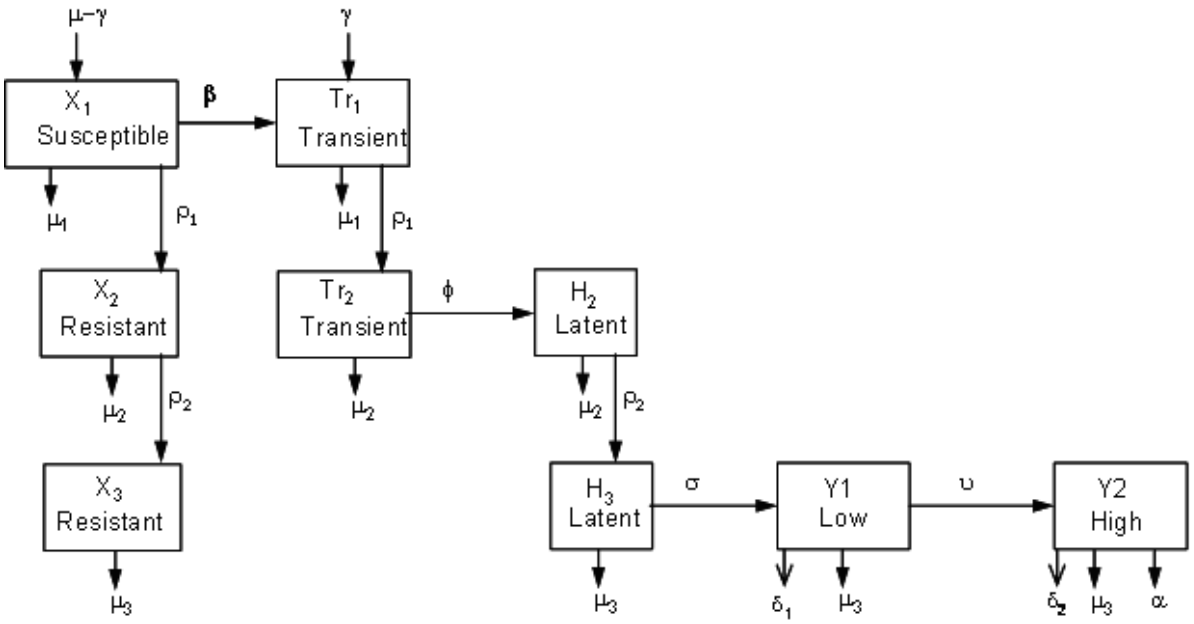


Fig. 2.3. Model C is the most complex state-transition model. Three age classes are calves (Class 1), heifers (Class 2) and cows (Class 3). Transition rates between states are represented as arrows and defined in Table 2.1. Animals move from susceptible calves (X_1) to either transient-shedding calves (Tr_1) at rate λ_C or resistant heifers (X_2) at rate ρ_1. Resistant heifers become resistant cows (X_3) at rate ρ_2. Transient calves (Tr_1) become transient heifers (Tr_2) at rate ρ_1 and then latent heifers (H_2) at rate ϕ. Latent heifers transition into latent cows (H_3) at rate ρ_2 and then low (Y_1) and high (Y_2) shedding cows at rates σ and ν, respectively. Animals enter the susceptible calves compartment (X_1) at a rate $\mu–\gamma$ and the transient-shedding calves compartment (Tr_1) at rate γ. Exit is age-class specific. Animals exit the herd from the calf class at rate μ_1, the heifer class at rate μ_2, and the cow class at rate μ_3. Disease-related exit at rate α is modeled in the high-shedding (Y_2) compartment. Intervention-related exit is from both low-shedding (Y_1) at a rate δ_1 and high-shedding (Y_2) compartment at rate δ_2.

		Infectious		
Susceptible	Age Class	1' Calves	2' Heifers	3' Cows
	1 Calves	10w	w	w
	2 Heifers	w	10w	w
	3 Cows	w	w	10w

Fig. 2.4. Contact-rate matrix for animals in model C by age class. Class 1, calves, are defined as less than 1 year old; Class 2, heifers, as 1–2 years old; and Class 3, cows, as greater than or equal to 2 years of age. Rate of contact ($RC_{n;n'}$) between non-infected (n) and infected animals (n') is presented as matrix values (n x n'). Although base rate of contact between members of different classes is unknown (w), it is assumed to be uniform.

Before t = 0 there were 100 hidden time steps that allowed the population to reach a stable age distribution (<0.0001% variation in the last 10 time steps before t = 100, limited only by accuracy specifications). Because this period was an artifact of arbitrary distribution of animals in the initial population and not reflective of dynamics on a real farm, it was not shown in model output. The population of 100 animals at t = 0 was uninfected, and 1 animal was replaced with an infectious (Y or Y_1) animal. All models were run for 100 years (1200 time steps) following introduction of intervention at 60 months to determine the time-frame to reach stable prevalence. Output for 20 years (240 time steps) post-intervention was presented because the 100-year time-frame is likely to be less informative given changes in herd dynamics over time. The 20-year time-frame allows comparison to previous papers that modeled prevalence following intervention. The test-and-cull intervention strategy modeled increased culling (δ_i) from the Y or Y_1 and Y_2 categories. Because models A and B did not differentiate between adult

and juvenile animals, in these models, all shedding animals were at risk of culling, regardless of age. During intervention, additional culling removed 25% of low-shedding animals ($\delta_1 = 0.25$) and 100% of high-shedding animals ($\delta_2 = 1$) annually. All calves from Y_2 adults were prevented from entering the population ($\gamma_{Y2} = 0.17 \rightarrow \gamma_{Y2} = 0$). This strategy attempted to represent a reasonable application of test-and-cull intervention in which sensitivity of detection for low-shedding animals was 20% for one fecal culture, and increased to a maximum of 25% for two fecal cultures annually (Whitlock et al., 2000). It was assumed that low shedders that were detected were then removed from the herd. In model A, a modified culling strategy was applied because there was only one infected category, Y. Here, a proportion of animals were removed from the infected category based on the calculated ratio of animals in H, Y_1 and Y_2 in model B ($\delta = 0.089$).

Intervention was modeled beginning at 5 years (t = 60 months) following herd infection by one animal (Y or Y_1). Delayed implementation of intervention strategies is more plausible than assuming herds are continually testing before the appearance of clinical disease in the population. Additional exit of infected animals due to known infection status is not simultaneous with detection even in those animals that are easily detectable (Y_2); the delay reflects current farm practices. These animals will most likely be present for several months before detection, especially if they are low shedders.

2.3.2. Simulation experiments

Simulation series one evaluated the impact of high shedders on the transmission of MAP when they were assumed to contribute several orders of magnitude more bacteria than low shedders.

All infectious cows that were not high shedders (<300 cfu/g) were defined as a homogenous population of low-shedding (Y_1) animals. High shedders (Y_2) were assigned a ratio of infectiousness (ε) relative to low shedders (Y_1). Because the relative infectiousness of low- and high-shedding adults is not known, models B and C were run with values of $\varepsilon = 1$, 10 and 100. A value of 1 indicated that all shedding animals were equally infectious and number of cow-to-calf contacts was the limiting factor for transmission. Relative values of 10 and 100 reflected additional contribution of high-shedding animals to the environment, expected to occur in the calving area (Whitlock et al., 2005).With this range of input values we could study the impact of high shedders. For each model the values of relative infectiousness were applied with and without a test-and-cull intervention. In model A, where high shedders could not be differentiated from other infected animals, increasing the infectiousness of high-shedding animals could not be addressed. In this simulation series, we assumed there was no vertical transmission ($\gamma = 0$) and no transmission between calves or heifers ($\beta_{12B} = \beta_{1C} = \beta_{2C} = 0$).

Simulation series two evaluated the impact of calves infected at birth ($\gamma > 0$) entering the herd when there was a test-and-cull intervention in place and no transmission occurred between calves or heifers ($\beta_{12B} = \beta_{1C} = \beta_{2C} = 0$). This series evaluated whether the presence of vertical and pseudo-vertical transmission could be sufficient to cause disease persistence in a herd undergoing test-and-cull intervention. Dam-to-daughter transmission of MAP at or before birth was addressed by introduction of a portion of calves directly into Tr_1 for models B and C (Figs. 2.1 and 2.2) and reflected in differential equations (Appendices A.2 and A.3).

Simulation series three evaluated the contribution of shedding by calves and heifers to the transmission of MAP (β_{12B}, β_{1C}, $\beta_{2C} > 0$). This series included only simulations with an active test-and-cull strategy. Infectiousness of transiently shedding animals was scaled relative to low-

shedding animals (reflected by different values of π). Due to the constraint of homogeneous mixing for all categories in model B, transient shedders (Tr) had rate of contact with susceptible that was equivalent to their rate of contact with low- or high-shedding animals. In model C, the rates of contact were assigned via values in the WAIFW matrix (Fig. 2.4).

3. Results

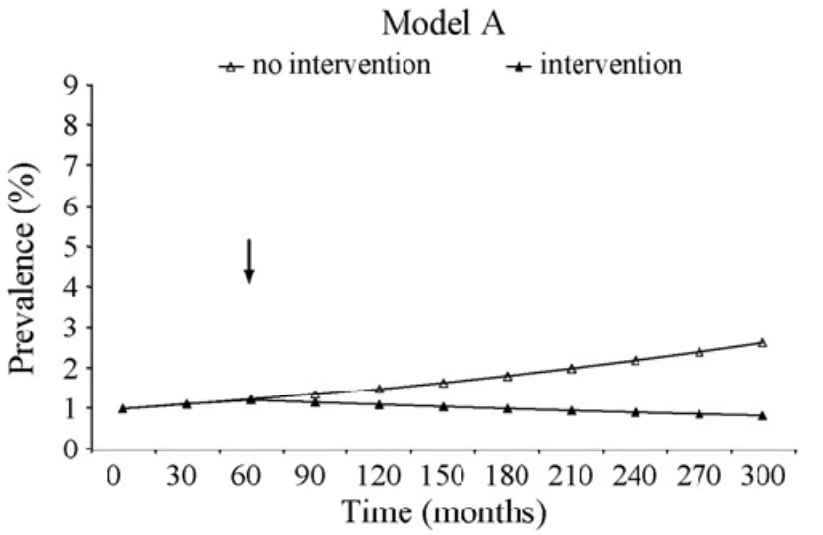
Individual simulations were usually solved within 2 s, whereas sensitivity analyses running all models with three separate input values for the variable of interest would typically take up to 20 s to complete.

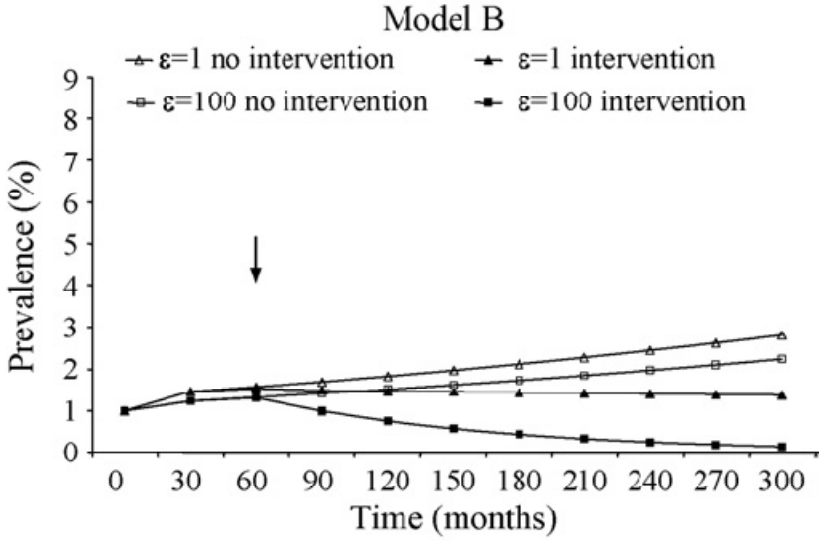
Because MAP is slow to progress in individual animals, requiring several years before animals become infectious, a long time interval was required to reach stable prevalence in herds. However, in previous papers as well as in our results, no models reached stable prevalence within 20 years following intervention.

With equivalent input values, all three models produced results that would be plausible for infection prevalence in herds when no test-and-cull intervention was in place (Fig. 2.5). Model A predicted the lowest infection prevalence of the three models within 20 years following intervention when compared to model B and C simulations where low and high shedders were equally infectious ($\varepsilon = 1$). Although simple models capture basic dynamics of systems and can be used to study complex mathematical phenomena, they are often insufficient to allow in-depth understanding of real world dynamics. Because model A does not allow study of multiple levels of infectiousness among infected animals, it was not considered further even though it produced plausible output. When high and low shedders were equally infectious ($\varepsilon = 1$) and with intervention as defined above, model B predicted declining infection prevalence in the herd,

while model C predicted increasing prevalence. Models B and C both predicted a decline in MAP prevalence for any value of epsilon over 10 (data for $\varepsilon = 100$ shown in Fig. 2.5).

Higher infection prevalence when high shedders were equivalent to low shedders relative to when high shedders were 100 times more infectious than low shedders was an effect of calculation of individual model β. At $\varepsilon = 1$, all infectious animals were considered equally infective at a weighted average of the contribution of the two low (Y_1) and high (Y_2) infectious shedding categories via the formula in Table 2.1. If $\varepsilon > 1$, then as high shedders became more infectious, low shedders became less infectious because the low-shedding infectious category no longer represented a weighted average between the two. The rate of contact of the high shedding (Y_2) class then limited transmission from highly infective animals.


Model A
no intervention
intervention
Prevalence (%)
0
1
2
3
4
5
6
7
8
9
0 30 60 90 120 150 180 210 240 270 300
Time (months)


Model B
ε=1 no intervention
ε=1 intervention
ε=100 no intervention
ε=100 intervention
Prevalence (%)
0
1
2
3
4
5
6
7
8
9
0 30 60 90 120 150 180 210 240 270 300
Time (months)

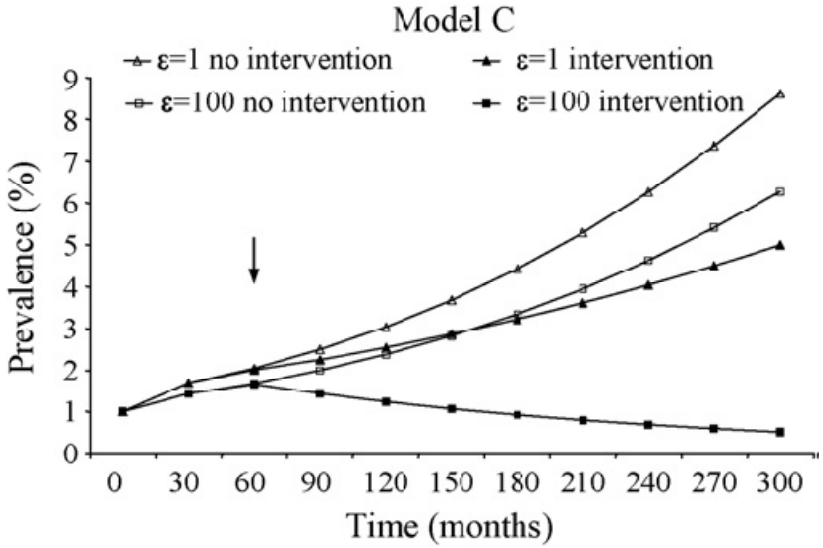

Model C
ε=1 no intervention
ε=1 intervention
ε=100 no intervention
ε=100 intervention
Prevalence (%)
0
1
2
3
4
5
6
7
8
9
0 30 60 90 120 150 180 210 240 270 300
Time (months)

Fig. 2.5. Infection prevalence with multiple levels of relative infectiousness of high shedders. Percent of the total herd infected (Y variable) includes animals of all ages. Time steps are in months for 20 years following intervention that begins at t = 60 months (indicated by an arrow). In this simulation there is no transient shedding ($\beta_1 = 0$), and all calves enter the population susceptible and uninfected ($\gamma = 0$). Triangles are from simulations with low shedders equally infectious as high shedders ($\varepsilon = 1$, open = no intervention, solid = intervention, $\beta_A = 2$, $\beta_B = 4.48$, $\beta_C = 5.21$). Squares are from simulations where high shedders are 100 times as infectious as low shedders ($\varepsilon = 100$, open = no intervention, solid = intervention, $\beta_B = 0.20$, $\beta_C = 0.25$). Since β is adjusted for total infectiousness, comparisons should be restricted to within triangles and within squares.

Simulation series two (Fig. 2.6) focused on the impact of vertical transmission (γ) on MAP prevalence. In simulations without interventions employed, horizontal transmission accounted for the majority of infections for all levels of contribution from high-shedding animals (not shown). In herds undergoing active intervention, vertical transmission permitted persistence of infection despite concurrent decrease in relative infectiousness of high-shedding animals.

Simulation series three (Fig. 2.7) included an infectious contribution from transiently shedding calves and heifers in model B ($\beta_{12B} > 0$) and model C (β_{1C} and $\beta_{2C} > 0$) in addition to vertical transmission ($\gamma > 0$). Model B required calves and heifers to be more infectious than low-shedding animals to sustain MAP-infection prevalence. Model C did not require infectious calves ($\pi = 0$) to maintain infection at an intermediate value of relative infectiousness of high shedders ($\varepsilon = 10$). For runs with a higher relative infectiousness of high shedders, calves less infectious than low-shedding adults were sufficient to cause persistence of infection. This model was extremely sensitive to transiently shedding calves, and transiently shedding animals quickly overwhelmed the system and caused a substantial increase in slope of prevalence.

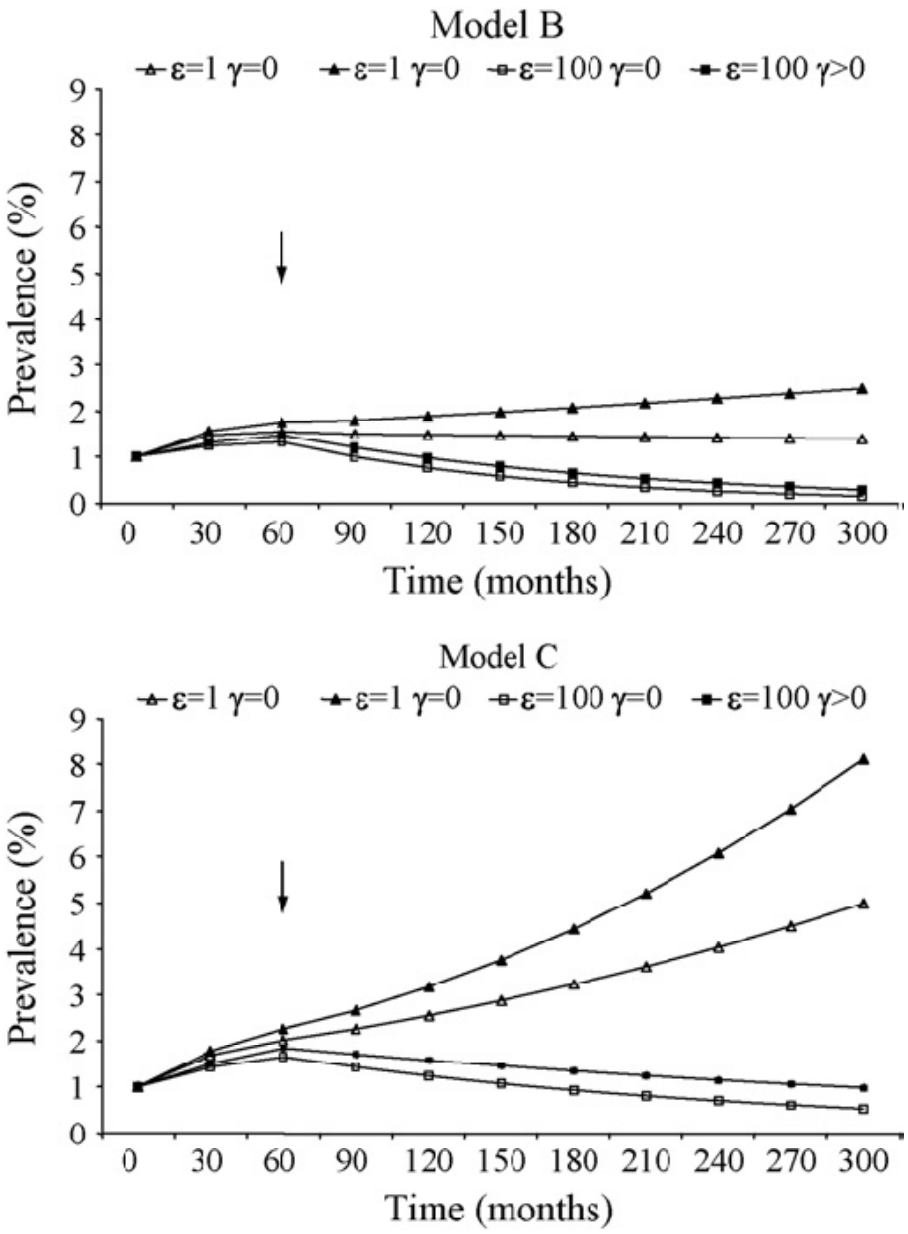


Fig. 2.6. Infection prevalence with ($\gamma > 0$) and without ($\gamma = 0$) vertical transmission. Percent of the total herd infected included animals of all ages. Time steps were in months for 20 years following intervention that begins at t = 60 months (indicated by an arrow). Triangles (open = without vertical transmission, solid = with vertical transmission) track herd-level prevalence when high shedders are equally infectious to low shedders ($\varepsilon = 1$, $\beta_B = 4.48$, $\beta_C = 5.21$), and squares (open = without vertical transmission, solid = with vertical transmission) track herd-level disease prevalence when high shedders are 100 times as infectious as low shedders ($\varepsilon = 100$, $\beta_B = 0.20$, $\beta_C = 0.25$).

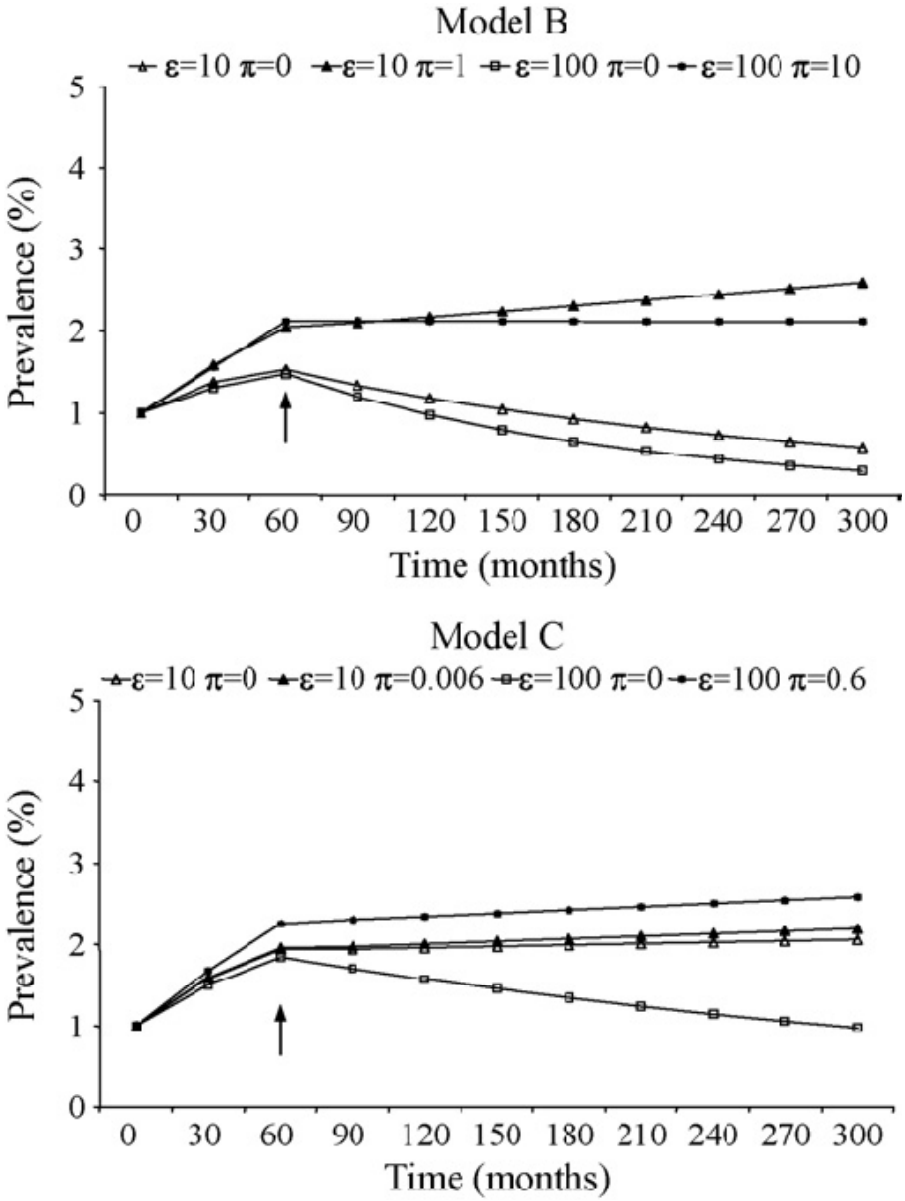


Fig. 2.7. Infection prevalence with vertical transmission ($\gamma > 0$) and transient shedding ($\pi > 0$) among calves and heifers. Infection prevalence was tracked in 1 month time steps for 20 years following intervention that begins at t = 60 months (indicated by an arrow). Triangles are from simulations when high shedders are 10 times as infectious as low shedders ($\varepsilon = 10$, $\beta_B = 1.53$, $\beta_C = 1.86$) and squares follow simulations when high shedders are assumed to be 100 times more infectious than low shedders ($\varepsilon = 100$, $\beta_B = 0.20$, $\beta_C = 0.25$). In model B, open symbols are from simulations when there is no infectious contribution from transient shedders ($\pi = 0$), and solid symbols are from simulations when there is infectious contribution from transient shedders ($\pi = 1$ as triangles or $\pi = 10$ as squares). In model C, the open symbols are from simulations when there is no infectious contribution from transient-shedding animals ($\pi = 0$) and the solid symbols are from simulations when there is infectious contribution from transient shedders ($\pi = 0.006$ as triangles or $\pi = 0.06$ as squares).

4. Discussion

If a complex model produces output equivalent to a simpler version, the simplest informative model should be selected to explore the system. In this series, model A is not informative concerning our parameters of interest. Although we know that test-and-cull interventions are not an optimal strategy for eliminating MAP, they do show a decrease in prevalence over time (Kalis et al., 1999; Caldow and Gunn, 2002). Models B and C produce similar outcomes, despite the lack of age-specific rates of contact in model B.

Environment is not considered as a source for MAP transmission in this series of models. Since MAP does not multiply in the environment, it can only serve as a mechanical vehicle. Presence of MAP in the environment increases the number of contacts between infectious animals and susceptible animals and might contribute to maintenance of infection if MAP is persisting for extended periods of time. There is evidence that MAP does not consistently persist past 6 months in the environment but might occasionally be recovered up to 1 year post-deposition (Whittington et al., 2004). Further models might be necessary to study the potential impact of environmental survival.

In this study we have employed deterministic models that provide average behaviors and require only point estimations of parameter values rather than distributions which are difficult to obtain. These models provide an excellent framework for studying the impact of transmission mechanisms and the role of control programs. Because control programs remove perceived infectious contacts, it is valuable to evaluate whether these targets are appropriately selected. Deterministic models can provide hypothetical average effects of control strategies. Limitations of these models include the inability to identify the range of different behaviors possible in a

system and to address the potential of stochastic fade-out in small populations. Future work on stochastic modeling will be necessary to add these components to our models.

4.1. Evaluation of the impact of high shedders

Bacterial load from MAP-shedding animals has a negative binomial distribution among infected adults (van Schaik et al., 2005; Whitlock et al., 2005). Moderate or high shedders constitute 20–30% of the shedding animals in a cross-sectional survey of herds (Crossley et al., 2005; NAHMS, 2005). Because high shedders can contribute $>10^4$ more MAP bacteria to the farm than low shedders, this population of animals could potentially drive transmission. If MAP were spread uniformly through the entire farm environment, high-shedding animals would be 10,000 times more infectious than low shedders, and the input of low shedders should be negligible in controlling disease spread.

When high-shedding animals are assumed to have infectiousness proportional to their MAP shedding levels (high shedders 10,000 times more infectious than low shedders), it becomes impossible to sustain infection under intervention. Neither model B nor model C is capable of sustaining infection with relative infectiousness of high shedders even 100 times that of low shedders (simulation series one). This outcome is not supported by field data. Historically, intervention attempts removing high-shedding animals (that are easily detected) have been insufficient to address long-term persistence of MAP (Jubb and Galvin, 2002; Benedictus et al., 2008). This would argue against high shedders as the sole source of infection in a dairy herd. Their rate of contract with susceptible animals might be limiting this transmission.

High shedders cause an important proportion of new infections because each contact with calves is more effective than contacts of calves with low-shedding adults. However, in a situation

where high-shedding adults are being removed at a reasonable pace (average time in herd of 1 year), these animals are not necessarily driving infection maintenance. Both models B and C show that if these animals were solely driving transmission, their removal would successfully eliminate disease. Alternative routes of transmission might be serving a primary role in sustaining a low prevalence in herds where high-shedding animals are successfully removed.

4.2. Evaluation of dam-to-daughter transmission

Addressing dam-to-daughter transmission from latent and low-shedding cows requires an elimination strategy that traces an animal's offspring to cull all surviving daughters of an infected dam. Because cows on average produce only a single female offspring that survives in the herd to first calving, this strategy should not be excessively costly in comparison to other aggressive test-and-cull regimens.

Importance of dam-to-daughter transmission was evaluated in simulation series two (Fig. 2.6, models B and C). The contribution of vertical transmission was insufficient to change whether intervention strategies predicted MAP persistence when high shedders were 100 times as infectious as low shedders (Fig. 2.6, models B and C: $\varepsilon = 100$). Intuitively, dam-to-daughter transmission alone would be insufficient to maintain infection in a population. This was quantified for Neospora caninum in dairy herds (French et al., 1999). However, when the effect of vertical transmission is combined with the presence of transient shedding (Tr_1 and Tr_2) of calves and heifers, then dam-to-daughter transmission appeared to have a real impact on infection spread.

If vertical transmission is highly successful, it becomes important to evaluate the plausibility of output from simulations without intervention. In this scenario, we observed only a small drop in infection prevalence in herds with aggressive intervention strategies (data not

shown). This is not consistent with the effects of current control strategies that successfully decrease prevalence of MAP in high-prevalence herds.

4.3. Evaluation of transient shedding

Following contact with cows at birth, calves are usually in much closer contact with other calves than cows for the next 12–24 months. If calves are excreting bacteria into the calf environment, there is a high risk of infection for susceptible animals. On most farms, calves will have a rate of contact with other calves up to several orders of magnitude greater than that of rate of contact with cows.

Biologic plausibility of active MAP shedding from calves has been shown in the available peer-reviewed literature (Rankin, 1959; Waters et al., 2003). Rankin (1961a) used an infection transmission experiment to determine whether and when shedding would occur following natural calf-to-calf infection. These animals might have been exposed to an elevated dose of MAP relative to farm levels (e.g. the equivalent of high-shedding adult animals), but the experiment showed that animals infected from other shedding calves can be culture-positive within a short time-frame. More recently, van Roermund et al. (2007) successfully illustrated calf-to-calf transmission among naturally infected animals. Although there are no peer-reviewed studies of calves with positive culture results in a farm environment, risk factors for MAP at a farm level include an association with group-housing calves (Wells and Wagner, 2000; NAHMS, 2005). While other factors associated with group housing might contribute to this finding, infected calves shedding MAP via active shedding or passive transport would explain this association.

The model outcome was sensitive to both the rate of contact (RC) and infectiousness (π) assigned to calves (Fig. 2.7). In model B, where age-based differences in mixing and herd

turnover were not addressed, transiently shedding animals needed to be as infectious as or more infectious than low-shedding adults to cause MAP persistence during intervention. In model C, age-based mixing and turnover were incorporated and rate of contact among calves was assumed to be 10 times that of calves and cows. For all model C simulations it was assumed that infectious shedding among calves was substantially below that of low-shedding adult animals (π). Based on culture data indicating that calves do not shed as frequently or as much as adults, this assumption is more plausible than that of model B. Increasing intra-class contact ($RC_{1;10}$) quickly led to calf-to-calf contacts being the primary source of infectious transmission and high MAP-infection prevalence (data not shown). We do not see this phenomenon in real life (Crossley et al., 2005), where average within-herd cow-level prevalence is low, and one can decrease prevalence of disease by focusing on reducing infectious adult animals. Transient-shedding heifers did not have increased rate of contact with calves relative to cows, and thus had a much smaller input into disease dynamics than did transiently shedding calves. At this point we do not have sufficient field data for the ratio of infectiousness between low- and high-shedding adults or the contribution of transient shedders to determine which model more accurately predicts herd prevalence.

5. Conclusion

It is clear from all models that high-shedding animals have an important impact on prevalence of MAP infected animals in a herd. However, more complex (and we argue more realistic) models used in this study illustrated that these animals are not alone responsible for infection persistence. Our models indicate that maintenance of infection in a low-prevalence herd that aggressively tests for shedding animals is caused by other (potentially multiple) factors.

Persistence of infection with a relative low level of prevalence is more easily predicted in models that incorporate dam-to-daughter transmission and calf-to-calf transmission.

6. Acknowledgments

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Appendix A. Differential equations for solving models

Differential equations for solving models

1 Model A

Equations 1-3 indicate rate of entry and exit for each category in model A.

(1) $dX_1 / dt = \mu N - (\mu_d + \rho + \lambda_A) X_1$
(2) $dY / dt = \lambda_A X_1 - (\delta + \mu_d + \alpha_1) Y$
(3) $dX_2 / dt = \rho X_1 - \mu_d X_2$

Equation 4 restricts the population to a constant size and equation 5 adjusts μ_d when there is disease-related culling.

(4) $dN / dt = 0$
(5) $\mu = \mu_d + (\alpha_1 + \delta) Y / N$

Equation 6 calculates for force of infection in model A.

(6) $\lambda_A = \beta_{3A} Y / N$

2 Model B

Equations 7-12 indicated rate of entry and exit for each category in model B.

(7) $dX_1 / dt = (\mu - \gamma) N - (\mu_d + \rho + \lambda_B) X_1$
(8) $dX_2 / dt = \rho X_1 - \mu_d X_2$
(9) $dTr / dt = \lambda_B X_1 + \gamma N - (\phi + \mu_d) Tr$
(10) $dH / dt = \phi Tr - (\mu_d + \sigma) H$
(11) $dY_1 / dt = \sigma H - (\delta_1 + \mu_d + \upsilon) Y_1$
(12) $dY_2 / dt = \upsilon Y_1 - (\delta_2 + \mu_d + \alpha) Y_2$

Equation 13 restricts the population to a constant size and equation 14 adjusts μ_d when there is disease-related culling.

(13) $dN/dt = 0$
(14) $\mu = \mu_d + \delta_1 Y_1 / N + (\alpha + \delta_2) Y_2 / N$

Equation 15 calculates force of infection.

(15) $\lambda_B = \beta_{12B} Tr / N + \beta_3 Y_1 / N + \varepsilon \beta_3 Y_2 / N$

3 Model C

Equations 16-24 indicate rate of entry and exit for each category in model C.

(16) $dX_1 / dt = (\mu - \gamma) N - (\lambda_C + \rho_1 + \mu_1) X_1$
(17) $dX_2 / dt = \rho_1 X_1 - (\mu_2 + \rho_2) X_2$
(18) $dX_3 / dt = \rho_2 X_2 - \mu_3 X_3$
(19) $dTr_1 / dt = \lambda_C X_1 + \gamma N - (\mu_1 + \rho_1) Tr_1$
(20) $dTr_2 / dt = \rho_1 Tr_1 - (\mu_2 + \phi) Tr_2$
(21) $dH_2 / dt = \phi Tr_2 - (\mu_2 + 2\rho_2) H_2$
(22) $dH_3 / dt = 2\rho_2 H_2 - (\mu_3 + \sigma) H_3$
(23) $dY_1 / dt = \sigma H_3 - (\delta_1 + \mu_3 + \upsilon) Y_1$
(24) $dY_2 / dt = \upsilon Y_1 - (\delta_2 + \mu_3 + \alpha) Y_2$

Equations 25-28 define populations in each age class. Equation 25 restricts the population to a constant size and equation 30 adjusts μ_3 when there is infection-related culling.

(25) $N_1 = X_1 + Tr_1$
(26) $N_2 = X_2 + Tr_2 + H_2$
(27) $N_3 = X_3 + H_3 + Y_1 + Y_2$
(28) $N = N_1 + N_2 + N_3$
(29) $dN/dt = 0$
(30) $\mu = \mu_1 N_1 / N + \mu_2 N_2 / N + \mu_3 N_3 / N + \delta_1 Y_1 / N + (\alpha + \delta_2) Y_2 / N$

Equation 31 calculates force of infection.

(31) $\lambda_C = \beta_1 Tr_1 / N + \beta_2 Tr_2 / N + \beta_3 Y_1 / N + \varepsilon \beta_3 Y_2 / N$

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CHAPTER 3

Survival of *Mycobacterium avium* subsp. *paratuberculosis* in bovine monocyte-derived macrophages is not affected by host infection status but depends on the infecting bacterial genotype[◊]

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Abstract

In this study we investigated the ability of different *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) strains to survive in bovine monocyte-derived macrophages (MDMs) of cows naturally infected with *M. paratuberculosis* and control cows. We tested the hypotheses that infection status of cows affects macrophage killing ability and that survival of *M. paratuberculosis* in macrophages is dependent on the strain. Peripheral blood mononuclear cells (PBMC) were obtained from Johne's disease-positive (n = 3) and age and stage of lactation matched Johne's disease-negative (n = 3) multiparious cows. Following differentiation, MDMs were challenged in vitro with four *M. paratuberculosis* strains of different host specificity (cattle and sheep). Two hours and 2, 4, and 7 days after infection, ingestion, and intracellular survival of *M. paratuberculosis* strains were determined by fluorescence microscopy. There was no effect of the origin of MDMs (Johne's disease-positive or control animals) on phagocytosis, survival of bacteria, or macrophage survival. In contrast, important strain differences were observed. These findings suggest that some *M. paratuberculosis* strains interfere more successfully than others with the ability of macrophages to kill intracellular pathogens which may make it important to include strain typing when designing control programs.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*M. paratuberculosis*) causes paratuberculosis (Johne's disease), a chronic, incurable granulomatous enteritis of ruminants (Buergelt et al., 1978; Chiodini et al., 1984). It is widely accepted that cattle usually become infected in utero or as calves through oral uptake of the organism from contaminated feed or the environment (Sweeney, 1996; Sweeney et al., 1992). Infected animals pass through two preclinical stages of the disease during an incubation period of 2–10 years. During the first, silent stage, *M. paratuberculosis* infection usually remains undetectable by currently available tests, while animals in the second preclinical stage may have increased antibodies detectable by available serological tests and/or may shed the bacteria in their feces (Whitlock and Buergelt, 1996).

To this day, our knowledge of host immune responses to *M. paratuberculosis* and the genetic constitution of the host potentially influencing infection as well as the genetic properties of the bacterium affecting its virulence remains incomplete (Coussens, 2001; Motiwala et al., 2006). To elucidate the interaction between *M. paratuberculosis* and the host immune system, a number of studies have investigated gene expression of PBMC from Johne's disease-positive and healthy control animals (Coussens et al., 2003, 2004a,b, 2005). Further, research has been carried out on the differences in cytokine profiles of PBMC and monocytes from *M. paratuberculosis*-infected animals and control animals (Khalifeh and Stabel, 2004; Stabel, 2000;Weiss et al., 2005). However, with the exception of a study by Weiss et al. (2005), none of the investigations have sufficiently addressed survival of the bacterium as a function of infection status of the animal.

Genetic predisposition to intracellular infections of macrophages with *Brucella abortus*,

M. bovis BCG, and *S. dublin* has been documented in cattle (Qureshi et al., 1996) and has been suggested for infections of sheep and cattle with *M. paratuberculosis* (Gonda et al., 2006; Nielsen et al., 2002; Reddacliff et al., 2005). These studies suggest that host genetics may play an important role in susceptibility to *M. paratuberculosis* infection and therefore are a possible reason for differences in the ability of macrophages to kill the organism between hosts of the same species.

Phenotypic strain differences between *M. paratuberculosis* isolates causing Johne's disease in cattle and sheep have been identified as early as the middle of the last century (Taylor, 1951). Only now have recent developments in the field of molecular biology and genomics provided the research community with tools that facilitate genotypic characterization of *M. paratuberculosis* isolates, representing a major advancement for epidemiological investigations of the disease (Dohmann et al., 2003; Harris et al., 2006; Motiwala et al., 2006). Multilocus short sequence repeat (MLSSR) typing has been used in genotypic analysis of *M. paratuberculosis* isolates derived from individual animals of a diverse range of hosts (Amonsin et al., 2004; Motiwala et al., 2004). This allows the analysis of virulence of different genotypes in vivo and in vitro, which will improve our understanding of the molecular pathogenesis of Johne's disease and may aid in the design of a better strategy for controlling the infection.

The objectives of this study were twofold. First, we evaluated potential differences in phagocytic and killing ability of monocyte-derived macrophages (MDMs) from cows that were test-positive by fecal culture and ELISA for *M. paratuberculosis* infection in comparison with matched cows of the same herd that had consistently shown negative results in both test categories. Second, we evaluated differences in intracellular survival in MDMs between different strains of *M. paratuberculosis.* Further, we studied the cytotoxicity of the different *M.*

paratuberculosis strains used in the experiment.

2. Materials and methods

2.1. Animals

The animals used in this study were in their third lactation and part of a 340-dairy-cow operation in New York State. The infection status of the animals had been monitored by serum ELISA (ParaCheck; CSL/Biocor, Omaha, NE) every 3 months and by fecal culture testing at least once every 6 months during a period of 16 months prior to initiation of the experiments. Fecal culture testing was conducted by the Johne's Research Laboratory, New Bolton Center, University of Pennsylvania, Kennett Square, PA, as described by Crossley et al. (2005). Johne's disease test-positive cows (n = 3) had been fecal culture-positive with between 0.75 and 50 CFU/tube for a minimum of three consecutive tests; they had also been positive by serum ELISA in at least one test. These animals showed no clinical signs of Johne's disease. Johne's disease test-positive animals had exclusively tested positive for infection with one *M. paratuberculosis* genotype (strain 1180, strain genotype described below) used in this study. The control animals (n = 3) had shown negative responses in all tests during the monitoring period. Control cows were matched with test-positive cows by days in milk, parity, and pregnancy status. None of the animals had shown clinical signs of any disease and had not been treated systemically with antimicrobial agents for at least 30 days prior to blood collection. Characteristics of the six animals in this study are presented in Table 3.1. The results of ELISA and fecal culture for all animals are detailed in Table 3.2.

Table 3.1 Characteristics of animals. Blood samples for the isolation PBMC were taken on different dates. One test-positive and one test-negative animal were matched and sampled per post-infection measurement.

	Animal ID					
	1180[a]	1184[a]	1160[b]	1194[b]	1179[c]	1038[c]
Test-positive	Yes	No	Yes	No	Yes	No
Date of birth	11/17/2000	11/26/2000	08/19/2000	12/06/2000	11/11/2000	06/12/1999
Days in milk	318	330	432	321	385	696
Days pregnant at sampling	216	238	0	0	0	0

[a]06/15/2005. [b]06/30/2005. [c]07/14/2005.

Table 3.2. The Paracheck ELISA (CSL/Biocor, Omaha, NE) was used in this study; samples were considered positive (marked with *) if the final OD was greater than kit control plus 0.1. The fecal culture test was performed as described by Crossley et al. (2005). Four tubes of Herrold's egg yolk medium (HEYM) were inoculated with aliquots of each sample. Individual value (a) represent colony forming units (CFU) per tube, while a range of value (b) describes the total of CFU for all four tubes. On some test dates the FC was not performed for test-negative animals (c).

ELISA and fecal culture (FC) test results

Test	Date	Animal ID					
		1180	1184	1160	1194	1179	1038
FC	02/2004	11,6,10,4 a	Neg	Neg	Neg	Neg	Neg
ELISA		0.462 *	0.053	0.057	0.050	0.063	0.068
ELISA	06/2004	0.756 *	0.067	0.056	0.054	0.053	0.068
FC	09/2004	75–150 b	Neg	38,22,32,25 a	Neg	0,2,0,1 a	Neg
ELISA		2.512 *	0.072	0.146	0.099	0.270*	0.075
FC	01/2005	75–100 b	– c	150–200 b	– c	1,1,0,1 a	– c
ELISA		1.334 *	0.064	0.537 *	0.064	0.160	0.067
FC	04/2005	150–200 b	Neg	75–100	Neg	3,7,7,3 a	Neg
ELISA		1.135 *	0.072	0.754 *	0.099	0.120	0.075
FC	07/2005	100–200 b	– c	155–250 b	– c	0,7,0,6 a	– c
ELISA		1.237 *	0.066	1.160 *	0.056	0.336 *	0.096

2.2. Blood collection, isolation of PBMC, and preparation of autologous serum

Approximately 800 ml of blood for the isolation of PBMC were obtained from the jugular vein of each cow using sterile blood collection bags J-520Q or J-520D with citrate phosphate dextrose adenine solution (CPDA-1) or acid citrate dextrose solution (ACD) as anticoagulant (Jorgensen Laboratories, Inc., Loveland, Co.). Additionally, between 150 and 200 ml of blood for the preparation of autologous serum were collected from the jugular vein or tail vein into 10-ml tubes without anticoagulant (VacutainerTM, Becton Dickinson, Franklin Lakes, NJ). All blood samples were stored at approximately 15 ºC for 4 h during transport to the laboratory.

Blood for the isolation of PBMC was transferred under sterile conditions from the blood collection bags into sterile glass bottles and subsequently into 50-ml centrifuge tubes. The blood was centrifuged at 1000 g and 22 ºC for 10 min. Buffy coats were harvested using sterile 5-ml plastic pipettes after discarding most of the plasma. Two buffy coats were pooled and resuspended in PBS (without Ca^{2+} and Mg^{2+}); the total volume of this mixture was adjusted to 50 ml. Twenty-five milliliters of cell suspension each were then carefully layered over 25 ml of Histopaque-1077 (Sigma–Aldrich, St. Louis, MO) in fresh centrifuge tubes. Cells were centrifuged at 400 g and 22 ºC for 30 min to separate erythrocytes and polymorphonuclear cells from mononuclear cells. PBMC were harvested from the PBS-Histopaque interface and washed three times with PBS at 4 ºC and 200 g for 10 min. The isolated mononuclear cells were resuspended in RPMI 1640 with Glutamax^{TM} I and 25 mM HEPES (Invitrogen, Carlsbad, CA). Except for cell cultures derived from cows 1180 and 1184, the growth medium was supplemented with 0.25 mg amphotericin B (Nitrogen, Carlsbad, CA)/ml. The cell density was adjusted to approximately 7 x 10^6 cells/ml, and autologous serum was slowly added to the cell

suspension. The final serum concentration of the growth medium was set at 12% as recommended by Campbell and Adams (1992). PBMC were incubated for 5 days at 37 ºC and 5% CO2 in 350-ml Teflon jars (Savillex, Minnetonka, MN) and 50-ml Teflon flasks (Nalgene Company, Rochester, NY) to allow monocytes in the unfractionated PBMC cultures to develop into macrophage precursor cells.

To obtain autologous serum, corresponding blood samples in collection tubes were allowed to clot for at least 6 h. Subsequently, the tubes were centrifuged at 4 ºC and 1800 g for 25 min, and serum was transferred into 50-ml centrifuge tubes and then micron filtered using 0.22-mm Steriflip-GP Filter Units (Millipore, Billerica, MA). The serum was aliquoted and stored at -20 ºC until use.

2.3. Cell culture conditions and infection

After the incubation period, cells in Teflon containers were resuspended by careful pipetting and centrifuged at 4 ºC and 200 g for 10 min in a fresh sterile tube. The cell pellets were resuspended in RPMI 1640 with Glutamax^{TM} I and 25 mMHEPES, and 2% of autologous serum (complete medium). Except for cell cultures derived from cows 1180 and 1184, the growth medium was supplemented with 0.25 mg amphotericin B/ml. Aliquots of cells were prepared in a 1:10 dilution in Trypan Blue, and a cell count was performed with an Olympus CK2 light microscope utilizing a hemocytometer. Macrophage precursor cells could easily be identified by their size and granularity, which allowed an overall estimation of the number of these cells per ml of cell suspension. Further aliquots of the cell culture were prepared for histological investigation by cytocentrifugation and subsequent staining of the cells on glass slides with Wright's stain. This procedure was completed to validate the above-described method

to determine the macrophage precursor cell fraction. The cell density was adjusted with growth medium to the correct seeding density of macrophage precursor cells. The vast majority of PBMC were utilized for experimental purposes not reported here. PBMC for the described assay were seeded at a density of 1 x 10^5 macrophage precursor cells into wells of 24-well culture plates containing 12 mm diameter glass cover slips. Cell cultures were challenged with bacteria at multiplicity of infection (MOI) of 5:1 the next day. To avoid removal of lymphocytes from the cultures, only part of the culture medium was replaced 3 h, 2 days, and 4 days after infection. Then, 0.2 ml of medium was carefully removed from the top of the undisturbed wells of the 24-well plates and 0.3 ml of fresh complete medium added.

2.4. Bacterial strains

Four *M. paratuberculosis* strains and one non-*M. paratuberculosis* positive control (strain 6043, member of the *M. avium-intracellulare* complex) as well as a negative control (medium alone) were used for the experiments. *M. paratuberculosis* strain 1018 (short sequence repeat [SSR] fingerprint: 7G4GGT) which had been isolated from a fecal sample of an individual animal at a dairy herd in Ohio where multiple strains of *M. paratuberculosis* were present. *M. paratuberculosis* strain 7565 (SSR fingerprint: 15G3GGT) had been isolated from an intestinal tissue sample of a sheep with Johne's disease. This strain showed typical ovine strain characteristics in culture. *M. paratuberculosis* strain 1180 (homologous farm strain) (SSR fingerprint: L1:7G L8:6GGT) was isolated from animal 1180 enrolled in this study. *M. paratuberculosis* strain 1099 (SSR fingerprint: L1:7G L8:5GGT) was also isolated from a Johne's disease-positive animal of the study farm, but none of the study animals were culture-positive for this heterologous farm strain. All strains were genotyped by a modified multilocus

short sequence repeat (MLSSR) described by Amonsin et al. (2004). Strains 1018, 7565, and 6043 are further characterized for differences in macrophage interaction elsewhere (Janagama et al., 2006).

All cell-infection experiments in this study were performed with bacteria derived from serial passages and dilutions of the respective bacterial stock cultures. Seven to 14 days prior to cell infection bacteria were grown in Middlebrook 7H9 broth (Difco laboratories, Detroit, MI) supplemented with 10% oleic acid albumin dextrose catalase (OADC; Becton Dickinson Microbiology System, Sparks, MD) and 2 mg/ml of Mycobactin J (Allied Monitor Inc., Fayette, MO). Bacterial density was determined using a hemocytometer count. In brief, each culture was vortexed for 10 s and subsequently incubated with 1:1 volume of 4% buffered formalin for 15 min after which it was syringed 10 times with a 23-g needle to break up clumps of bacteria. Based on the average of two hemocytometer counts, sufficient medium containing live bacteria was syringed 10 times with a 23-g needle and then diluted with complete medium to produce an MOI of 5:1 for cell infections. Negative control PBMC cultures received complete medium with an average volume of Middlebrook 7H9 medium supplemented with 10% OADC and 2 mg/ml of Mycobactin J to control for the effect of Middlebrook 7H9 broth on cell cultures.

2.5. Fluorescence microscopy

Samples for fluorescent microscopy were collected four times: 2 h and 2, 4, and 7 days after experimental infection. First, 100 mg carboxyfluorescein diacetate (CFDA) (Invitrogen, Carlsbad, CA) were dissolved in 1000 ml DMSO, and 220 ml of this master mix were diluted 1:25 in infection medium. Then, 150 ml of CFDA solution were added to each well of the 24-well plate after removal of the growth medium. Cells were incubated for 20 min at 37 ºC.

Thereafter, coverslips were transferred into new 24-well plates. Coverslips were fixed with 0.5 ml of 4% formaldehyde each for a minimum of 15 min at 4 ºC. MDMs on the coverslips were subsequently permeabilized with 100%methanol for 20 min at -20 ºC. Methanol was removed and the cells were counterstained with 0.5% Evans Blue in PBS for 20 min in the dark at room temperature, after which the coverslips were washed twice with PBS. The coverslips were removed from the wells, washed in PBS, and mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes, Eugene, OR) on glass slides. To prevent the mounting medium from dehydration coverslips were sealed using clear nail polish. Slides were stored in the dark at 4 ºC. Slides containing three coverslips each (triplicate set-up) were assessed at 400x magnification with an Olympus System Microscope BX41 and the FITC filter (excitation [ex] 480 nm, band pass [BP] 40, emission [em] 535 nm, BP 50); TRITC filter (ex 545, BP 30, em 620 nm, BP 60), and DAPI filter (ex 365 nm, BP 10, em 460 nm, BP 50). Images were captured by a MicroFireTM camera, Model S99809 (Optronics, Goleta, CA) and analyzed with Picture-FrameTM, Version 2.1 software (Optronics, Goleta, CA). Five defined visual fields were captured per coverslip (as read on a clock face, 12 o'clock, 3 o'clock, 6 o'clock, 9 o'clock and the center of the slide). TRITC (cytoplasm ofMDMs) and FITC (viable bacteria) filter images were merged in PictureFrameTM, and cells were evaluated on the screen (Fig. 3.1). Only MDMs that were located entirely within the boundaries of the picture were considered. MDMs were classified in five different categories: MDMs with zero, 1–10, 11–20, 21–50, and >50 bacteria per cell.

2.6. Flow cytometric analysis

PBMC cell phenotypes were assessed for each animal by single-color flow cytometric analysis on the day after isolation and on the day after completion of the incubation period in Teflon jars. The monoclonal antibodies employed in this study were directed against CD4 (CACT138A), CD8 (CACT80C), the γδ T cell receptor 1-N24 (GB21A), CD14 (MM61A) and a yet unspecified surface molecule which is expressed on bovine B cells and is recognized by the antibody BAQ155A. All antibodies were obtained from VMRD, Pullman, WA. Before fixing with 2% formaldehyde and subsequent antibody staining, cells were treated with ethidium monoazide (EMA; Molecular Probes, Eugene, OR) according to a protocol by De Rosa (2004) to mark dead PBMC. Individual cell culture aliquots (1 $x10^6$ cells/100 ml aliquot) were stained with unconjugated primary antibodies and a FITC-conjugated secondary antibody. Briefly, samples were incubated for 15 min on ice with (concentration 15 mg/ml) of the primary antibody, washed with FACS buffer (PBS with 5% FBS and 0.02% sodium azide) and finally stained with a secondary FITC-conjugated antibody for 20 min at a concentration of 10 mg/ml (goat anti-mouse IgM + IgG + IgA (H + L); SouthernBiotech, Birmingham, AL). After a final washing step, cell pellets were resuspended in 500 ml of FACS buffer and analyzed on a LSR II cytometer (BD Biosciences, San Jose, CA). The data generated were analyzed for 30,000 EMA negative cell events. Analysis was performed with FlowJo software Version 4.6.2 (Tree Star Inc., San Carlos, CA).

2.7. Statistical analysis

Results of the three separate phagocytosis experiments with a pair of one test-positive and one test-negative animal were combined. A linear mixed model was used for analysis of

data. The proportion of MDMs and the number of bacteria per infected MDM were calculated for each of 5 viewing fields in the 3 cover slips on a slide during 4 time periods with 5 strains and one control in a total of 6 cows (3 test-positive and 3 test-negative) for a total of 2160 observations. To estimate the average number of bacteria per infected cell, the number of cells per category (1–10, 11–20, 21–50, and >50) were multiplied by 5, 15, 35, and 75, respectively. Means were calculated for each relevant subgroup. Standard error estimates of the mean were obtained using the square root of the usual (binomial and normal) variance divided by the sample size. The comparison of test-positive versus test-negative animals and the comparison of strains were performed in generalized linear mixed models. The proportion of infected cells or the number of bacteria per infected MDM was the outcome variable, while the experiment numbers (1–3), test-positive versus test-negative animals, strain indicator, and potential interactions were the predictor variables. Correlation within viewing field and within cover slip was accounted for using hierarchical random effects. The phagocytic index (percent infected cells times bacteria per infected cell) was used to combine parameters for bacteria per infected cell and percent infected cells in the raw data set as described by Zurbrick and Czuprynski (1987).

Flow cytometric data were checked for outliers. The Wilcoxon rank sum non-parametric method was used to compare cell populations between test-positive and test-negative animals and between the results on day 1 and day 5.

Statistical Analysis System (SAS) Version 9.1 (SAS Institute Inc., Cary, NC) was used to analyze the dataset. We controlled for experiment number in the analysis of the results. Statistical significance was set at $p = 0.05$.

3. Results

3.1. Johne's disease status of cows did not influence macrophage infection and survival of bacteria

Fluorescence microscopy was applied to visualize phagocytosed bacteria and to calculate the phagocytic index (Fig. 3.1). At 2 days post-infection, the phagocytic index was highest for all strains except strain 1099, which peaked 4 days post-infection. The positive control showed a consistently high phagocytic index in all experiments. Table 3.3 shows the results of the parameters for percent of infected cells and bacteria per infected cell for the cells derived from test-positive and test-negative animals. Results are presented for the four measurement times (2 h and 2, 4, and 7 days after infection) and for the five strains used. Results of generalized linear models showed no evidence of significant differences between infection results in cells derived from test-positive compared to cells derived from test-negative animals ($p > 0.9$) for any of the five bacterial strains. Fig. 3.2 shows the results of the individual cows with the phagocytic index at 2 days after experimental infection for the homologous farm strain 1180 and the bovine strain 1018. There was no significant difference between the test-positive and test-negative animals.

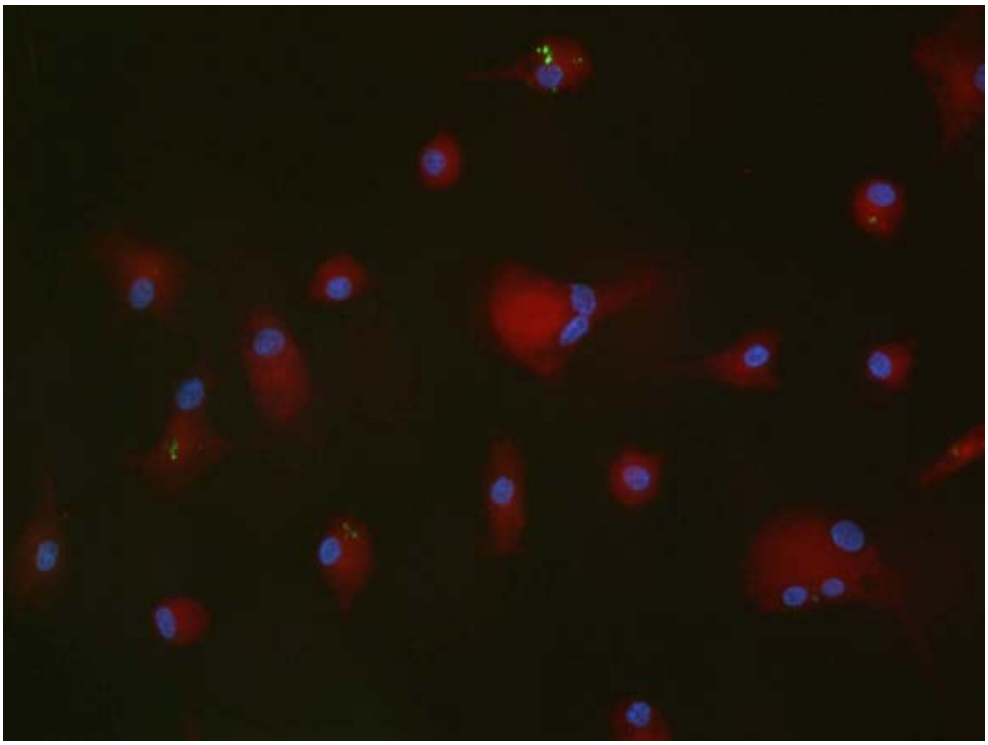

Fig. 3.1. Image of macrophages of cow 1194 infected with strain 1180 at post infection measurement time 3.Visual fields were assessed at 400x magnification with an Olympus System Microscope BX41 using the FITC, TRITC, and a DAPI filter. Three images were captured using different filters and merged in PictureFrameTM to be evaluated on the screen. MDMs (red) located completely within the boundaries of the picture were counted and assessed for bacteria (green). Infected macrophages were classified in five different categories according to the number of bacteria (zero, 1–10, 11–20, 21–50, and >50 bacteria) per cell. For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.

Table 3.3. Percent of infected cells and bacteria per infected cell summarized for each strain by measurement time and for the Johne's test-positive (n = 3) and test-negative (n = 3) cows. Bovine MDMs were incubated with the indicated strains at an MOI of approximately 5 in the presence of autologous serum for 2 h or 2, 4, or 7 days. At each post-infection measurement time, the cells were fixed, stained, and evaluated with fluorescence microscopy. The percentage of infected cells was calculated, and the numbers of bacteria per infected cell were estimated at each measurement time. Results are expressed as the mean ±S.E.M. from five coverslips per experiment with three replicates for each experiment. There was no significant difference between test-positive and test-negative animals with any of the bacteria tested ($p > 0.90$). Thus, the above values represent the average per group (test-positive [n = 3] and test-negative [n = 3]).

Strain	Time after experimental infection	Johne's test-negative (n = 3)		Johne's test-positive (n = 3)	
		Percentage of infected cells	Bacteria/cell	Percentage of infected cells	Bacteria/cell
1018 (bovine; Ohio farm)	2 h	23 ±4	11 ±2	32 ±4	12 ± 2
	2 days	39 ±3	13 ±1	48 ±4	12 ± 1
	4 days	36 ±4	13 ±2	32 ±3	12 ± 1
	7 days	19 ±3	12 ±1	24 ±4	13 ± 2
1099 (heterologous farm strain)	2 h	26 ±4	22 ±3	33 ±5	20 ± 3
	2 days	42 ±5	23 ±3	33 ±5	20 ± 4
	4 days	41 ±5	22 ±3	45 ±4	25 ± 3
	7 days	37 ±4	20 ±2	38 ±5	19 ± 3
1180 (homologous farm strain)	2 h	33 ±5	24 ±2	47 ±6	26 ± 3
	2 days	42 ±6	20 ±2	46 ±5	26 ± 3
	4 days	38 ±6	19 ±2	28 ±5	19 ± 3
	7 days	36 ±5	17 ±2	25 ±5	19 ± 2
6043 (positive control)	2 h	58 ±5	19 ±2	70 ±6	19 ± 2
	2 days	71 ±5	31 ±3	76 ±3	21 ± 3
	4 days	82 ±3	32 ±3	50 ±5	15 ± 2
	7 days	80 ±3	31 ±3	42 ±5	15 ± 2
7565 (ovine)	2 h	46 ±4	17 ±2	52 ±5	21 ± 3
	2 days	59 ±4	19 ±2	82 ±4	27 ± 2
	4 days	58 ±4	14 ±1	52 ±5	15 ± 2
	7 days	36 ±3	15 ±2	42 ±5	16 ± 2

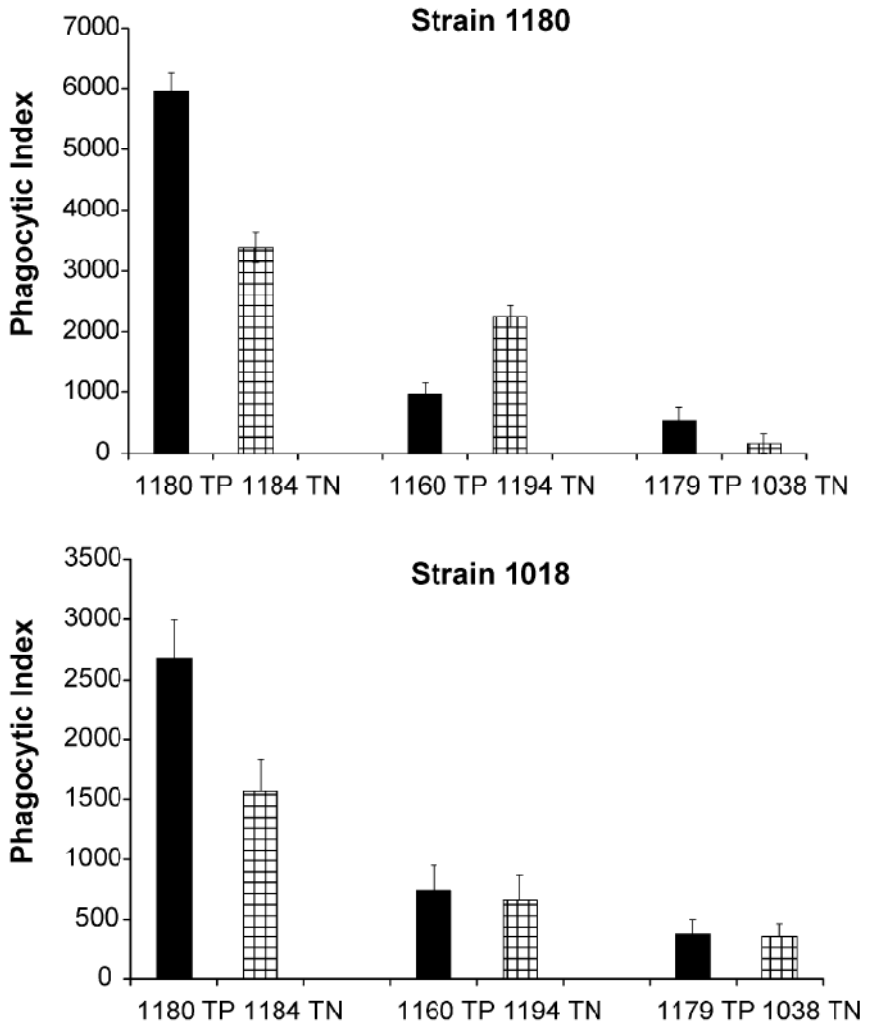

Fig. 3.2. Phagocytic index of MDMs infected with the indicated strains of *M. paratuberculosis* after 2 days of incubation. The test-positive (cow number followed by TP) and the test-negative (cow number followed by TN) animals are indicated along the horizontal axis. The animals are grouped in pairs reflecting the actual set-up of the experiment. No significant difference was observed between test-positive and test-negative cows (value ± SE).

3.2. M. paratuberculosis genotype affected the percent of infected MDMs and the total numbers of phagocytosed bacteria per infected cell

Differences between strains were observed in both the percentage of infected MDMs and the number of bacteria per infected MDM. Consistently, the homologous and heterologous *M.*

paratuberculosis strains 1180 and 1099 from the source farm were closest in growth pattern to the positive control strain 6043, whereas bovine strain 1018 was consistently the least successful in both percent of infected MDMs and bacteria per infected MDM. Results are presented in Table 3.4. The differences in the phagocytic index of the three bovine *M. paratuberculosis* strains are given in Fig. 3.3A. The source farm strains were significantly different (generalized linear mixed model result, $p < 0.05$) from strain 1018, and were not significantly different from one another. The ovine strain initially showed a very high number of bacteria per MDM, but subsequently was the only strain that showed a significant decline in bacteria per infected cell over time (Fig. 3.3B).

3.3. Number of MDMs per visual field continuously increased over time

The average number of MDMs per visual field for most strains and the negative control constantly increased over time ($p < 0.001$). Test-negative animals tended to have higher numbers of MDMs per visual field ($p < 0.03$) than test-positive cows. Among the infected MDMs, those infected with ovine strain 7565 showed a pattern similar to that of the MDMs in non-infected wells, with a rapid increase between 4 and 7 days. In contrast, MDMs infected with the bovine strains and the positive control strain 6043 did not show marked proliferation at measurement time 4 (Fig. 3.4).

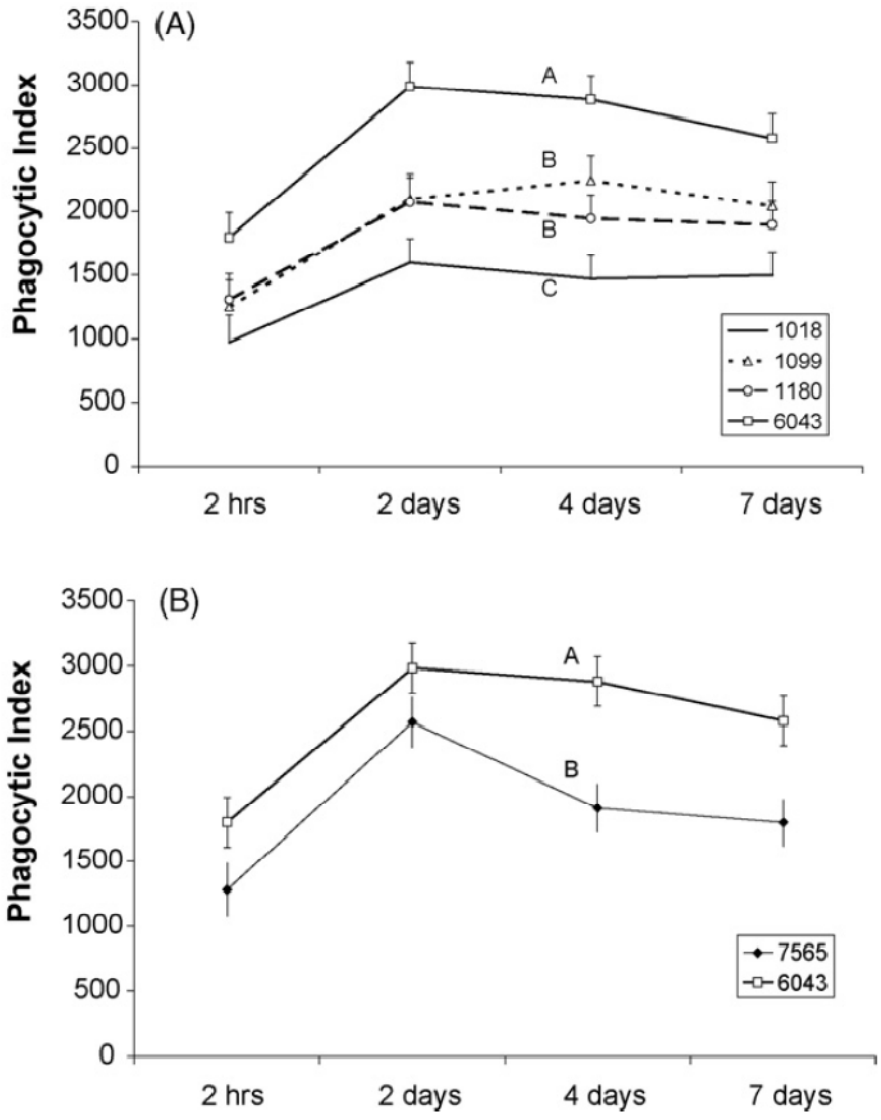

Fig. 3.3. Survival of the indicated strains of *M. paratuberculosis* and the positive control strain 6043 (member of the *Mycobacterium avium-intracellulare* complex) by MDMs over a period of 7 days. (A) Strain 1099 is the heterologous farm strain, strain 1180 is the homologous farm strain, and strain 1018 is an isolate from an Ohio dairy herd. Significant differences were observed between strains 6043, 1099, and 1180 and 1018, as indicated by different letters. (B) Strain 7565 is the ovine strain. Significant differences were observed between strains 6043 and 7565, as indicated by different letters. The results for (A) and (B) are presented as the phagocytic index (percent of infected cells times bacteria per infected cell).

Table 3.4. Least square means of percent infected cells, bacteria per infected cell and phagocytic index summarized for each strain by measurement time after experimental infection. MDMs were incubated with the indicated *M. paratuberculosis* strains at an MOI of approximately 5 in the presence of autologous serum for 2 h, 2, 4, and 7 days. At each measurement time, the cells were fixed, colored, and evaluated with fluorescence microscopy. The percentage of infected cells was calculated and the numbers of bacteria per infected cell were estimated for each measurement. The phagocytic index is shown in the last column. Results are expressed as the least square mean ±S.E.M. from a generalized linear mixed model that included intercept, strain, test-positive vs. test-negative animals, experiments (1–3) and measurement time. Significant differences between strains are indicated by different letters in columns.

Strain	Time after infection	Percentage of infected cells	Bacteria/cell	Phagocytic Index
1018 (bovine; Ohio farm)	2 h	26 ±13 D	15 ±2 C	973 ±211 C
	2 days	70 ±4	20 ±1	1599 ±187
	4 days	55 ±9	20 ±2	1472 ±186
	7 days	40 ±7	22 ±1	1499 ±185
1099 (heterologous farm strain)	2 h	37 ±4 C	24 ±3 AB	1252 ±212 B
	2 days	59 ±4	29 ±3	2100 ±194
	4 days	66 ±5	30 ±3	2246 ±191
	7 days	64 ±3	28 ±2	2045 ±189
1180 (homologous farm strain)	2 h	42 ±6 CD	24 ±2 B	1310 ±203 B
	2 days	60 ±8	27 ±2	2075 ±196
	4 days	58 ±8	28 ±2	1945 ±187
	7 days	55 ±6	26 ±2	1897 ±183
6043 (positive control)	2 h	78 ±3 A	22 ±2 A	1798 ±192 A
	2 days	89 ±3	32 ±3	2985 ±190
	4 days	87 ±4	31 ±3	2882 ±190
	7 days	82 ±5	29 ±3	2581 ±191
7565 (ovine)	2 h	51 ±7 B	20 ±2 B	1281 ±205 B
	2 days	87 ±2	29 ±2	2569 ±194
	4 days	78 ±3	22 ±1	1909 ±186
	7 days	62 ±5	23 ±2	1796 ±178

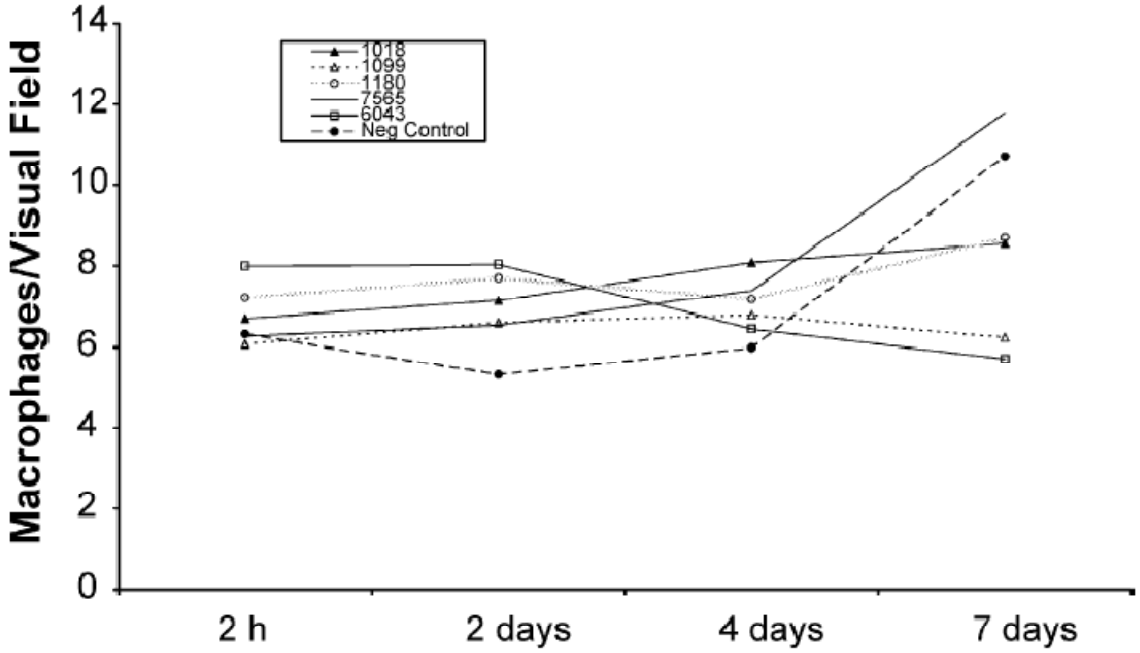

Fig. 3.4. Number of MDMs per visual field over a period of 7 days. All the bacterial strains and the negative control (medium only) are shown. MDMs infected with strains 6043 and 1099 had decreased proliferation of adherent cells between measurement times 3 and 4 relative to the other strains and the negative control.

3.4. Unfractionated PBMC flow cytometry results changed after 5 days of incubation

First, EMA-positive cells were gated to exclude dead cells. Remaining cells were further gated in the forward scatter (FSC) and sideward scatter (SSC) to exclude cell debris. Finally, specific antibody staining of the preselected cells was analyzed in the green fluorescence channel. Final results are presented in Fig. 3.5, which shows the cell profile separately for test-positive and test-negative animals on either day 1 or day 5 of PBMC culture. Non-parametric statistical testing indicated that the proportion of cells did not differ between test-positive and test-negative animals, but that there was a significantly different profile on day 5 compared to the starting distribution on day 1. A significant (Wilcoxon rank sum test, $p < 0.05$) increase was observed in the proportion of CD4-positive and CD8-positive cells over the 5 days of incubation. A significant decrease was observed for CD14-positive

cells. There was no difference in the relative distribution of γδ T cells and B cells.

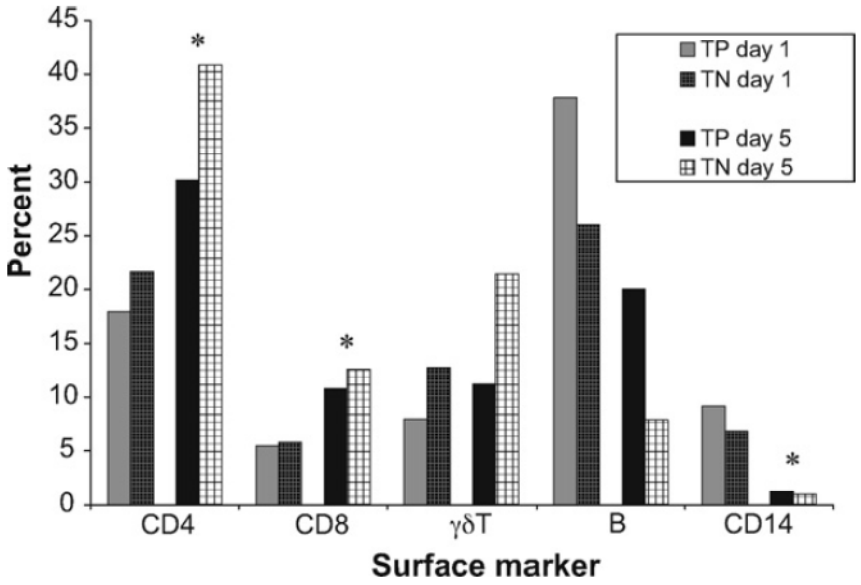

Fig. 3.5. FACS results of PBMC 1 day after isolation and after 5 days of incubation with autologous serum in Teflon containers just before experimental infection with mycobacteria. There was no significant difference between test-positive (TP) and test-negative (TN) animals in any of the cell fractions on either day 1 or day 5. However, the relative distribution between day 1 and day 5 changed significantly ($p < 0.05$) for CD4+, CD8+, and CD14+ cells.

4. Discussion

The interaction between host and infectious agent determines the eventual outcome of the infection. In this study we investigated the influence of host infection status (test-positive versus test-negative for Johne's disease) and strain differences (five strains, including the positive control) on the outcome of *in vitro* infection in MDMs.

Despite a century of research, Johne's disease remains one of the most mysterious and challenging infectious diseases of cattle. Intriguingly, only a small percentage of animals exposed to *M. paratuberculosis* can later be detected as infected with this pathogen (Johnson-Ifearulundu and Kaneene, 1999). This could be due to a difference in the ingested dose of the pathogen (Rankin, 1959) and/or the age of the animal at the time of exposure (Taylor, 1953). However, there is strong evidence that host factors are exceptionally important to

susceptibility to mycobacteria (Bellamy, 2003). Several genes have been identified as potential determinants in *M. tuberculosis* infection. These include human natural resistance-associated macrophage protein gene (NRAMP1, recently renamed Slc11a1), and susceptibility to tuberculosis 1 locus (sst1). Mouse studies indicate there may be several additional genes associated with resistance to mycobacteria (tbs1 and tbs2, tuberculosis severity loci; Trl-1, Trl-2, Trl-3, Trl-4, tuberculosis resistance loci) (Bellamy, 2006). In *in vitro* experiments with multiple *M. tuberculosis* strains, Li et al. (2002) found substantial host-to-host variability in bacterial propagation. As with *M. tuberculosis*, patterns of resistance to *M. paratuberculosis* may also be host-genetic related (Qureshi et al., 1996). There is evidence for inheritance of susceptibility to *M. paratuberculosis* infection in animals raised under the same conditions (Gonda et al., 2006; Koets et al., 2000; Mortensen et al., 2004; Nielsen et al., 2002). Furthermore, Reddacliff et al. (2005) found an association between host MHC-II and NRAMP alleles with *M. paratuberculo*sis clinical status in sheep.

In this study we compared intracellular survival of different *M. paratuberculosis* strains isolated from cattle and sheep in MDMs from peripheral blood of cows. We chose two groups of subjects to isolate MDMs from: animals that had repeatedly tested positive for Johne's disease and matched cows with negative results in all tests prior to the onset of experiments. To investigate potential variations in genetically determined susceptibility between cows of different infection status, we chose animals raised under the same management conditions from the same farm. As these animals had grown up in the same environment, we assume them to have been exposed to a similar microbial population. If this was true, then animals which were test-negative had either eliminated the agent or suppressed shedding to a level below the threshold of detection and thus are classified as resistant. Animals which shed *M. paratuberculosis* are less capable of fighting off infection and are

termed susceptible.

We assumed that all MDMs used in this study were equally capable of phagocytosing bacteria. We accounted for the differences due to the experimental run by always pairing a test-positive with a test-negative animal and by using a linear mixed model for statistical analysis. Due to the experimental set-up we cannot estimate the proportion of live and dead bacteria using our counting data. However, comparison of real time PCR data with the microscopy data will allow addressing the question of whether there might have been a higher proportion of dead bacteria in the MDMs of test-negative animals compared to the cells of test-positive animals because in the PCR assay bacterial DNA of live and dead cells will be accounted for. Thus, potential differences might be demonstrated when interpreting the PCR data in the light of the findings of the currently reported experiments. In the described assay we only kept track of live (green fluorescing) bacteria by utilizing carboxyfluorescein diacetate as an indicator of bacterial metabolic activity. The diacetate groups of the CFDA molecules are hydrolysed by intracellular esterases which results in the formation of the fluorescein molecules carboxyfluorescein; these are retained in cells. There is no esterase activity in nonviable cells, thus these cells do not fluoresce (Hoefel et al., 2003). Bacteria used for the assays were always prepared in the same manner. Thus, we assumed a high viability of bacteria and an equal proportion of live bacteria in the different infection media.

There was no effect of the origin of MDMs (resistant or susceptible animals) on phagocytosis, survival of bacteria, or macrophage survival in this dataset. This is in agreement with the findings of Weiss et al. (2005), who investigated the survival of one *M. paratuberculosis* strain in peripheral blood monocytes from infected and control animals from the same farm. However, this contrasts with findings of Khalifeh and Stabel (2004) which indicated that macrophages from infected cows are less capable of killing *M. paratuberculosis* than those from negative control animals.

The Weiss et al. study used a pure monocyte culture with no contact with autologous lymphocytes, while Khalifeh and Stabel used an unfractionated culture system with both macrophages and lymphocytes. Because our culture system closely resembled that of Khalifeh and Stabel, with a mixed culture at time of infection, we hypothesized that macrophages from subclinically infected animals would be less successful in killing *M. paratuberculosis* bacteria compared to cells from control cows.

The difference in the findings could be due to the small sample size in our study (n = 3 animals/group), resulting in a low power of statistical tests, or to the fact that we used only a population of subclinically infected animals, while Khalifeh and Stabel used both subclinical and clinical animals. In addition, we cannot rule out the possibility that our test-negative animals had previously been exposed to or were infected with farm-specific *M. paratuberculosis* strains. It has been reported that between 30% and 47% of cattle that were consistently fecal culture negative but originating from infected herds will have culture-positive tissues at the time of slaughter (Meyer zu Vilsendorf, 1995; Whitlock and Buergelt, 1996).

In the context discussed here, it may be that previous exposure to *M. paratuberculosis* is more important than current shedding status. Weiss et al. (2005) report that their control animals originated from the same farm as Johne's disease-positive animals, but that those cows did not show any signs of infection with *M. paratuberculosis* at necropsy (indicating a population that would have been classified as resistant in our study). Because Khalifeh and Stabel (2004) used animals from premises without any history of *M. paratuberculosis* as their control animals, those animals were not necessarily more resistant to infection, but had not been exposed. All of the positive animals in that study would have been classified as susceptible by our definition. A difference in cellular and humoral immunity of exposed and non-exposed animals was previously reported (Huda et al., 2004), but has not been evaluated

in terms of killing capacity of macrophages.

M. paratuberculosis strains exhibit *in vivo* virulence differences at a host species level (Motiwala et al., 2006; O'Brien et al., 2006; Saxegaard, 1990). Although there are no specific proteins identified which explain species specificity, recent sequencing of the M. paratuberculosis genome (Li et al., 2005) and microarray analysis showed genome level differences between sheep and cattle strains (Marsh et al., 2006). Shin et al. (2006) identified by insertion sequence mutagenesis of several genes responsible for lower survival and distribution of *M. paratuberculosis* mutants in a mouse model. There are three conserved regions present in *M. avium* subsp. *avium* containing 24 open reading frames which are present in cattle strains of *M. paratuberculosis* but lacking in sheep strains, and this may play a role in host specificity (Marsh et al., 2006). Host cytokine profiles, matrix metalloproteinases, and tissue inhibitor of matrix metalloproteinases differed when MDMs from naive young cattle were infected with *M. paratuberculosis* strains isolated from human, ovine, or bovine hosts (Janagama et al., 2006). There are few data on virulence differences between bovine-specific *M. paratuberculosis* strains in vivo or in vitro. Janagama et al. (2006) noted a trend towards association of some MLSSR strain types with low morbidity.

We were interested in survival characteristics of *M. paratuberculosis* in MDMs from animals which had previously been naturally exposed to *M. paratuberculosis*. MDMs were infected with two *M. paratuberculosis* strains from the host farm, a bovine-specific strain which was from a different farm as well as an ovine specific strain, and a non-*M. paratuberculosis* positive control (member of the *M. avium-intracellulare* complex). Three of the five strains had previously been analyzed in naive control animals (Janagama et al., 2006). In that study, bovine strain 1018 survived better than ovine strain 7565. Our additional bovine strains had SSR fingerprints (7G/6GGT and 7G/5GGT) which were distinct from that of strain 1018 (7G/4GGT). The assumption that the 7G/4GGT profile is one of reduced

pathogenicity was testable and we found that the bovine strains were more successful at survival than the ovine and the positive control strains. Farm-specific strains were very similar in behavior to each other and more successful than bovine strain 1018.

Such strain-specific survival differences are well characterized in other mycobacterial species, with *M. tuberculosis* strains classified by their virulence in cell culture or in experimental infections of mice. Li et al. (2002) found that there is not a strong correlation between virulence as measured by CFU counts of bacteria in PBMC pure culture experiments and in whole mouse models. Since we utilized a mixed culture MDM system, we expect that our results are more reflective of the immune status of the animal than when purified MDM cultures alone are used. Strain differences may result in some strains showing high levels of virulence or high contagiousness. However, these two characteristics (virulence and contagiousness) are likely independent and not necessarily both present in one specific strain. Tuberculosis strain CDC1551 is a highly contagious, but relatively avirulent, strain of *M. tuberculosis*, resulting in a high rate of contacts testing positive for exposure, but few individuals developing clinical signs (Manca et al., 2001). This is the first study showing potential virulence differences among bovine-specific *M. paratuberculosis* strains. Increased understanding of strain-specific behavior will make it possible to design more effective control strategies and could help answer some of the still open questions in Johne's research. If there are multiple strains present on a farm (Harris et al., 2006), there is the potential for strain competition, with multiple strains competing for the same niche in the host system. Due to the nature of our visual assays, we likely do not observe definite bacterial counts. However, all comparisons between animals and strains were made using the same technique, so we feel comfortable that our conclusions are valid.

Cytotoxicity of mycobacterial strains has been linked to virulence. Highly virulent strains of *M. tuberculosis* can suppress apoptosis of host macrophages, allowing bacterial

survival and propagation (Raja, 2004). In our experiment, the number of macrophages for all cultures (including the uninfected culture) increased over time, probably as a result of continued maturation of monocytes to become adherent macrophages over the course of the experiment. It is possible that antigenic stimulation also caused proliferation in differentiated macrophages (Luo et al., 2005). MDMs infected with bovine *M. paratuberculosis* strains (1018, 1180, and 1099) had a decrease in proliferation of adherent cells between measurement times 3 and 4 relative to both controls (negative and positive control strain 6043) and MDMs infected with the ovine strain (7565). This suggests that bovine *M. paratuberculosis* strains reduce fitness or maturation of host macrophages.

Although our study suggests that there are no obvious differences in macrophage killing capacity in test-positive and test-negative cows, the data cannot be used to draw conclusions on the animals' respective macrophage killing capacity as calves or as naive adults exposed to the pathogen. It is the latter that more likely determines eventual infection status. This question could be efficiently explored with long-term longitudinal studies. This study demonstrated differences in phagocytosis and intracellular survival of four different genotypes of *M. paratuberculosis* isolates utilized in PBMC infection assays. The bacterial isolates from the farm on which the study animals were housed were more successful in invasion and survival in MDMs than bacterial strains to which the animals had not been previously exposed. We found no important differences in in vitro macrophage killing abilities between cows currently shedding *M. paratuberculosis* and matched test-negative controls. However, important differences were observed between strains of *M. paratuberculosis.* Nevertheless, the presented results need to be interpreted with caution due to the small number of animals used in our experiments. Further studies in this area would be essential to understand differences in pathogenesis and transmission characteristics of *M. paratuberculosis* strains and their associated impact on infection control programs.

5. Acknowledgements

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CHAPTER FOUR

Quantification of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) survival in monocyte-derived macrophages[◊]

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Abstract

Real-time PCR assays were developed to quantitate *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in bovine monocyte-derived macrophages. We measured the absolute number of both host cells and bacteria in *in vitro* challenge assays. Results obtained from real-time quantitative PCR (qPCR) DNA copy counts were compared to visual quantitation of fluorescent-stained MAP in macrophages. Conclusions from our original visual analysis were supported by the second (qPCR) methodology; however, the qPCR assay proved to be more consistent between samples and was easier to perform. There was a strain-to-strain difference in growth curves between fluorescent quantitation (FQ) and qPCR that we believe to be a consequence of bacterial growth characteristics in FQ. In summary, real-time PCR assays provided a more accurate and precise method for evaluating intracellular growth dynamics when comparing strains of MAP.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes chronic granulomatous enteritis in multiple ruminant species (Sweeney, 1996). MAP is estimated to cost the US dairy industry alone upwards of 200 million dollars annually (Ott et al., 1999).

Strains of MAP are host adapted at a species level, and cross-species infections tend to have clinical manifestations that diverge from infections due to host-specific strains (Stabel et al., 2003, Stewart et al., 2007). For this reason, it is important to have reliable assays for quantifying survival and growth characteristics of strains of multiple host specificities, including bovine-specific MAP strains. Until recently, we have lacked reliable methods to type and investigate strains beyond the level of host specificity (Motiwala et al., 2003). Recent addition of multilocus short sequence repeat (MLSSR) strain typing techniques to the arsenal of typing tools allows better differentiation among bovine-specific isolates (Amonsin et al., 2004, Harris et al., 2006).

Recently Janagama et al. (2006) reported increased uptake and survival of a bovine-specific MAP strain by bovine monocyte-derived macrophages (MDMs) in comparison to both ovine- and human-specific MAP strains. These data were similar to our previous work (Gollnick et al., 2007) that indicated that host specificity of MAP strains was associated with bacterial replication and macrophage survival in bovine MDMs. Bovine-specific MAP strains showed significantly better survival in bovine MDMs compared to ovine-specific strains. Host infection status (test-positive or test-negative) did not influence bacterial survival irrespective of MAP strain used, indicating that previous exposure of MDMs to MAP did not affect *in vitro* bacterial survival (Gollnick et al., 2007).

We previously used microscopic fluorescence-based assays to semi-quantify both the number of host MDMs and the number of MAP bacteria in *in vitro* challenge assays. This method was very time-consuming and, as with all microscopy-based assays, somewhat

subjective. Because cells do not distribute uniformly within sample wells, it often happens that there is high within-sample variability in number of cells per visual field and number of bacteria per cell. In addition, we observed that if coverslips were not oriented with the maximum density distribution in the same location from one sample to the next on the same slide, there was a low reproducibility of data between slides. To improve on the precision and reproducibility of these assays we have developed a quantitative real-time PCR (qPCR) assay for both host cell and bacterial genome copies. The qPCR assay evaluates total number of bacterial and host genomes within a defined sample, but does not provide the same depth of information about bacterial and host cell interactions as the FQ assay. FQ provides information on number of bacteria within individual cells as well as percent of macrophages infected over time.

The objective of this report is to evaluate data from a single experimental setup using these two quantitation systems. The resulting data demonstrate that qPCR is a more rapid method, with significantly less inter-strain variability in comparison to fluorescent quantitation (FQ).

2. Materials and methods

2.1. Animals, cells, and culture

For a thorough description of animals, monocyte-derived macrophage culture methods and information on MAP strains used, we refer to our previous report by Gollnick et al. (2007). In brief, age-matched pairs of MAP-test-positive and MAP-test-negative 5–6-year-old lactating cows from a single farm were selected for peripheral blood mononuclear cell (PBMC) collection. All pairs of cows had been tested for MAP semi-annually by fecal culture and quarterly by serum ELISA for a minimum of 7 quarters. Buffy coat cells were separated via centrifugation in Histopaque media and allowed to mature. Cells were quantitated visually

and separately by FACS analysis to determine the population of live cells at time of infection. Cells were plated in both 24-well tissue culture plates with glass coverslips for visual quantitation experiments and 6-well tissue culture plates for quantitative real-time PCR experiments.

Macrophages were infected with a multiplicity of infection (MOI) of five bacteria to each macrophage. Four different strains of MAP (strains 1018, 1099, 1180, 7565) and one strain of *M. avium intracellulare* (MAI) were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 10% oleic acid albumin dextrose catalase (OADC; Becton Dickinson Microbiology System, Sparks, MD) and 2 μl/ml Mycobactin J (Allied Monitor, Inc., Fayette, MO). Two of the MAP strains were assumed to be bovine-specific and isolated from the same farm as the cow cell donors used for the experiment (strains 1099 and 1180), one was a bovine-specific strain from a farm in Ohio (1018), and one was a sheep-specific strain (7565). The MAI strain with two IS900 insertion sequences (strain 6043) was used as a positive control, negative control was infection media alone. For additional strain information we refer to our previous work (Gollnick et al., 2007).

2.2. Microscopic fluorescent quantitation

Methods for FQ have been previously published in detail (Gollnick et al., 2007). In brief, macrophages on coverslips were permeabilized with alcohol and stained with carboxyfluorescein diacetate (Invitrogen, Carlsbad, CA). Destaining removed the background from macrophages. A 4′-6-diamidino-2-phenylindole (DAPI) stain was used to color both bacteria (green) and macrophage nuclei (blue). Macrophages and bacteria per macrophage were counted via fluorescent microscopy. Phagocytic index was defined as the average number of bacteria per cell weighted by number of macrophages present. As mentioned in our initial work (Gollnick et al., 2007), bacteria per cell was a categorical measurement (0, 1–10,

11–20, 21–50, >50). Total number of bacteria was calculated by multiplying cell population by midpoint value of each category except >50, which was assigned a value of 75 bacteria per cell.

2.3. Quantitative real-time PCR methods

2.3.1. DNA isolation

Prior to collecting adherent cells from the 6-well plates, media was removed and cells were washed twice with culture media to remove non-adherent cells. One ml of cold aqueous buffer (10 mM Tris pH 8,1 mM EDTA pH 8) was placed in each well and incubated for 10 min at 4 °C. Cells were removed using a 25-mm cell scraper (Sarstedt, Newton, NC) into a 2-ml tube with 500 µl of 0.1 mm glass beads (Biospec products, Bartlesville, OK). These samples were then frozen until further processing.

Samples were thawed and 750 µl of phenol:chloroform:isoamyl alcohol pH 6.7 (Fisher Scientific, Pittsburgh, PA) was added to each 2-ml tube. Tubes were shaken for 3 min in a MiniBeadbeater-8 (Biospec Products, Bartlesville, OK) to lyse both macrophages and MAP. All liquid from the 2-ml tubes was placed in a 2.0 Heavy Phaselock tube (Eppendorf, Hamburg, Germany) and spun for 5 min at 16,000 × *g* at room temperature. 500 µl of the top phase from the Phaselock tubes was transported to a new vial with 50 µl Proteinase K (Qiagen, Inc., Valencia, CA) and 500 µl Qiagen buffer AL (Qiagen, Inc., Valencia, CA). The QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA) was then used as directed in the manufacturer's protocol with several listed modifications. Following introduction of buffer AL and proteinase K, lysates were incubated 30 min at 70 °C. DNA was then bound, washed and extracted as per manufacturer's instructions. Two separate incubations at room temperature for 10 min in 150 µl of ddH2O followed by a 3-min centrifuge at 6000 × *g* were

used for elution. Both an extraction and a media blank were included as a negative control for each extraction series and pair of animals.

2.3.2. qPCR parameters

All PCR tests were run in Fast Optical 96-well reaction plates (Applied Biosystems, Foster City, CA). Thermocycling was performed in a 7500 Fast machine (Applied Biosystems, Foster City, CA) using the standard heat block.

2.3.2.1. Bovine cells

GenBank sequences of bovine gapdh (XM617666, XM606919, XM618013, U85042, AJ000039, XM598051) were compared in Lasergene software to produce a 880-bp consensus that was used to select primers for a SYBR green PCR assay (Lasergene, Madison, WI). A 108-bp segment of the bovine GAPDH gene was selected based on high replication efficiency and low background amplification (Forward Primer: 5'-GGC CCC TCC CGT GCT TTT CCT T-3', Reverse Primer: 5'-TCC CCC GCT TCC CCT GCT TTC AA-3'). Primers were used at a final concentration of 500 nM in a 25-µl master mix that included 12.5 µl of iTaq SYBR green supermix with Rox (Roche, Indianapolis, IN), 5 µl of ddH2O, and 5 µl of genomic DNA. Following a 2:15 minute denaturation at 95 °C, samples were run for 35 cycles of 15 s denaturation at 95 °C, 30 s annealing/extension at 60 °C. Melt curves were run from 95 °C to 60 °C.

A DNA standard for quantitation was extracted from buffy coat cells of one cow (cow ID 1192). DNA was isolated using the same protocol as used for experimental samples, with the addition of Qiagen RNase and a 2-min incubation at room temperature prior to addition of buffer AL during the QIAamp DNA extraction protocol. Cells were not counted prior to DNA extraction, and sequential dilutions following DNA extraction were based on genome equivalents of DNA present. DNA concentration was determined on a SmartSpecTM3000

spectrophotometer (Biorad, Carlsbad, CA) and diluted to a maximum concentration of 5×10^4 genome equivalents per 5 µl (Vinogradov, 1998). Although the standard was created via two-fold dilutions, only 2.5e4, 6.25e3, 3.125e3, 390.6 and 97.7 genome equivalents/5 µl were run in triplicate on all plates to allow maximum number of samples. Aliquots of standards were made and frozen at −20 °C and thawed only once to prevent DNA degradation.

2.3.2.2. MAP

To identify appropriate primers for qPCR in the multiple MAP strains and MAI, a 1652-bp segment of the HSP65 gene was amplified on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA) using primers (102406Forward 5'-GCG GTT TCG GCG GCT TGT A-3', 102406Reverse 5'-CGG CGG GCG AGT TGC GGA CCT T-3') from Integrated DNA Technologies (IDT, Coralville, IA). The PCR products were sequenced via Applied Biosystems Automated 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) at the Cornell Life Sciences Core facility. Four primers were used for sequencing: (102406Forward, 112806seq1: 5'-TGC GGT TCG ACA AGG GTT ACA TCT-3', 112806seq2: 5'-GAC GAA GTA GCC CGA GAT GTA ACC-3', and 112806altR: 5'-CGG TAC CGG CGG GCG AGT TG-3'). A consensus sequence was formed with N substitution for all heterogeneous areas in DNAstar (Lasergene 6, Madison, WI) prior to use of PrimerExpress 2.0 software (Applied Biosystems, Foster City, CA) to identify potential targets for both primers and probes.

Real-time PCR amplification of a 109-bp segment of the HSP65 gene was performed using the primers (HSP65F 5'-TGA CGA AGT AGC CCG AGA TGT-3') and (HSP65R 5'-GGC AAC GAG GGC GTC AT-3') from Integrated DNA Technologies (Coralville, IA). A minor groove binding probe was selected with a 5'FAM tag and a 3' non-fluorescent quencher (5'-CCT TGT CGA ACC GCA-3') (ABS, Foster City, CA). Primers were used at a final

concentration of 900 nM and probe at a concentration of 250 nM in a 20-µl master mix that included 10 µl of TaqMan Universal master mix without UNG (Applied Biosystems, Foster City, CA), and 5 µl of genomic DNA. Following an initial activation at 95 °C for 10 min, quantitation was performed via 40 cycles of a two-step assay with denaturation at 95 °C for 30 s, and annealing and amplification at 60 °C for 60 s.

The standard for this assay was developed from extraction of 200 µl of each of 7H9 culture broth from the 5 MAP and MAI strains grown for 11 days in liquid media as listed above. DNA was isolated using the same protocol as experimental samples with the addition of Qiagen RNase (100 units/µl, 4 µl/200 µl sample) in the QIAamp kit. DNA concentration was determined on a Nanodrop 1000 (Thermoscientific, Wilmington, DE) and diluted to a maximum concentration of 10^8 genome equivalents per 5 µl of standard. Ten-fold dilutions of MAP genomic DNA from 10^7 to 10^1 genome equivalents per 5 µl were created for this assay. Only 10^7, 10^6, 10^4, 10^2, and 10^1 genome equivalents per 5 µl were used in the final standard run in triplicate on all five plates in order to maximize range while limiting the number of sample wells consumed.

2.4. Statistical analysis

All statistical analyses were performed in SAS version 9.1 (SAS Institute Inc., Cary, NC). For an in-depth description of analysis methods used for trends in macrophage survival and bacterial propagation, see our previous report (Gollnick et al., 2007). Phagocytic index, macrophage growth and number of bacteria were used as common outcomes for both qPCR and FQ. The qPCR assay did not allow differentiation of bacteria per cell or percent of cells infected although these parameters were measured in the FQ experiments. For qPCR, all adherent host cells were captured by the assay, thus phagocytic index is bacteria per adherent host cell rather than bacteria per macrophage and macrophage count reflects all adherent cells.

In FQ, where morphology allowed confirmation of macrophages as the predominant adherent cell type, phagocytic index and host cell number reflect only cells with macrophage phenotype. Less than 0.1% of all adherent cells observed in the FQ did not have typical macrophage morphology (data not shown).

For both methods, macrophage number was log transformed prior to running regression models. Linear mixed models equivalent to the models described previously (Gollnick et al., 2007) were used to evaluate phagocytic index and macrophage numbers. Phagocytic index and macrophage total number were modeled using as predictor variables experiment number (1–3), host-shedding status, a MAP strain indicator variable, and two-way interactions of these predictor variables. For FQ, phagocytic index weighted based on number of macrophages per field to control for the difference in variability in average due to a small (large variance) or large (small variance) number of macrophages. For qPCR, number of macrophages was sufficiently large and consistent that variances were not skewed by small numbers of cells in samples and this adjustment was not performed.

To evaluate split-sample repeatability within a testing technique, the null linear mixed model was run with sample as a repeated measure and a compound symmetric correlation matrix. Multiple measurements from the same sample (qPCR) or coverslip (FQ) were averaged prior to running this model.

To evaluate agreement between the quantitation methods, all measurements on a given time point from the same cow and bacterial strain were averaged by quantitation method. This provided 144 unique datapoints that could be compared pairwise. To put the two experimental methods on the same scale, all categories (bacteria, macrophages, bacteria per cell) were divided by the maximum observed value in a category. Correlation coefficients were not influenced by this scaling. Correlation analyses were performed on all paired measurements for macrophages, bacteria and PI. Normal probability plots were used to determine whether

macrophages and PI were normally distributed within and between strains (data not shown). Log transformation of number of macrophages allowed parametric comparison between the two methods using Pearson correlations. The FQ and qPCR values were compared by Spearman correlation to account for non-normal distribution of data points. Strain-specific correlations and host-infection-status correlations were also evaluated with identical methods.

3. Results and discussion

3.1. qPCR standards

The bovine genomic standard was linear over the entire range of concentrations evaluated, with an average amplification efficiency of 1.40 (range: 1.09–1.75, std dev: 0.141) and an average correlation coefficient of genomic copies and cycle number of 0.993 (range: 0.985–0.999, std dev: 0.003). There was no amplification in the negative control. The MAP genomic standard was linear over the entire range of concentrations evaluated, with an average amplification efficiency of 1.85 (range: 1.74–2.05, standard deviation: 0.086) and an average correlation coefficient of genomic copies and cycle number of 0.995 (range: 0.987–0.999, standard deviation 0.003). Any amplification of the negative control was always below the lowest standard dilution and was therefore reported as negative.

3.2. Experimental results

Overall, there was an acceptable level of agreement between the bacterial and cell numbers assessed by the two divergent methods. Macrophage numbers increased over time in both quantitation methods. The effect of cow, strain, and experiment number was consistent between techniques. Experiment number, cow, timepoint and strain predicted phagocytic index and bacterial numbers in both quantification methods. There were no significant interactions between the primary parameters in either regression model. There was no impact

of shedding status of cow on survival of MAP. In both experimental designs, MDMs infected with bovine-specific strains of MAP were more similar to non-infected cells than those MDMs infected with non-bovine-specific strains of MAP.

Although all main effects were consistent between the two models, the relationship between PI and strain was influenced by timepoint in FQ but not in qPCR. Neither analysis found a relationship between shedding status of animal and phagocytic index or bacteria count.

3.3. Split-sample repeatability

In our assay execution, we focused on decreasing the variability due to processing variation among equivalent samples. Correlation between repeated samples for macrophages (samples from the same strain, cow and timepoint) was 0.47 for qPCR and 0.37 for FQ. Correlation between bacterial samples was 0.66 for qPCR and 0.42 for FQ, and correlation between phagocytic index measurements for repeated samples was 0.59 for qPCR and 0.57 for FQ. Results indicated that both systems had an adequate level of agreement between repeated samples, but the qPCR technique showed more consistency among repeated samples than the FQ technique. This may have contributed to the absence of the timepoint-by-strain interactions in qPCR as presented above.

3.4. Agreement between assays

3.4.1. Macrophages

Log-macrophage numbers in the two assay techniques were evaluated using Pearson correlations and the two assay methods produced similar results (Pearson $R = 0.864$, $n = 144$, $P = < 0.0001$) (Fig. 4.1).

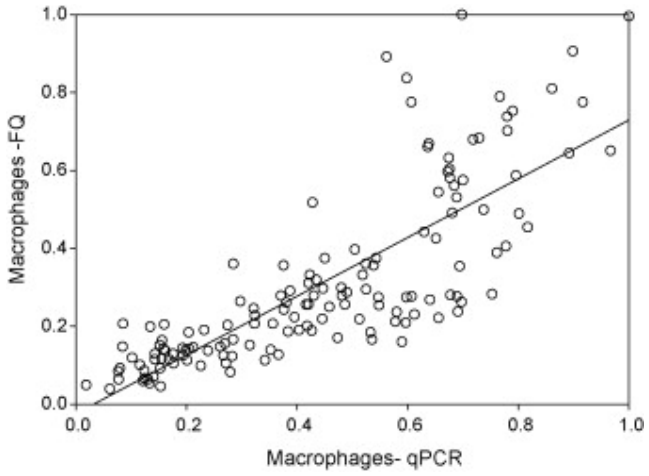

Fig.4.1. Macrophage count by qPCR (*x*-axis) vs FQ (*y*-axis). All values have been divided by the maximum value for each respective quantitation methodology. A perfect correlation would have a slope of one.

3.4.2. Phagocytic index/bacteria

For phagocytic index, the correlation between two assay methods was 0.68 (Spearman rank correlation, $n = 120$, $P < 0.0001$). Univariate descriptive analysis rejected the normal distribution for most strains (KS < 0.05). Strain-specific Spearman correlation coefficients between the methods were 0.74 (strain 1018), 0.91 (strain 1099), 0.62 (strain 1180), 0.94 (strain 6043) and 0.89 (strain 7565). All these correlations were statistically different from 0, with $P < 0.001$. No adjustments were made for multiple comparisons. It is worth noting that strain 1180, one of the bovine-specific strains, had a poor correlation between fluorescent and qPCR phagocytic index (Fig. 4.2).

Absolute bacterial numbers had the same trend as phagocytic index, but the correlations between assay methods were generally lower. Strain-specific Spearman correlation coefficients between the methods were 0.73 (strain 1018), 0.40 (strain 1099), 0.27 (strain (1180), 0.83 (strain 6043) and 0.66 (strain 7565). All correlations were again significant with $P < 0.001$ with the exception of strain 1099 ($P = 0.06$) and strain 1180 ($P = 0.20$).

3.5. Interpretation

From these results we conclude that for MDMs, FQ and qPCR produced data that are broadly comparable. However, variations in the phagocytic index between the different bacterial strains was a major driver in interpretation of the infection assays. Variation between assays in determining the absolute number of bacteria indicate that one or both methods may have reproducibility issues, but the correlation between the two methods was robust and remained strong on a per strain basis. Several options have been considered as potential contributors to the high but not perfect correlation between methods. If there were a population of non-macrophages

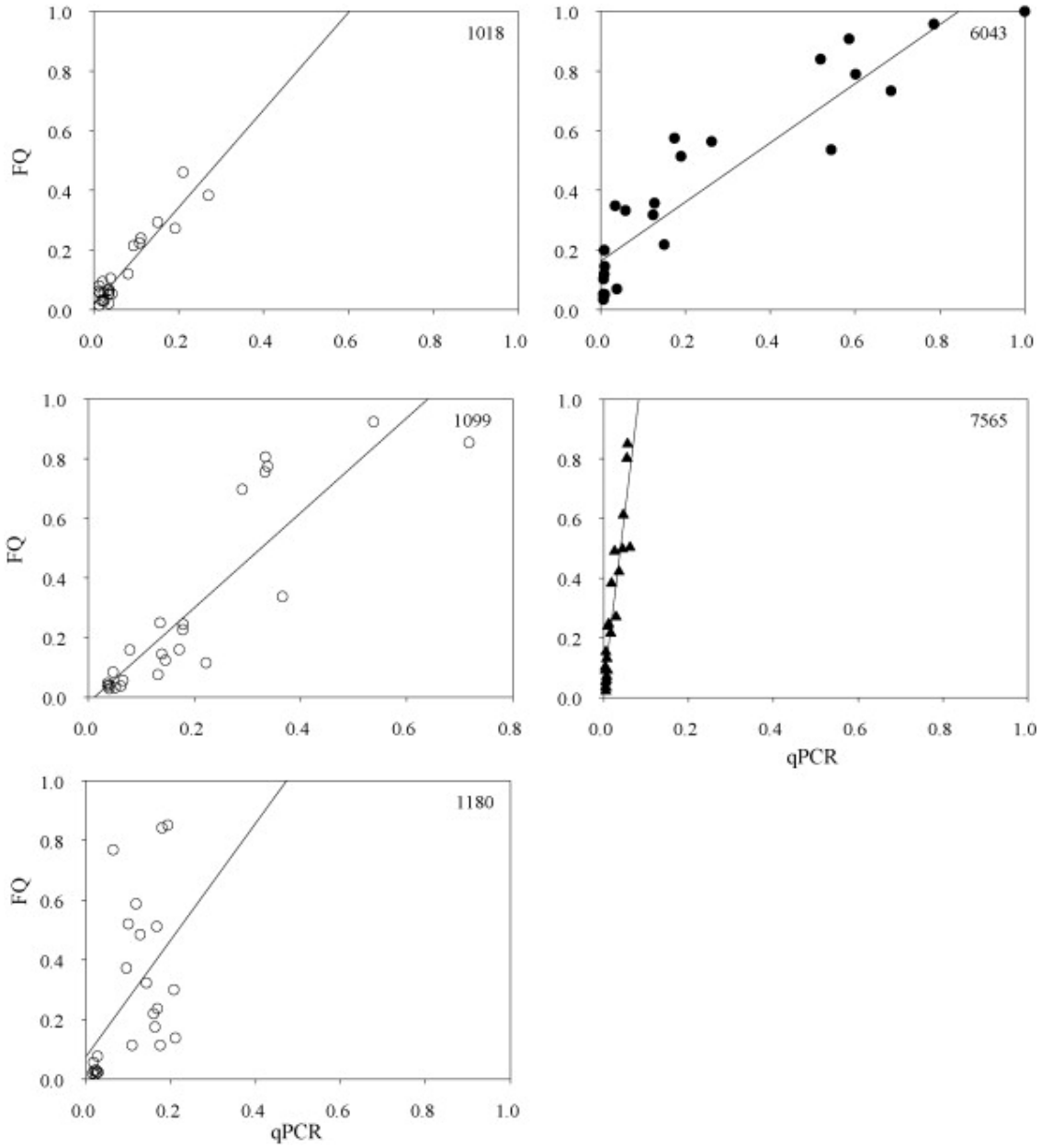

Fig. 4.2. Correlation between phagocytic index (PI) measurements using qPCR (*x*-axis) and FQ (*y*-axis) by strain. Trend lines represent least squares estimation. All values have been divided by the maximum value for each respective strain and quantitation methodology as in Fig. 4.1.

adhering to the culture plates they would have been eliminated in the FQ assay but not the qPCR assay where all adherent cells were analyzed. Based on the very low number of non-macrophage adherent cells in the FQ assay, this is an unlikely hypothesis. Dead bacteria can still have sufficient recoverable DNA to be detectable by PCR, but the cells no longer have

intact cell walls to maintain fluorescence (van der Vliet et al., 1994). This would elevate the total count of DNA in the qPCR data, but should not have a strain-to-strain bias. Heterogeneity of primer or probe binding sites could cause differential amplification of strains of MAP; however, the sequencing of the entire region prior to primer selection ensures that this is not a concern in our assay.

Fluorescent quantitation might be influenced by a strain-specific tendency of bacteria to clump so that more than one bacterium appeared in this microscopic assay as a single bacteria while the qPCR correctly identified the number of bacterial copies (Schleig et al., 2005). If clumping is consistent within strain, but variable between strains, then we would expect a pattern where individual strain analyses have fairly high correlation coefficients, but there is a wide range of between strain variation. This effect could explain our findings. Indeed, we see a greater level of agreement between strains from the same host (and more similar growth characteristics) than we do between strains from different hosts. We therefore suggest that the qPCR assay is likely to provide us with more accurate results as compared to the FQ assay.

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CHAPTER FIVE

Within-farm strain dynamics of *Mycobacterium avium* subsp *paratuberculosis*: Evidence for limited vertical transmission

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Abstract

MAP-infected dairy cattle are assumed to be a high risk for transmitting infection to their daughters. Alternatively, if both dam and daughter are genetically more susceptible to MAP, they may be both infected but not necessarily due to vertical transmission. Using strain typing techniques including multi locus short sequence repeat (MLSSR) typing allows a potential distinction between vertical transmission and genetic susceptibility. Analyzing strain diversity in longitudinal datasets provides additional insight into within-herd infection dynamics, including the transmission of MAP from dams to daughters.

To investigate the importance of vertical transmission, we identified 12 pairs of dams and daughters for which both animals are known MAP infected from the Regional Dairy Quality Management Alliance (RDQMA) study herd in NY. All adult animals on the farm were tested for MAP via fecal culture semi-annually for seven years. Tissue samples were available on a subset of cull animals. Animals were considered MAP-infected if they ever cultured positive or if any of their tissues cultured positive at slaughter. Cultures were performed at University of Pennsylvania on HEYM solid media. Positive cultures were substreaked and processed for MLSSR typing. Following genotyping, isolates from each dam-daughter pair were compared to determine whether they shared the same MAP genotype. Environmental MAP burden at birth was assessed via typing of MAP-positive environmental samples (collected four times a year) and known MAP-infected animals present on the farm during the high-risk first year of life.

Of the 12 infected dam-daughter pairs, 9 had identical strains shared between the dams and daughters. In addition, 2 daughters had the dam's strain as well as another circulating strain. Overall, there were 8 strains represented in the daughters that did not come from dams (2 daughters had multiple strains which did not originate from the dam). These results lend additional importance to the impact of genetics on susceptibility, as 5 of 12 daughters carried

different strains of MAP than their dams, even when concurrently infected with the dam's strain.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of an incurable, infectious, chronic, granulomatous enteritis in ruminants, called Johne's disease or paratuberculosis. The direct effects of Johne's disease on animal welfare and productivity may appear insignificant and are often masked by the largely subclinical character of the disease in domesticated livestock. The incubation period prior to the onset of clinical signs ranges from 2 to 10 years (Lepper, et al., 1989, Chiodini, et al., 1984). However, the impact of Johne´s disease on the US dairy cattle industry is substantial, including decreased milk production (Smith, et al., 2009), increased risk of being culled and a decreased calving rate in high-shedding animals (Smith, et al., 2010). Estimated costs of decreased weight gain, milk production loss, premature culling and reduced carcass value add up to an annual amount of $250 million (USD) in the United States industry alone (Ott, et al., 1999). In addition to the proven economic impact of MAP infection on ruminants, on-going controversy concerning the possible role of MAP in Crohn's disease (human inflammatory bowel disease) suggests that MAP itself might be of public health significance (Behr and Kapur, 2008; Feller, et al., 2007).

Cows are most susceptible to infection as calves from birth to a few months of age (Taylor, 1953; Windsor and Whittington, 2010), but can also be infected as adults (Doyle and Spears, 1951; Rankin, 1961). Evidence of intrauterine transmission and shedding of MAP in milk and colostrum has been reported (Seitz, et al., 1989; Sweeney, et al., 1992; Nielsen, et al., 2008). Although calf-to-calf transmission has been demonstrated in several experimental settings where calves were being housed together (Rankin, 1961; van Roermund, et al., 2007), horizontal infection from cows to calves and vertical infection from dam to calf are believed to be the most common and most important infection routes in dairy cattle (Marce, et al., 2010). Previous work also described that calves born from latent dams who would go on to

shed culturable levels of MAP or dams which were currently shedding MAP in their feces were more likely to shed MAP than calves without this risk-profile (Benedictus, et al., 2008). Calves growing up with a future high-shedder and calves being born shortly following the freshening of a MAP infected dam were at higher risk of being infected than those not exposed to future high-shedders or possibly contaminated calving environments (Benedictus, et al., 2008).

There is no satisfactory treatment for MAP infection. No antimicrobials are approved for the treatment of Johne's disease because of their lack of efficiency and their failure to provide bacteriological cure. A vaccine for MAP exists but is not commonly used because it offers only partial protection and interferes with interpretation of tuberculosis tests (Muskens, et al., 2002). Consequently Johne's disease is currently controlled via improving management practices and test-and-cull programs. The most cost-effective option for reducing the prevalence of MAP infection in herds is application of management measures to disrupt known transmission routes (Groenendaal and Galligan, 2003; Kudahl and Nielsen, 2009). Specific management recommendations for reducing within-herd MAP transmission focus on removing high-risk animals (Nielsen and Toft, 2007) and minimizing the exposure of newborn calves to potential MAP sources (Pillars, et al., 2011). Currently, none of these control measures address genetic susceptibility to Johne's disease.

Heritability of susceptibility to infection with MAP has been estimated to be between 0.06 and 0.159 (Koets, et al., 2000; Mortensen, et al., 2004; Hinger, et al., 2008; Attalla, et al., 2010). These heritability estimates are affected by the definition of infection, diagnostic methods used in the studies (blood or milk ELISA, fecal cultures, tissue cultures), study sample sizes, study population (breed) and statistical models (mixed animal model, sire model) used in these studies. It is therefore difficult to provide a precise estimate of heritability, other than that it appears to be greater than zero. Population-based heritability

estimates have resulted from host genome based studies to identify genetic markers for MAP susceptibility. Two genome-wide studies identified loci for MAP resistance (or conversely, susceptibility) to *MAP* infection using Illumina Bovine SNP50 assay (Settles, et al., 2009; Minozzi, et al., 2010). A SNP on chromosome 9 (BTA9) is associated with shedding (being fecal-culture positive) when truly infected (tissue-culture positive) (Settles, et al., 2009) and being ELISA positive (Minozzi, et al., 2010). Zanella et al. performed an association study on the same dataset as Settles et al. and found BTA6 to be associated with tolerance to paratuberculosis (Zanella, et al., 2011). Several candidate genes identified by these genome-wide studies have been examined for further association with infection. However, attempts to locate genes specifically associated with susceptibility or resistance to paratuberculosis have had limited success. Caspase recruitment domain 15 (CARD15) variants were associated with infection (Pinedo, et al., 2009a) in cattle, but not with clinical disease as reported in a comparative analysis of bovine, murine, and human CARD15 transcripts (Taylor, et al., 2006).Toll-like receptor 4 (TLR4) SNPs were associated with infection in one of two recent genomic analyses (Mucha, et al., 2009; Pinedo, et al., 2009b). The risk of MAP infection was associated with alleles of the SLC11A1gene (formerly NRAMP1) in two (Pinedo, et al., 2009a)(Ruiz-Larranaga, et al., 2010) but not in a third (Hinger, et al., 2007) recent study. Differences in diagnostic methods used in these studies may have contributed to the conflicting findings.

To investigate the importance of vertical transmission, studies have reported on the prevalence of fetal infection in cattle and thereby estimated the incidence of calves infected via *in utero* transmission. Based on a meta-analysis of the published literature, it was estimated that 9% of fetuses from subclinically MAP-infected cows (fecal-culture positive) and 39% of fetuses from MAP-infected cows with clinical signs of Johne's disease were infected with MAP prior to birth (Whittington and Windsor, 2009). True prevalence of fetal

MAP infection could be higher than reported in these studies due to low test sensitivity (Eamens, et al., 2000;Whittington and Windsor, 2009). Incidence of *in utero* transmission therefore depends on within-herd prevalence and the ratio of sub-clinical to clinical cases among infected cows (Whittington and Windsor, 2009).

Current methods in molecular genetics make it possible to discriminate between different bovine-specific MAP strains (Motiwala, et al., 2006). These molecular genetics techniques can also be used as a tool to investigate the possibility of vertical transmission by comparing MAP strains in samples from the dam with strains in samples from the daughter. When comparing different MAP fingerprinting techniques, the multi locus short sequence repeat (MLSSR) technique using all 11 loci selected by Amonsin et al. (2004) appeared to have the highest discriminatory power with a Simpsons diversity index of 0.967 (Motiwala, et al., 2006). Four of these identified repeats (locus 1, locus 2, locus 8 and locus 9) were used to describe MAP strains in a US-wide collection (Harris, et al., 2006) and to study within-herd transmission dynamics of MAP (Pradhan, et al., 2011).

Although it remains difficult to distinguish between the effects of heredity of susceptibility and vertical transmission in a dataset, modern strain typing techniques applied to precise longitudinal studies can serve as a precise method to address this distinction. The objectives of this study were to assess the most likely route of transmission in positive dam-daughter pairs and the importance of vertical transmission, through analysis of precise MAP strain typing results.

2. Materials and methods

2.1. Sampling

MAP isolates used in this study were part of a longitudinal dataset gathered by the Regional Dairy Quality Milk Alliance (RDMQA) on one of three commercial dairy farms in

the northeast US (Pradhan, et al., 2009). Complete details of the longitudinal study including methods of sample collection and preliminary microbial analyses are available in previous work (Pradhan, et al., 2011). Briefly, fecal samples were collected from all cows semiannually and cultured for MAP on Herrold's Egg Yolk Media (HEYM) with Mycobactin J at the University of Pennsylvania using their standard sample processing methods. Environmental samples were collected four times a year and also cultured for MAP using the same method. In addition, when animals were sold for slaughter, four intestinal tissue samples (lymph nodes and ileum) and a slaughter fecal sample were collected by USDA Food Safety Inspection Service personnel at the slaughterhouse and shipped to University of Pennsylvania on ice for culture.

MAP shedding levels were quantified based on growth of colonies on four HEYM slants (multiplied by a correction factor of 4.3) (Pradhan, et al., 2011). For a subset of samples that were initially qualified as too numerous to count (TNTC) additional dilutions of original fecal material were performed to obtain an accurate count. Individual positive samples were substeaked onto HEYM slants containing mycobactin J and were shipped to Quality Milk Production Services (QMPS) at Cornell University for molecular typing. Because samples often had many colonies per unit, the majority of samples were shipped as substreaks of multiple colonies, rather than representing a pure culture of only one colony per animal.

2.2. Selection of samples

The evaluation of the most likely route of transmission was assessed by strain-typing isolates from dam-daughter pairs. A dam-daughter pair was selected if both a dam and daughter that had at least one of the following: (A) any MAP positive fecal sample (B) any MAP-positive tissue or fecal sample collected at the slaughterhouse. To evaluate the

horizontal transmission probability, all MAP-positive samples identified on Farm A were further evaluated and any identified MAP strain was assigned a strain type. At least one tissue from every tissue-culture positive animal was strain-typed.

2.3. DNA extraction

DNA was extracted from the substreaked HEYM slants as described by Pradhan et al. (Pradhan, et al., 2011). Briefly, a sterile plastic 10 ul loop was used to remove a portion of the bacterial lawn from the substreaked samples. The bacteria were resuspended in sterile water (Invitrogen Corporation, Carlsbad, CA USA) and 250 ul of sterile 0.1 mm zirconia/silica beads (BioSpec Products, Inc. Bartlesville, OK USA) in a 2 ml beadbeater screwcap vial (Biospec Products, Inc., Bartlesville, OK USA). Cells were homogenized for 5 minutes at maximum speed in a Beadbeater-8 homogenizer (Biospec Products, Bartlesville, OK, USA). Following homogenization, DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA USA) following the manufacturer protocol with a few modifications. DNA was homogenized for 30 minutes at 70 °C prior to removing 600 µl to combine with 600 µl AL buffer and 60 µl of proteinase K (Qiagen, Valencia, CA, USA). This aliquot was mixed thoroughly and incubated for a further 30 minutes at 70°C. The remainder of the manufacturer protocol was followed exactly until elution in 150 µl sterile water. Eluted samples were stored at -20°C until used for PCR typing. Samples were extracted in small batches from 4 to 10 samples and an extraction negative was always processed alongside to ensure no contamination.

2.4. MLSSR

Initially the four loci described by Harris et al. (Harris, et al., 2006) were selected because of their diversity index and the existence of previous work using these loci to which

we could compare our findings. We elected to add five loci also described by Amonsin *et al.* (locus 3, locus 5, locus 6, locus 10 and locus 11) to increase specificity of our strain-typing methodology. The additional discriminatory value of these additional loci was evaluated after processing all isolates from the identified dam-daughter pairs and a subset of the all isolates available from farm A. When we did not observe diversity in this subset of samples (minimum of 60 samples/locus), the locus was not processed for the remainder of the samples. Locus 2 was not used in this analysis due to the difficulty in interpreting sequence reads when more than nine G repeats were detected.

PCR amplification was carried out with the extracted DNA for all isolates using the primers described in Amonsin *et al.* 2004(Amonsin, et al., 2004). Alternate primers for locus 1 were used on a subset of samples: 5'-GTG TTC GGC AAA GTC GTT GT-3' and 5'-GCG GTA CAC CTG CAA G-3'. Information about modified primers for locus 1 was obtained from the Center for Genomics and Veterinary Population Medicine Department, University of Minnesota, St. Paul, Minnesota. The 25-µl PCR amplification reaction mixture for each SSR contained: 2X GoTaq Green Master Mix (Promega Corporation, Madison, Wisc.), 0.625 µl of 10 µM upstream and downstream primers (Integrated DNA Technologies, Coralville, Iowa), 9 µl of distilled water, 1.25 µl of DMSO (Dimethylsulfoxide) and 1 µl of genomic DNA. As in our previous work, PCR amplification was performed using the following conditions: an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min. The PCR amplification for the modified locus 1 primer set was performed using the same conditions as all other primer pairs, but for 35 cycles instead of 40. A PCR master mix blank was included as a negative control for each amplification and the extraction negative was processed for at least one locus for all extraction batches. Two µl of PCR product was electrophoresed at 105 V for 30 min on a 1.5% (wt/vol) agarose gel with 1.25 ul

EtBr in 0.5X TBE buffer (0.45 M Tris-Borate, 0.01 M EDTA, pH 8.3). PCR products were then visualized through UV transillumination. Amplicons were purified with either a PureLink PCR purification kit (Invitrogen, Carlsbad, CA USA) or a QIAquick PCR spin columns (Qiagen, Valencia, CA USA) and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware). PCR amplicons were sequenced using standard dye terminator chemistry on a 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, Calif.) at the Cornell University Life Sciences Core Laboratories Center (http://cores.lifesciences.cornell.edu/brcinfo/).

2.5. Genotyping

Chromatograms were read in SeqMan (DNASTAR Inc., Madison, WI). Number of short sequence repeats were assigned for each locus, and each isolate was assigned a strain-type based on the pattern of repeats across all loci. All isolates with greater than or equal to 11 G repeats at locus 1 were considered equivalent (marked >11) (Clarke, et al., 2001; Amonsin, et al., 2004; Fazekas, et al., 2010). When there were multiple strains in one MAP sample (confirmed by homopolymer forward and reverse sequencing), isolates were assigned both potential strain types, and CFUs per gram were equally divided between those different strains. When we were unable to distinguish the combinations of these different strains due to multiple loci with ambiguous designation, the isolate was assigned a "mixed" designation. Complete MLSSR types were assigned after determining the allele combinations on locus 1, locus 6, locus 8, locus 9 and locus 10. Following genotyping, strain types of all isolates from each dam-daughter pair were compared to determine whether they shared the same MAP genotype.

2.6. Statistical analysis

SAS v9.2 was used for statistical analysis (SAS Corporation, Cary NC). To estimate the horizontal MAP transmission pressure, all available environmental samples and all MAP-positive fecal samples collected within 12 months after birth of the daughter and the most recent fecal sampling before birth were analyzed. Samples were weighted using a semi-quantitative logarithmic scale by CFUs per gram of feces. Weighting factors were: 10 for samples with 1-50 CFU's per gram of feces, 20 for samples with 51-2000 CFU's per gram of feces and 30 for strains found in samples with >2000 CFU's per gram of feces. Independently, unweighted categories were created for cows that were ever-positive by fecal and/or tissue culture, with each cow contributing all strains that were cultured at any point throughout its entire lifetime.

The source of all daughter strains can be subdivided into three categories: "Horizontal": the daughter's strain does not match any of the dam's strains but is found on the farm (defined as circulating strains, based on fecal samples of currently-shedding animals present on the farm and contemporary environmental samples), "Dam Strain": the daughter's strain matches one of the dam strains or "Introduced": the daughter's strain does not reflect strains found on the farm or any of the strains found in her dam.

To determine the probability that an infected daughter carries the same strain as her dam by horizontal transmission, strain prevalence was calculated by summing the total number of strains in individual cows over the course of the study. The expected probability of horizontal transmission of specific strains (independent of the potential vertical transmission) was calculated using the above defined unweighted strain prevalence. In addition, the number of expected dam-daughter MAP-positive pairs was calculated by evaluating the probability of testing positive by each test and the probability of being tested. The probability of vertical

transmission was then defined as the observed strain identity between dam and daughter, above and beyond the expected identity from horizontal transmission from either method.

3. Results

3.1 Data analysis

On farm A, 572 dam-daughter pairs were identified with fecal-culture information on both dam and daughter and 20 dam daughter pairs were identified with tissue-culture information for both dam and daughter, for a total of 574 dam-daughter pairs with at least one MAP test for each animal (Table 5.1). MAP prevalence among dams was 17% (101/574 MAP-positive dams, 51/572 by FC, 63/105 by TC) and MAP prevalence among daughters was 8% (48/574 MAP-positive daughters, 16/573 by FC, 35/91 by TC) on Farm A (Table 5.1).

The expected number of MAP-positive dam-daughter pairs on Farm A, assuming no vertical transmission between dam and daughter, (calculated based on the MAP prevalence amongst daughters and dams) was 1.5% (expect 8.4 dam-daughter MAP-positive pairs from 574 total pairs). The observed number of MAP-positive dam-daughter pairs on Farm A is 13, which is higher than expected given random mixing (Poisson distribution, P=0.05).

Daughters with fecal-culture-positive dams were at more than 5-times increased risk of becoming fecal-culture-positive as daughters of fecal-culture-negative dams (Relative risk 5.6, CI 1.7-12.8, P=0.01). There was a trend toward being MAP test-positive when born to a dam with a positive fecal and/or tissue culture (Any+ vs All-) (chi-square value 3.25, P=0.07, Figure 5.1). Daughter MAP tissue-infection-status was not influenced by dam MAP tissue-status (Fisher's exact test, P value =0.43, Figure 5.1), although sample size was small (n=18).

Table 5.1. Dam and daughter test status by fecal culture (FC), tissue culture (TC) or by either method ("Any"). Latent daughters are tissue-culture positive but fecal-culture negative throughout their lifetimes. Negative dams are animals that did not test positive by all method listed. Slow-latents are animals which never shed but are tissue-culture positive. Fast latents are not shedding prior to or up to the first fecal test following calving, but shed after the birth of the daughter classified. Shedding dams are fecal-culture positive before the calving of the identified daughter or at the first fecal culture post-parturition.

	Daughter FC			Daughter TC			Daughter Any			Latent
	+	-	sum	+	-	sum	+	-	sum	FC- TC+
Dam Fecals										
FC+	5	46	51	4	6	10	8	43	51	3
FC-	11	510	521	31	42	73	40	482	522	29
Sum	16	556	572	35	48	93	48	525	573	32
Dam Tissues										
TC+	2	61	63	5	9	14	7	56	63	1
TC-	0	42	42	1	3	4	1	41	42	5
Sum	2	103	105	6	12	18	8	97	105	6
Dam Any										
Any+	6	95	101	8	14	22	13	88	101	7
Any-	10	462	472	27	42	69	35	438	473	25
Sum	16	557	573	35	56	91	48	536	574	32
Potential Negatives										
Lifetime FC-	3	127	130	32	44	76	33	97	130	30
Lifetime FC- TC-	0	36	36	1	2	3	1	35	36	1
FC- before birth	1	24	25	0	2	2	2	23	25	1
Slow Latents (untested before birth)										
FC-TC+ after birth	1	33	34	4	7	11	5	29	34	4
FC-TC+ ever	1	47	48	4	7	11	5	43	48	4
Fast Latents (FC- before birth)										
FC+ after birth	0	11	11	3	5	8	3	11	14	3
Shedding Dams										
FC+ before birth	0	1	1	0	0	0	0	1	1	0

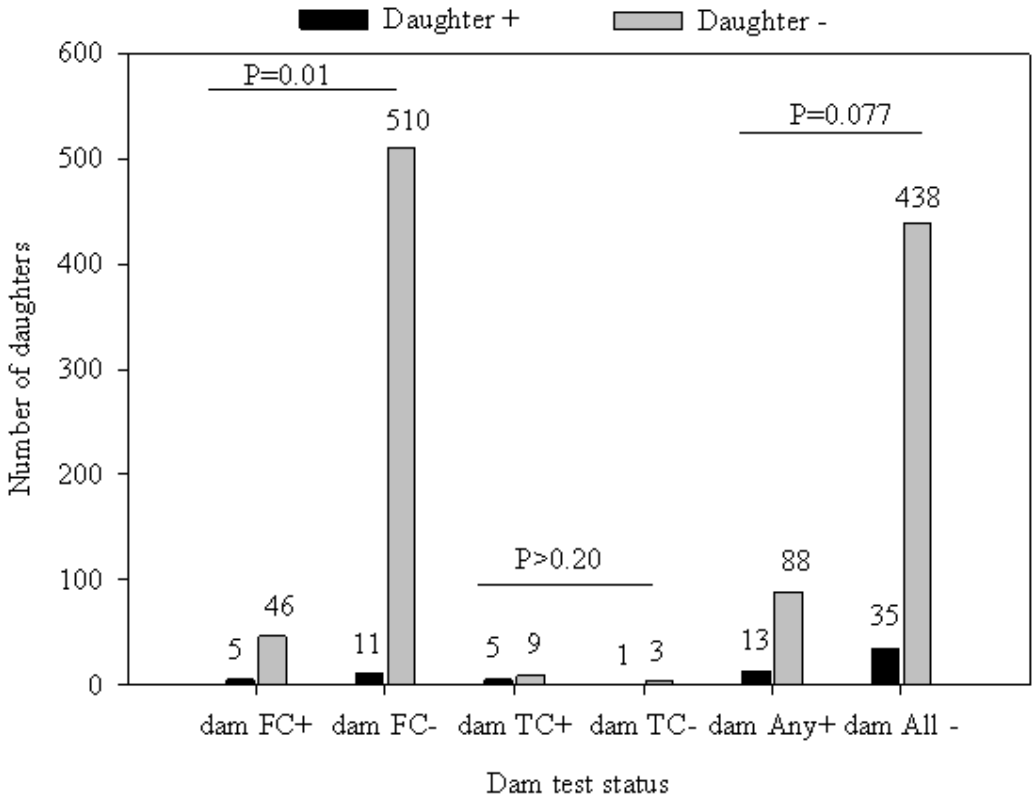

Figure 5.1. Relationship between dam fecal and tissue culture status and dam fecal and tissue culture status. FC= Fecal culture, TC= Tissue culture, Any = parallel testing using all available test results (so that any positive will classify an animal as infected). Black bars indicate daughters with positive tests, grey bars indicate daughters with negative tests. Dam FC status is compared to daughter FC status, dam TC to daughter TC and dam any-test status to daughter any-test status. Number of animals in each category is noted on the graph.

3.2 Genotyping

Diversity was observed at loci 1,6,8,9 and 10 of the 8 loci analyzed. All fecal and environmental isolated from Farm A were MLSSR-typed using the 5 loci for which there was diversity in the 12 dam-daughter pairs. Nine (75%) of dam-daughter pairs had identical MAP strains between dams and daughters (Table 5.2). Two of these nine daughters also carried additional MAP strains. In three pairs, daughter strains did not match any of the strains identified in the dam. Among all the 19 strains identified in the 12 daughters, eleven of these

strains (58%) matched a strain present in the dam and eight (42%) present in the daughters did not originate from the dam.

Strains of dominant horizontal MAP exposure could be estimated for four daughters who were born within one year of the initiation of the RDQMA whole-herd samplings (Figure 5.4). The remaining eight daughters were born greater than one year prior to any of the sample dates. Therefore most of the birth events were not associated with contemporary environmental or fecal culture samples and consequently exposure from on-farm sources other than dams could not be measured directly.

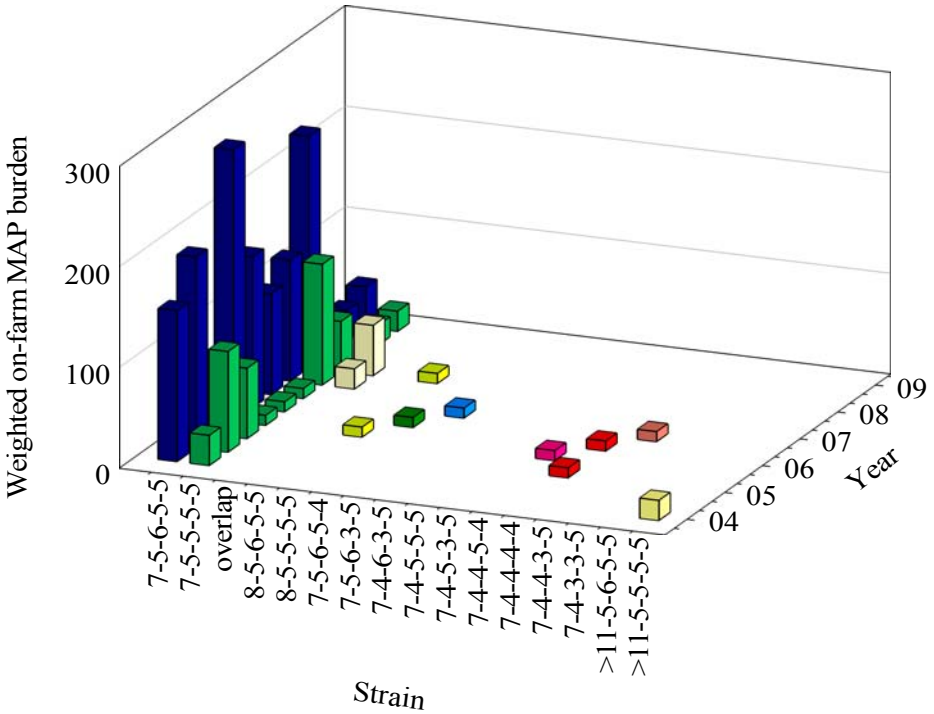

Figure 5.4. On-farm MAP burden at each sampling time-point based on fecal samples of currently shedding animals present on the farm and contemporary environmental samples weighted by CFU/g categories. Tissue culture data are not reflected in this graph.

There was more diversity in MAP strains in tissue cultures relative to fecal cultures despite the larger number of animals tested by fecal culture (Figure 5.5, Panels A and B). Seven strains which were in tissues were never recovered on the farm, indicating low prevalence of fecal shedding. MAP strain 7-5-6-5-5 was dominant in both fecal-positive and any-positive animals.

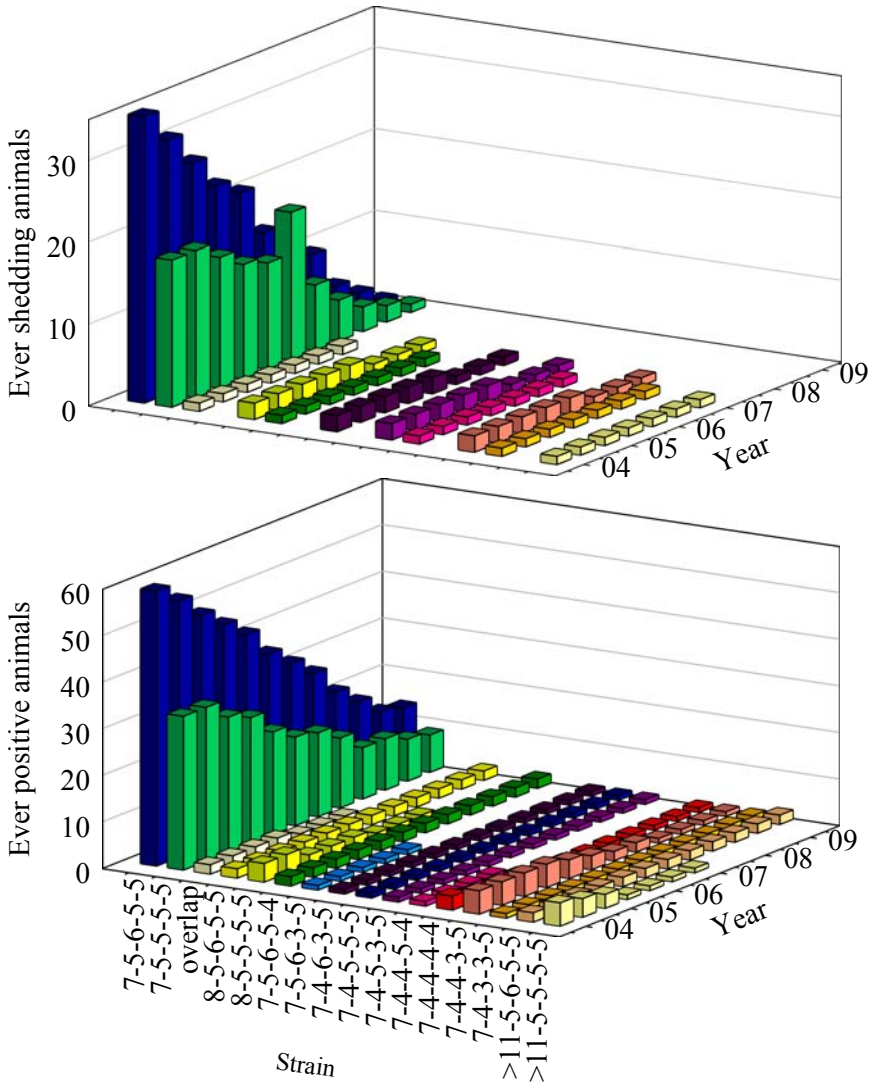

Figure 5.5. On-farm MAP burden assuming lifetime shedding of all fecal-culture positive animals (A) or fecal-culture and tissue-culture positive animals (B). Every animal that is FC+ or TC+ is represented for each strain identified in that animal for the duration of life. Each six month increment is calculated individually, and animals that are culled are included in the increment in which they exit the population.

Table 5.2. Strain types in daughters, dams, and the farm environment/herdmates which are shedding within one year following the birth of an infected daughter. Each pair is assigned an ID. All strains present in any of the three sources (dam, daughter, environment/herd) are listed for each pair. Each row represents samples of the same strain. Sample type (tissue or fecal) is indicated for samples from daughters and dams. Any strain that is common to dam and daughter is highlighted in grey, and any strain shared by daughters and the environment is italicised. Herd and environmental samples are weighted as described in the methods section. Mixed strains recovered from single samples which could not be differentiated are listed as mixed strains and any combination of alleles which is represented at least once on the farm is considered a possible strain from these samples. Because the majority of daughters were born previous to the initiation of the study, these animals do not have an environmental burden assigned.

		Daughter				Dam			Herd and Environment
Pair	Strain	ID	Birthdate	Sample	Isolates	ID	Sample	Isolates	Weighted contribution
1	7-4-6-3-5	1751	Aug 4, 2004	Tissue	2				
1	7-4-4-3-5	1751	Aug 4, 2004	Tissue	2				
1	7-5-5-5-5					1255	Fecal	2	22.4%
1	7-5-6-5-5								64.2%
1	>11-5-5-5-5								7.5%
1	7-(5/6)-(5/6)-5-5								4.5%
1	7-4-4-4-4								1.5%
2	7-4-4-3-5	1683	Oct 10, 2003	Fecal, Tissue	3				
2	7-4-5-3-5	1683	Oct 10, 2003	Tissue	1				
2	*7-5-6-5-5*	*1683*	*Oct 10, 2003*	*Tissue*	*1*				*72.5%*
2	8-5-5-5-5					774	Tissue	1	
2	7-4-4-4-4					774	Tissue	1	
2	7-4-5-5-5					774	Tissue	1	
2	7-5-5-5-5								15%
2	>11-5-5-5-5								12.5%
3	*7-5-6-5-5*	*1645*	*Oct 25, 2003*	*Fecal*	*1*	*1342*	*Fecal*	*1*	*72.5%*
3	7-5-5-5-5								15%
3	>11-5-5-5-5								12.5%
4	*7-5-6-5-5*	*1628*	*Sept 20, 2003*	*Fecal*	*2*	*1127*	*Fecal, Tissue*	*4*	*80%*
4	7-5-5-5-5					1127	Tissue	1	12%
4	>11-5-5-5-5								8%
5	7-5-6-5-5	1494	Oct 11, 2002	Fecal, Tissue	8	1099	Fecal	2	

5	7-5-5-5-5					1099	Fecal	2
6	7-5-5-5-5	1429	Apr 28, 2002	Tissue	1			
6	7-(4/5)-(5/6)-5-5					1091	Fecal	1
6	7-(4/5)-(5/6)-5-(4/5)					1091	Fecal	1
6	7-(4/5)-(4/5)-(5/4)-(4/5)					1091	Fecal	1
7	7-5-6-5-5	1415	Apr 20, 2002	Tissue	1	1120	Tissue	1
8	7-5-6-5-5	1372	Jan 10, 2002	Fecal	1	2126	Fecal	1
8	7-5-5-5-5					2126	Fecal	1
9	7-5-5-5-5	1300	Jul 17, 2001	Fecal, Tissue	3	1127	Tissue	1
9	7-5-6-5-5	1300	Jul 17, 2001	Tissue	2	1127	Fecal, Tissue	4
9	7-5-6-5-4	1300	Jul 17, 2001	Tissue	2			
10	7-5-6-5-5	1257	Mar 12, 2001	Tissue	2			
10	7-5-4-4-4					711	Tissue	1
11	7-5-6-5-5	1189	Nov 27, 2000	Tissue	3	655	Tissue	2
11	7-5-5-5-5	1189	Nov 27, 2000	Tissue	1	655	Tissue	1
12	7-5-6-5-5	1099	Dec 11, 1999	Fecal	2	693	Fecal	1
12	7-5-5-5-5	1099	Dec 11, 1999	Fecal	2			

3.3 Vertical transmission of MAP strains

All dam-daughter pairs which shared the same MAP strain shared one of the two dominant strains (7-5-6-5-5 and 7-5-5-5-5) which together consistently comprised more than half of the weighted CFU burden on the farm as well as more than half of all infected animals (Figures 5.4 and 5.5). Of the additional strains recovered in daughters of known-infected dams during the longitudinal study, three were not recovered from the environment during the calves' high-risk first year of life (7-4-4-3-5, 7-4-5-3-5, 7-4-6-3-5).

For the four daughters from the MAP-positive dam-daughter pairs born within one year of initiation of fecal sample collection, we evaluated the presence of the MAP strains in relation to the exposure of the daughter to these strains in her environment during her first year of life. The MAP strains in daughters, dams and on the farm are shown in Table 5.2. For example, for the 3rd daughter in Table 5.2, the conditional probability of uptake of strain 7-5-6-5-5 from her environment, conditional on that she was going to be MAP infected was 0.725 while uptake of any other strain circulating on the farm at that moment was 0.275. For the 4th daughter in Table 2, the conditional probability of uptake from the environment of strain 75655 was 0.80 while uptake of any other strain circulating on the farm at that moment was 0.20.

4. Discussion

The observed number of MAP-positive dam-daughter pairs on Farm A exceeds the expected number of MAP-positive dam-daughter pairs. Vertical transmission can be responsible for the additional MAP-positive dam-daughter pairs observed; however, there is also substantial transmission of strains which are not commonly found in the farm environment and are not from the dam in the daughters. It is possible that the large number of strains in the daughters which are

unique on the farm represent new introductions of MAP from external sources rather than within-farm transmission.

Increased prevalence of MAP-positive daughters among latent dams found in this study was consistent with previous work. Benedictus and colleagues described increased risk of infection for daughters born to infected dams versus daughters born to negative dams based on the same type of single-farm longitudinal data collection as this study. Daughters born to dams that tested positive by fecal culture within two years following birth (latent dams) were at increased risk of being infected versus daughters from test-negative dams. There was no difference in transmission rates between latent dams who shed shortly following calving or those that remained latent for at least two years after calving (Benedictus, et al., 2008). Aly and Thurmond studied the relevance of dam (serologic) status in a retrospective longitudinal study of 625 animals using ELISAs. Daughters of seropositive dams were 6.6 times more likely to be seropositive compared to daughters of seronegative dams (Aly and Thurmond, 2005), but as serology does not correlate well with low shedding, these could have been latent or shedding animals. Although the number of strains shared between dams and daughters suggest vertical transmission as transmission route of infection, genetic susceptibility could also account for the additional positive dam-daughter-pairs observed on farm A.

A concern with strain typing techniques that rely on genomic areas with high genetic variability is their stability over time. Mutation rates at these areas might be too high for usage in strain typing over several years. Recent work on *Yersinia pestis* and *Escherichia coli* short repeat sites indicates that some bacteria have relatively rapid evolution at SSR sites (Girard, et al., 2004; Vogler, et al., 2006; Vogler, et al., 2007). Evolutionary rates are unknown for *Mycobacterium avium* subspecies *paratuberculosis,* especially at sites of MLSSR analysis.

Stability of short sequence repeats was investigated by Harris et al. by performing two experiments. In the first experiment, three MAP isolates were serially grown for ten passages *in vitro*. For every isolate, Locus 1, 2, 8 and 9 were sequenced for each serial passage. In the second experiment, Harris et al. harvested DNA from ten colonies from a single isolation of each of the three isolates for SSR analysis. Both experiments showed no SSR genotype changes. In studies of *Mycobacterium tuberculosis*, variable number tandem repeats (VNTR) methods are frequently used for strain typing. However, there is debate about the exact rate of evolution for VNTRs. Savine *et al.* examined the mutation rate for 12 VNTR loci, where 1.8% (1/56) of isolates from patients showed a change at one locus over an average period of time of 1.37 years, while 98.2% remained stable (Savine, et al., 2002). This finding reflects a mutation rate of 10^{-3} per locus per year. A mathematical modeling study estimated the average mutation rate of the same 12 VNTRs to be $2.3*10^{-8}$ (Grant, et al., 2008). Other estimates vary between $7*10^{-4}$ and $1.5*10^{-2}$ per locus per year (Reyes and Tanaka, 2010). Although the real mutation rate of MAP remains unknown, evolutionary rates close to those estimated for *Mycobacterium tuberculosis* inspire confidence in SSR stability. A more precise estimate of the MAP mutation in SSR's demands serial passages of several different isolates over a period of longer duration to capture sufficient mutations at SSR sites to determine mutation rates.

In *Mycobacterium tuberculosis* the use of molecular characterization of different strains with VNTR-methods demonstrated infections with multiple strains of *Mycobacterium tuberculosis* occurs in humans (Richardson, et al., 2002; Garcia de Viedma, et al., 2003; Garcia de Viedma, et al., 2004; Warren, et al., 2004; Garcia de Viedma, et al., 2005; Shamputa, et al., 2006; Dickman, et al., 2010). Several studies reported individuals harbouring more than one strain in their sputum (Chaves, et al., 1999; Richardson, et al., 2002; Warren, et al., 2004; Garcia

de Viedma, et al., 2005; Shamputa, et al., 2006). Multiple strains have also been found in different anatomical sites within an infected patient (Garcia de Viedma, et al., 2003; Garcia de Viedma, et al., 2004). Multiple infections were increasingly observed in high tuberculosis-incidence communities (Richardson, et al., 2002) and were even more evident in other very-high tuberculsis-incidence settings such as prisons (Nardell, et al., 1986; Chaves, et al., 1999; Chaves, et al., 1999; Sonnenberg, et al., 2001; Shamputa, et al., 2006), mines (Sonnenberg, et al., 2001), and homeless shelters (Nardell, et al., 1986). This suggests there could be a connection between crowded settings with high contacts between individuals and the prevalence of mixed infections in *Mycobacterium tuberculosis*. HIV prevalence which depresses immune function contributes to an increased number of mixed infections, which could be paralleled by effects of high milk production or concurrent BLV or BVDV infections in herds.

The similarity between conditions of these high-prevalence settings in *Mycobacterium tuberculosis* and dense housing and high contact rate among dairy cows is evident. In this study multiple strains were observed in different samples from one cow but also within a single sample (confirmed by repeated culture, extraction and PCR amplification). Mixed infections will be further investigated in future work by determining the ratio/proportion of each strain within a sequence of the isolate and the result of amplifying the different strains within an isolate following dilutions. Of particular interest is whether having more than one MAP strain predisposes animals to become shedders or have clinical disease.

The completeness of this unique longitudinal dataset on a well-managed dairy herd with long follow-up (7 years of biannual sampling) combined with the additional value of tissue culture results on a subset of the culled animals provides us with the confidence that the correlations observed in this study reflect reality. To get a stronger estimate of MAP pressure of

the different strains from on-farm sources, more frequent environmental samples should be taken around the freshening events. Additional discriminatory power of the MLSSR typing technique will be available with the addition of locus 2. Preliminary cloning experiments by the authors of this study show promising results on readability of locus 2 sequences. In addition to getting a better understanding of vertical transmission, a longer follow-up could provide us with a more complete display of MAP strain type diversity and evolutionary rates.

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CHAPTER SIX

A meta-analysis of the effect of dose and age-at-exposure on shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in experimentally infected calves and cows. ◊

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Abstract

A meta-analysis was performed using all published and one unpublished long-term infection-challenge experiments to quantify the age- and dose-dependence of early and late shedding of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in cattle. There were 194 animals from 17 studies fulfilling the inclusion criteria, of which 173 received a known dose of MAP and 21 were exposed naturally. Results from parametric time-to-event models indicated that challenging older calves or using multiple exposure experimental systems resulted in a smaller proportion and shorter duration of early shedding as well as slower transition to late shedding from latent compartments. Calves exposed naturally showed infection progression rates that were variable but similar to other infection routes. The log-normal distribution was most appropriate for modelling infection-progression events. The infection pattern revealed by the modelling allows better understanding of low-grade endemicity of MAP in cattle, and the parameter estimates are the basis for future transmission dynamic modelling.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a bacterial pathogen of the ruminant gastrointestinal tract that can cause persistent diarrhoea and eventual death due to malnutrition (Whitlock and Buergelt, 1996; Clarke, 1997). The potential link between MAP and human Crohn's disease makes the high prevalence of MAP in meat- and milk-producing animals of concern to human health (Shulaw and Larew-Naugle, 2003). Sensitivity of bacterial-culture-based screening tests is limited in early infection stages due to low or intermittent shedding of bacteria. Immune-response-based serum tests are also limited due to the substantial delay between infection and consistently positive serology (Collins and Zhao, 1994; Stewart, et al., 2007). Because the contribution of these early-shedding animals to infectious transmission is potentially important, understanding shedding patterns of recently infected animals is essential for designing and predicting the impact of control strategies.

Age at exposure and dose received could influence pathogenesis of MAP infection in cattle (Rankin, 1959; Larsen, et al., 1975). Calves artificially challenged with high doses of bacteria have been reported to become infectious more rapidly relative to calves challenged with low-doses of bacteria (Rankin, 1959; Collins and Zhao, 1994). However, the relationship between dose of challenge and progression to infectiousness has not been fully quantified. Dose-dependent or age-dependent response to infection is common for many pathogens, including MAP (Medema, et al., 1996; French, et al., 2002; Windsor and Whittington, 2010). Previous work with other pathogens illustrates the potential impact of changes to pathogenesis due to age at exposure subsequently resulting in a change in infection transmission dynamics in a population (Medley, et al., 2001).

Transmission of MAP is assumed to be primarily from infectious adults to susceptible

young animals, and control of MAP within farms currently involves a two-prong approach of testing and removing infected adult animals and increasing hygiene on the farm to limit contact between calves and faecal material of adult animals (Collins, et al., 2006). It is generally assumed that poor diagnostic-test sensitivity (Whitlock, et al., 2000) leading to incomplete culling where some infectious animals remain in the herd and environmental persistence of MAP bacteria (Larsen, et al., 1956; Whittington, et al., 2004) contribute to the sustained prevalence of MAP in herds despite intervention attempts. However, our previous series of mathematical models indicated that, with current assumptions of shedding patterns, infectious contributions of adult shedders alone were insufficient to explain infection persistence and the observed pattern of infection prevalence (Mitchell, et al., 2008). The models indicated that an additional contribution from infectious calves could be important and in some situations dominate within-herd transmission in low-prevalence herds.

In ordinary differential equation (ODE)-based mathematical models of infectious dynamics within herds like our previous work (Mitchell, et al., 2008), modellers assume a constant rate of exit from each infectious compartment. This simplifying assumption limits model complexity and results in exponential decay of the population within each infection stage over time. Changing the assumption of constant rate of exit can have a dramatic effect on the stability of infections within simulated populations. A variable rate of exit more accurately predicts the basic reproduction ratio (R_0) and risk of stochastic fadeout (Keeling and Grenfell, 1998). The nature and heterogeneity of infection progression are highly influential on infection dynamics (Keeling and Grenfell, 1998). This heterogeneity may change the efficacy of intervention strategies being evaluated.

To evaluate current assumptions of constant rate of exit and to estimate age- and dose-dependent shedding patterns, we performed a meta-analysis of all available experimental infection trials of MAP in cattle. We determined the rate of entry and exit from shedding and non-shedding compartments following a challenge infection of calves and cows with known quantities of MAP. We assessed the effect of age-at-infection, challenge strategy and dose on time-to-shedding and duration of MAP shedding. We also analyzed the fit of exponential time-to-event models and compared these to other distributions to ascertain whether the assumption of constant exit rates from compartments was valid. Finally, we discussed the application of the current research to adapt the mathematical model of MAP transmission on commercial US dairy farms (Mitchell, et al., 2008) to age- and dose-dependent durations of shedding categories.

2. Methods

2.1. Data sources

Literature search on PubMed (www.pubmed.org) identified 16 studies that fulfilled inclusion criteria outlined below. All literature searches and evaluations to identify, appraise and select primary studies were performed by a single author. Papers were identified in PubMed using the following combinations :'paratuberculosis' or 'Johne's' or 'johnei' and 'experimental' and 'cow' or 'cattle' or 'calf'. Abstracts identified by these searches were used to screen for papers in which authors performed experimental infections in cattle. Papers with evidence of experimental infection of cattle in the abstract (even if the faecal shedding was not the focus of the study) were screened in detail to evaluate whether inclusion criteria were met. If insufficient details were present in the abstract to determine whether papers tracked faecal shedding over time, the entire text and figures were evaluated. If multiple papers were identified using the same group of

experimentally infected cattle, these animals were only represented once. Review papers were not eligible for inclusion, but papers referenced in a review paper were eligible to be included even if they did not appear in the original search criteria. Studies published in languages other than English were eligible for inclusion if found in the original PubMed search or from citations in review articles. Those papers which were not identified in the PubMed search but were cited in reviews were requested from the Cornell University Library network and screened with the same criteria. Additionally, a cited-reference search was carried out in Web of Knowledge (http://www.isiwebofknowledge.com/) to identify other eligible papers which cited early experimental infection studies. We contacted MAP experts (including primary study authors) to identify other studies with available data. Personal communication and the Proceedings of the International Colloquium on Paratuberculosis provided data from one additional study (see Table 6.1).

2.2. Inclusion/Exclusion criteria

Because of limited correlation between quantitative value of serological tests and infectiousness as assessed by colony-forming units per gram of faecal material in recently infected animals (Collins and Zhao, 1994; Stewart, et al., 2007), studies were included only if faecal-culture data were presented. Minimum study duration to be included in our analysis was 5 months. Data had to be presented in the paper for intervals less than or equal to one month duration, or data had to be presented for the month at first positive sample for animals sampled at intervals less than or equal to one month duration. If data were collected monthly at initiation of the study and subsequently the culture intervals were increased, the dataset was truncated to only include the monthly data points. Contaminated culture data were treated as no value rather than as positive or negative. If there was a transition between categories which included contaminated

Table 6.1. Challenge and animal characteristics in the studies used for our analyses

Study	N	Used	Dose range	Method	MAP strain	Age	Sex	Breed	Duration
(Taylor, 1953)	12	6	100 mg	IV	5 recently collected isolates	1 d, 1 mo, 6 mo	M	A	23-26 mo
(Taylor, 1953)	12	6	100 mg	PO	5 recently collected isolates	1 d, 1mo, 6 mo	M	A	20-27 mo
(Rankin, 1958)	6	6	100 mg	IV	Taylor1953	1 mo	F	A	10-46 mo
(Rankin, 1959)	50	16	100 mg	IV	From a "typical case"	1 mo	M,F	A,F	7-12 mo
(Rankin, 1959)	50	16	10 mg	IV	Dilution from 100 mg	1 mo	M,F	A,F	7-12 mo
(Rankin, 1959)	50	16	1 mg	IV	Dilution from 100 mg	1 mo	M,F	A,F	7-12 mo
(Rankin, 1959)	50	2	0.1 mg	IV	Dilution from 100 mg	1 mo	M,F	A,F	7-12 mo
(Rankin, 1961a)	6	6	100 mg	IV	Taylor1953	3 yr	F	A	21 mo
(Rankin, 1961b)	9	9		NE	Taylor1953	1 d, 1 mo,6 mo	F	A	4 yr, 36-47 mo
(Rankin, 1962)	7	7		NE	ND	3+ yr	F	A	16-48 mo
(Larsen et al., 1974)	16[b]	8	180 mg	PO	Strain "virulent to cattle"	26 d	ND	ND	134 d
(Larsen et al., 1975)	10	10	180 mg	PO	Recently isolated strain	1 mo, 9 mo, adult	ND	HF	1 mo-11 yr
(Larsen et al., 1977)	14	14	100 mg	PO,IV,SQ	Recently isolated strain	3 wk	ND	HF	150 d
(Thorel et al., 1985)	28[c]	4	$1.6x10^8$ cfu	IV	7912 Thorel et Valette 1976	3-4 wk	ND	H,N,F	12 mo
(Milner et al., 1987)	16[a]	9	200 mg	PO	ND[d]	3-7 wk, 17 mo	M,F	Mixed	3-21 mo
(Lepper et al., 1989)	28[a]	20	200 mg	PO	ND	2-6 wk	ND	ND	3-33 mo
(Collins and Zhao, 1994)*	16[a]	5	$3x10^6$ cfu	PO	Clinical animal	1 mo	F	H	35-51 mo
(Collins and Zhao, 1994)*	16[a]	5	$3x10^8$ cfu	PO	Clinical animal	1 mo	F	H	35-51 mo
(McDonald et al., 1999)	29[a,b]	3	6 grams	PO	Recent field isolate	<2 mo	ND	J,F, JF	25-27 mo
(McDonald et al., 1999)	29[a,b]	2	20 gm Int	PO	Clinical animal	<2 mo	ND	J,F, JF	25-27 mo
(McDonald et al., 1999)	29[a,b]	5		NE	Faecal-culture positive dam	birth	ND	J,F, JF	23-29 mo
(Waters et al., 2003)	3	3	$1.6x10^7$ cfu	PO	ND	2-5 wk	M	H	320 d
(Stabel et al., 2003)	12[c]	6	$3x10^{10}$ cfu	PO	Clinical animal	>3 mo	ND	Beef	6 mo
(Stewart et al., 2007)	30[a,c]	5	60-80g	Int	Clinical animal	6 weeks	ND	HF	52 mo
(Stewart et al., 2007)	30[a,c]	5	4-$8x10^{10}$ cfu	PO	Clinical animal	6 weeks	ND	HF	23-52 mo

N: number of animals in the study.
Used: number of animals used in this analysis.
[a] additional animals were uninfected controls
[b] additional animals were vaccinates

[c] strains from different species used on the remaining calves

Dose range: Either a grams wet weight or colony forming units (cfu) depending on individual study.

Animals dosed with ground intestine (Int) from clinical animals were considered distinct from animals dosed with cultured bacteria.

Method: Method of dose delivery.

Intravenous (IV), subcutaneous (SQ), natural exposure-contact with clinical animals (NE), Oral (PO)

Strain: MAP strain was often unspecified or noted as a recently isolated strain only. Taylor1953 is a strain used for the Rankin studies isolated previously.

[d] no data published

Age: age at initial infection.

Duration: amount of time animals were enrolled in the study.

Sex: Male (M), Female (F) or not designated (ND)

Breed: Ayrshire (A), Friesian (F), Holstein (H), Holstein-Freisian (HF), Jersey (J), Normandy (N), Jersey-Friesian crossbred (JF), Mixed, not designated (ND),

Beef calves (non-specified)

* Collins, M. T. Lifetime faecal culture data on all animals from [2]. Personal communication to RM Mitchell on 1 June 2006.

cultures, the midpoint of the time period between two available cultures was assumed to be the time of transition. In studies which included multiple different challenge strains, only those animals identified as infected with bovine-specific MAP strains were included. Animals from farms with known MAP-positive animals or animals born from infected dams were excluded from the dataset with two exceptions; Taylor *et al* (Taylor, 1953) and McDonald *et al* (McDonald, et al., 1999). In the McDonald study, dams of calves enrolled in the study were test-negative at the time of study initiation but calves were not specifically from a MAP-free herd. Calves from dams that were subsequently test-positive are removed from the known-dose analysis. In the Taylor study, animals are cited as from a herd with very low incidence of clinical disease (Taylor, 1953). Animals receiving vaccinations against MAP were excluded.

To evaluate on-farm infections compared to experimental infections, studies in which animals were exposed to clinical or shedding animals (defined as 'natural exposure') were included for a secondary analysis provided they met all other inclusion criteria. The calves in the McDonald study (McDonald, et al., 1999) born to culture positive dams were included in the natural exposure portion of the study.

2.3. Standardization of experimental data across studies

If faecal cultures were recorded at intervals more frequent than monthly, all samples within a month were included in the determination of binary shedding/non-shedding status for that interval. For the Collins study which sampled animals every 28 days (Collins and Zhao, 1994), time intervals were converted to months. Faecal-culture technique depended on author and year of study. The sensitivity of culture was expected to be influenced by culture method and laboratory; however, quantitative information on culture sensitivity by laboratory/specific

method is lacking and changes in culture sensitivity were therefore not accounted for in the analysis. If animals were given two or more infectious doses of MAP in a short period of time and faecal cultures were reported in months post exposure, we assumed that values of time-since-infection were reported as time since first dose (Milner, et al., 1987; Lepper, et al., 1989; Collins and Zhao, 1994; McDonald, et al., 1999; Stabel, et al., 2003; Waters, et al., 2003; Stewart, et al., 2007).

2.4. Model of MAP infection compartments

Infection compartments (Figure 6.1) were defined by culture status of animals based on Mitchell *et al*. (Mitchell, et al., 2008) with the addition of a slow-progressing latent compartment. This slow-progressing latent compartment accounted for animals which never entered the early shedding stage. A very similar model structure has been used to analyze bovine tuberculosis experimental-infection data and in human tuberculosis (Pollock and Neill, 2002; Cohen, et al., 2007; Kao, et al., 2007). Presence of two distinct risk periods for shedding MAP in experimentally infected calves, one shortly after inoculation which is not associated with clinical disease (Collins and Zhao, 1994; Stewart, et al., 2007) and one period later in life which often is associated with clinical disease (Whitlock and Buergelt, 1996), provides the basis for our assumption of early and late shedding stages. The possibility that animals do not experience early (detectable) shedding but enter a slow-progressing latent period before late shedding is considered based upon findings that some infected animals remain tissue culture positive following an extensive period of time without detectable MAP shedding (Rankin, 1961a; McDonald, et al., 1999).

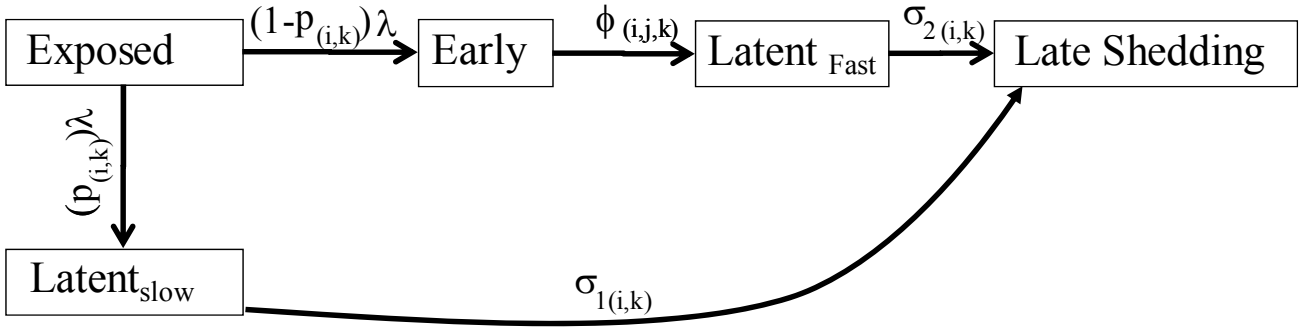


Figure 6.1. Graphical representation of MAP infection compartments. Exit from exposed (rate λ) is divided into a portion becoming early shedders (1-p) and a portion becoming slow-progressing latents (p). Exit from exposed is dependent on age at infection (i) and dose method/strategy (k). Exit from early shedding (ϕ) is dependent on age (i), dose (j) and method (k), while exit from both latent categories, fast-progressing latent (σ_2) and slow-progressing latent (σ_1), is only dependent on age (i) and dose method (k).

For this analysis, shedding must have occurred within 12 months following challenge infection to be termed early. Exit from early shedding (ϕ) into the fast-progressing latent state was defined as a two-month period with negative culture data. Any shedding following entry into the fast-progressing latent compartment was interpreted as entering the late-shedding compartment (σ_2), even if it began within the first 12 months following exposure. Entry into late shedding was the last event considered for this analysis.

2.5. Classification of animals across multiple studies

There were a total of 194 eligible animals from 17 studies, of which 173 were infected with a known dose of bacteria and 21 were exposed via cohabitation with infectious animals (natural exposure) (Table 6.1). Number of animals differs by compartment based both on individual study duration and infectious progression. Studies used multiple classification schemes to quantify challenge exposure: milligrams wet weight of bacteria, colony forming units

(cfu), grams of intestine from clinically ill animals. Similarly, delivery methods of infectious doses of bacteria depended on the individual study and included intravenous (IV), subcutaneous (SQ) and oral (PO). Although animals that were challenged with ground intestine from clinical animals as infectious material were dosed orally, this dosing method was treated as distinct from other forms of oral dosing due to inability to precisely quantify cfus delivered. Dose was analyzed initially both as a continuous and a categorical variable. We categorized challenge doses into low ($\leq 10^7$ cfu, n=23), medium ($>10^7$ to $<10^9$ cfu, n=28) and high ($\geq 10^9$ cfu, n=122) by converting milligrams wet weight of bacteria into cfu via the conversion published by Whittington (Whittington, et al., 2004). The categories were assigned based on data distribution, with the majority of animals receiving between 100 and 300 mg wet weight bacteria as an infectious dose. Dosing method was included as a categorical variable in the regression models with intravenous dosing as the baseline. Sixty-three animals were given multiple (3-5) doses of MAP over 1 to 7 day intervals (3-28 day total time between first and last dose received). Multiple exposure was included in all initial regression models (see below) as a categorical variable with single exposure as the baseline. Age was evaluated as both a continuous and a categorical variable with categories based on age at first exposure. The selection of categories was based on previous assumptions that as animals age they become more resistant to infection. Age category 1 was animals < 3 months old (n=145), age category 2 was animals $\geq$3 months but <3 years (n=18), and age category 3 was all animals $\geq$3 years (n=10). In the models with age as a continuous variable, animals identified as adult (Rankin, 1962) without specific age definition were assigned an age of 36 months. Calves identified as weaned beef calves (Stabel, et al., 2003) were assumed to be between 5 and 12 months old and were assigned an age of 6 months (category 2).

2.6. Time-to-event statistical models

Data were described using non-parametric Kaplan-Meier curves in Intercooled Stata 10 (Statacorp, College Station, Texas, 2008). Univariate tests of equality were performed by age category, dose category and multiple exposure (yes/no) for each infection compartment. Animals were treated as independent without regard to within-study clustering. Wilcoxon-Breslow-Gehan tests of equality were used to identify differences at a univariate level.

Parametric time-to-event regression models were used to determine the most likely distribution of transition rates between compartments (Stata command: streg). The youngest age, highest dose, single exposure infections with intravenous bacteria were baseline categories for all preliminary models. To evaluate the population that never experienced early shedding, exit from exposed was modelled in a split-population time-to-event regression model (Stata command: lncure). This divided the population into animals at risk of becoming early shedders while in exposed (1-p) and the remaining animals that enter directly into the slow-progressing latent compartment by default (p).

The majority of the published studies used in our analysis did not provide quantitative information on infectiousness of animals; typically only presence/absence of cultured MAP bacteria was reported. Therefore we determined shedding as a binary event without including additional information on quantity of shedding during infectious periods.

Study was perfectly correlated with age and dose in most instances, thus including study as a fixed effect in the analysis to account for differences in culture techniques and challenge-dose quantification would not meaningfully correct for experimental techniques used. Rather, intra-study correlation was addressed by including robust standard errors in all models. Robust

variance was estimated through the Huber-White sandwich estimate of variance (Rogers, 1993). Using this method, individual observations were assumed to be correlated within clusters, but clusters themselves were assumed to be independent of one another (i.e. no between-study correlation) (Cleves, et al., 2008). Using the robust clustering method, a single study with aberrant findings had less leverage than if individuals from that study were included without adjustment for intra-study correlations.

Time-to-event for individual *i* (T_i), depended on explanatory variables ($\beta_k X_{ik}$) including the intercept (i.e. constant, β_0) and the expected distribution of residuals for a baseline individual for each distribution tested ($\sigma\varepsilon_i$). In a log-normal model, ε_i has a normal distribution with constant mean and variance and ε_i are independent (Allison, 1995). For the exponential model, σ is fixed at 1.0 and ε_i are also independent but in this particular model, ε_i had an extreme-value distribution. Clustering was accounted for by evaluating correlation matrices within studies, so that $\sigma\varepsilon_i$ was a complex error that represented the correlation matrix multiplied by a residual term (Wooldridge, 2002). Time-to-event models evaluated were accelerated failure-time models (AFT) with the generic formula for expected value shown in equation ⟨1⟩.

$$\langle 1 \rangle \qquad \mathbb{E}[T_i] = e^{(\beta_0+\beta_1 X_{1i}+\beta_2 X_{2i}+\cdots+\beta_n X_{ni}+\sigma\varepsilon_i)}$$

For the mixed-population model to model exit from the exposed category, two distributions were added: one for a population with no risk of becoming early shedders (p), and one for a population at risk (1-p) of becoming early shedders. Animals in the population that transitioned into the early shedding compartment (1-p) exited exposed at a rate dependent on the log-normal distribution and their covariates. All those remaining in the exposed category at 12 months (p) moved into the slow-progressing latent category. Therefore expected time-to-event

for an individual (T_i) was either 12 months with a probability (p) or $e^{(\beta_0+\beta_1 X_{1i}+\beta_2 X_{2i}+\cdots+\beta_n X_{ni}+\sigma\varepsilon_i)}$ with probability (1-p) as in equation

$$\langle 2 \rangle \qquad \mathbb{E}[T_i] = 12p + (1-p)e^{(\beta_0+\beta_1 X_{1i}+\beta_2 X_{2i}+\cdots+\beta_n X_{ni}+\sigma\varepsilon_i)}$$

2.6. Evaluating model distributions

For the split-population model for exit from exposed, only the log-normal distribution could be evaluated due to software limitations. For all other models, the hazard function was estimated using maximum-likelihood estimators to select the best representation of duration of stay in a compartment. Five statistical distributions were evaluated: exponential, Weibull, log-logistic, log-normal and generalized gamma. Age, dose and method of infection were included in all initial models and non-significant parameters were removed in a stepwise backward fashion. If any age or dose category was significant, then all three age or dose categories were maintained in the final model. When models were nested, model fit was hierarchically tested by the likelihood ratio statistics. The Akaike Information Criteria (AIC) and plots of cumulative hazard vs. Cox-Snell residuals were used to select the most appropriate overall model distribution.

2.7. Single or multiple-dose infection strategies relative to natural-exposure studies

One of the applications of our regression model is the evaluation of experimental doses relative to that of an unknown dose provided through chronic exposure which occurs in natural-exposure studies. Three natural-exposure studies were used for estimation of challenge dose (Rankin, 1961b; Rankin, 1962; McDonald, et al., 1999). In two of these studies susceptible animals were housed with multiple clinical and high shedding animals (Rankin, 1961b; Rankin,

1962). In the most recent study, calves were born to and/or nursed by shedding dams (McDonald, et al., 1999). Progression through infection compartments was graphically compared between experimental infection strategies (single- and multiple-exposure) and the unknown challenge of natural exposure. Kaplan-Meier (K-M) curves were constructed to evaluate the rate of transition between infection compartments. Separate curves were plotted for known experimental doses and unknown doses via natural exposure.

3. Results

3.1. Description of the study population

Median follow-up in the exposure category was 3 months (range 1-12 months), and 74% of animals (n=128/173) entered early shedding within this time. Median follow-up in the early shedding compartment was 2 months (range 1-38 months), and 44% (56/128) of animals entered the fast-progressing latent state within this time. Median follow up in the fast-progressing latent compartment was 4 months (range 2-48 months), and 41% (23/56) of the fast-latent animals entered late shedding. Median follow-up in the slow-progressing latent compartment was 10 months (range 2-42 months), and 36% (9/25) of these animals entered late shedding. The proportion of animals shedding at each sampling time ranged from zero to nearly half of animals depending on time post-exposure, with peaks at 3 and 36 months post-exposure (Figure 6.2).

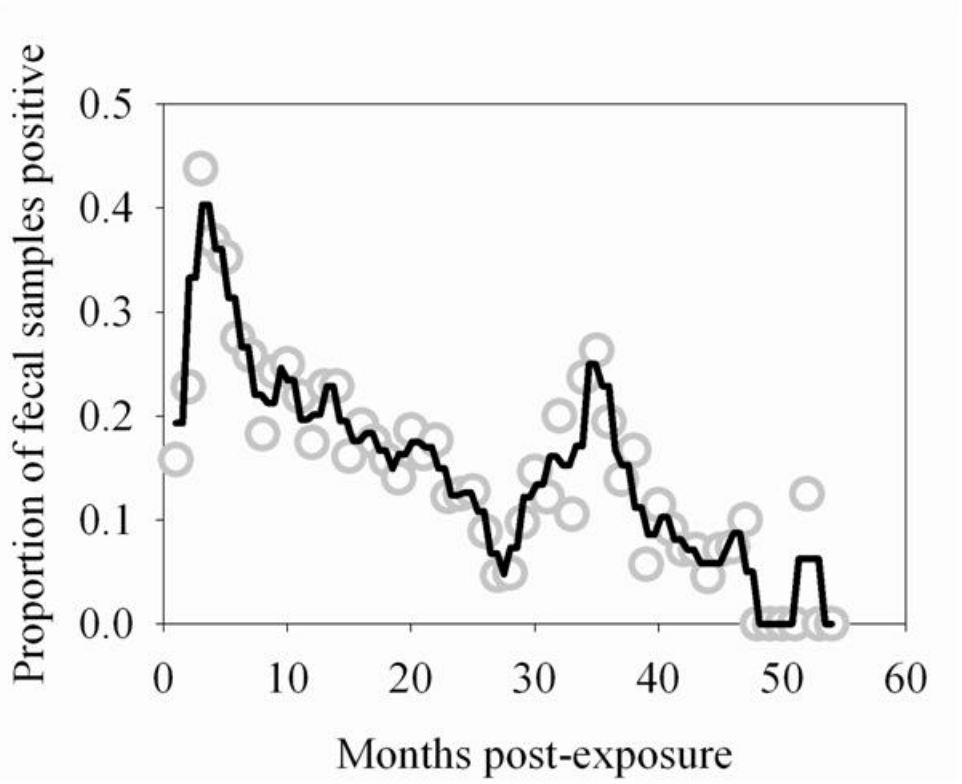


Figure 6.2. Proportion of animals with a positive faecal sample each month following exposure across all studies. Grey circles are monthly proportion of shedding animals. Black line is the smoothed running average (nearest neighbours). Contaminated samples are excluded from the denominator.

3.2. Time-to-event models

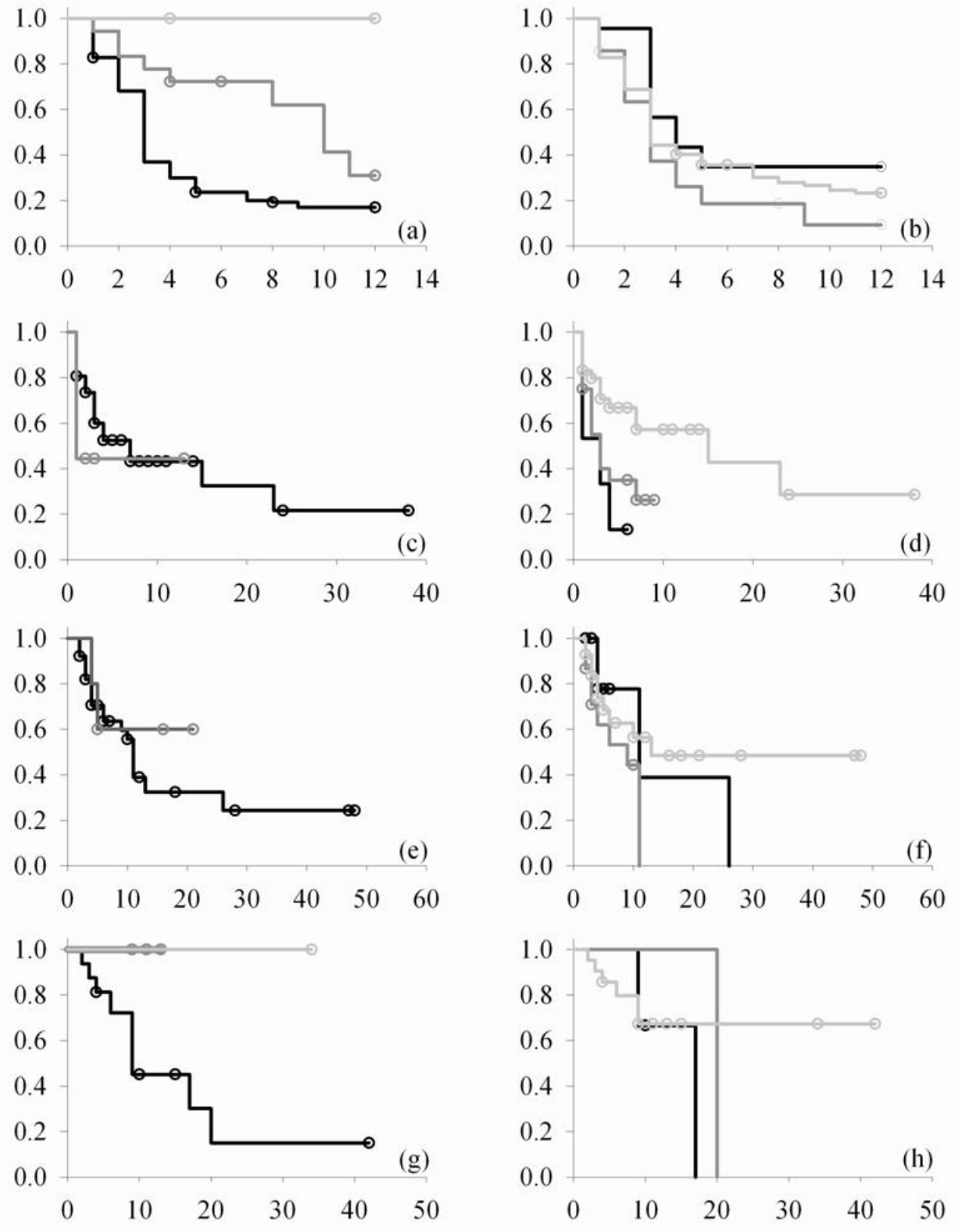
In non-parametric analysis of duration in infection categories using K-M curves (Figure 6.3) as well as univariate Wilcoxon-Gehan-Breslow statistics (Table 6.2), age at infection played a role in rate of exit from all categories except exit from fast-progressing latent. Challenge dose was only important in early shedding duration. Multiple exposure increased rate of exit from exposed and rate of exit from slow-progressing latent compartments (Figure 6.3). The majority of young and intermediate aged animals shed MAP within 12 months of exposure (Figure 6.3a).

In parametric time-to-event models, animals exposed at a young age (category 1, black line)

Table 6.2. Description of data and univariate analysis. N is number of animals that enter the category. The number which exit due to a shedding event (begin or stop shedding, depending on the compartment) is indicated for each shedding compartment. P value is for a univariate Wilcoxon-Breslow-Gehan test of equality for each category of interest (age, dose or multiple exposure).

	Age			Dose			Multiple exposure	
Category	**1**	**2**	**3**	**1**	**2**	**3**	**Y**	**N**
Exit from exposed								
N	145	18	10	23	28	122	110	62
Begin shedding	119	9	0	15	24	89	85	43
P value	(<0.001)			(0.12)			(<0.001)	
Exit from Early								
N	119	9	-	15	24	89	85	43
Stop shedding	51	5	-	13	15	28	42	14
P value	(0.05)			(0.001)			(0.92)	
Exit from Fast Latent								
N	51	5	-	13	15	28	42	14
Begin shedding	21	2	-	4	9	10	15	8
P value	(0.62)			(0.24)			(0.11)	
Exit from Slow Latent								
N	16	3	6	3	1	21	10	15
Begin shedding	9	0	0	2	1	6	1	8
P value	(0.02)			(0.99)			(0.04)	

exited rapidly from exposed (with rate λ) and from slow-progressing latent (with rate ν_1) (Fig. 6.3a and 6.3g) and spent longer in the early shedding category (Fig. 6.3c) than animals exposed at older ages. Challenge dose only increased time in the early shedding category (Table 6.3b). Multiple dosing decreased the time spent in the early shedding category and increased time spent in all non-shedding categories. Oral dosing caused more rapid entry into early shedding, and, in addition, greatly increased the duration of the non-shedding slow-progressing latent compartment. Subcutaneous dosing increased rate of exit from early shedding and decreased rate of exit from the fast-progressing latent category. Overall the changes related to subcutaneous dosing decrease the total portion of time that a recently exposed animal would be shedding.


1.0
0.8
0.6
0.4
0.2
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0 2 4 6 8 10 12 14
(a)
(b)
0 10 20 30 40
(c)
(d)
0 10 20 30 40 50 60
(e)
(f)
0 10 20 30 40 50
(g)
(h)

Figure 6.3. Panels *(a)-(h)* are Kaplan-Meier plots of duration within infection categories: *(a)* and *(b)* = exposed, *(c)* and *(d)* = early shedding, *(e)* and *(f)* = fast-progressing latent, and *(g)* and *(h)* = slow-progressing latent. X axis is months and Y axis is proportion of animals still in the infection state. Left panels (a,c,e,g) are stratified by age at challenge (category 1: <3 mo, category 2: 3 mo-1 yr, category 3: >1 yr). Right panels (b,d,f,h) are stratified by challenge dose (category 1: $\leq 10^7$ cfu, category 2: $>10^7$-10^9 cfu , category 3: $\geq 10^9$ cfu). Dark line = category 1, intermediate grey =category 2, pale grey line = category 3

The split-population model estimated that 55% of animals did not develop detectable early shedding (p) or shed only for a short time period, and the remainder of animals (1-p) from exposed entered early shedding in an age-dependent manner (Table 6.3a). Highest risk of entry into early shedding was among the youngest animals and there was a zero risk of early shedding in adults.

Models using dose and age as continuous variables rather than categorical variables produced estimates that were broadly similar with respect to age and challenge dose except in the case of exit from fast-progressing latent (data not shown) for which age and dose were significant only in the continuous models. Values of predictors were not linear with age or dose for any categorical model and therefore results presented are for categorical models.

3.3. Goodness-of-fit and comparison of statistical distributions

Goodness-of-fit statistics for the best-fitting distributions for each state transition are presented in Table 6.4. Gamma distributions did not converge for all models except exit from slow-progressing latent, whereas log-logistic and Weibull typically showed a poor fit to the data. Log-normal models had lower AIC than exponential models for all categories, indicating a superior fit despite the extra degree of freedom in log-normal models relative to exponential models. Transition from slow-progressing latent (σ_1) was best fit by a log-logistic distribution.

Table 6.3. Regression coefficients, robust standard error and statistical significance for all log-normal time-to-event models. σ is the shape parameter for log-normal exit rates, π is the proportion of animals which are not eligible for early shedding by virtue of being on the slow-latent pathway.

Parameter	Coef.	RSE	Z	P>|z|	95% CI
a) Exit from exposed (λ)		Number of subjects=173			RSE adjusted for 15 studies
Age category 2	0.85	0.40	2.13	0.034	0.07, 1.63
Age category 3	16.8	.	. .		.
Oral dosing	-0.37	0.18	-2.07	0.038	-0.72, -0.02
Multiple dosing	0.69	0.20	3.41	0.001	0.29, 1.09
β_0, intercept	0.85	0.16	5.27	<0.001	0.53, 1.16
Ln σ	-0.57	0.10	-5.50	<0.001	-0.77, -0.37
Ln π (entering slow-latent)	-0.56	0.02	-28.0	<0.001	-0.64, -0.56
b) Exit from early shedding (ϕ)		Number of subjects= 128			RSE adjusted for 14 studies
Age category 2	-1.27	0.46	-2.77	0.006	-2.17, -0.37
Dose category 2	-0.91	0.24	-3.87	<0.001	-1.37, 0.45
Dose category 1	-1.41	0.24	-5.96	<0.001	-1.87, -0.94
Subcutaneous dose	-1.37	0.23	-5.92	<0.001	-1.83, -0.92
Multiple dose	-0.68	0.27	-2.53	0.011	-1.22, -0.15
β_0, intercept	2.32	0.24	9.62	<0.001	1.85, 2.79
Ln σ	0.11	0.09	1.23	0.22	-0.07, 0.28
c) Exit from fast-latent (σ_2)		Number of subjects=56			RSE adjusted for 10 studies
Age category 2	0.55	0.19	2.88	0.004	0.18, 0.92
Subcutaneous dose	4.27	0.88	4.87	<0.001	2.56, 5.99
Multiple doses	0.87	0.41	2.12	0.034	0.07, 1.67
β_0, intercept	1.99	0.21	9.71	<0.001	1.59, 2.40
Ln σ	-0.04	0.19	-0.23	0.82	-0.41, 0.32
d) Exit from slow-latent (σ_1)	Number of subjects=25				RSE adjusted for 7 studies
Age category 2	6.61	1.58	4.18	<0.001	3.51, 9.72
Age category 3	7.97	1.61	4.96	<0.001	4.82, 11.1
Oral dosing	1.81	0.13	14.0	<0.001	1.55, 2.06
Multiple doses	1.32	0.13	10.2	<0.001	1.06, 1.57
β_0, intercept	-0.42	0.13	-3.25	0.001	-0.68, -0.17
Ln σ	-0.57	0.38	-1.49	0.14	-1.31, 0.18

Table 6.4. Goodness of fit of modelled distributions in time to exit models for each of the four infection categories. Values presented are consistent with the categorical models from Table 6.3. Evaluation of log-likelihood (ll) in both the null and full models, number of observations (Obs), degrees of freedom (df), and Akaike Information Criteria (AIC) from all final models.

Category	Model	Obs	ll(null)	ll(full)	df	AIC
Exit from Exposed (λ)	Log-normal	173	-448	-428	6	868
Exit from Early Shedding (ϕ)	Exponential	128	-142	-124	6	260
	Log-normal	128	-132	-119	7	252
Exit from Fast-latent (σ_2)	Exponential	56	-55.5	-54.1	2	112
	Log-normal	56	-50.2	-47.2	5	104
Exit from Slow-latent (σ_1)	Exponential	25	-25.7	-14.3	5	39
	Log-normal	25	-24.6	-10.3	6	33
	Log-logistic	25	-25.1	-10.2	6	33

Plots of cumulative hazard vs. Cox-Snell residuals of the exponential and log-normal models were compared by visual inspection (Figure 6.4). Because most of the residuals were small, the distributions of residuals were heavily skewed towards the origin. There was little difference between the residual plots from exponential and log-normal hazards (Figure 6.4a, b, c). The only substantial difference observed in model fit was for exit from slow-progressing latent, where the exponential model produced high estimates for duration of slow-progressing latent well beyond the lifespan of a cow. Note that when comparing log-normal and exponential models with the same mean, the median is higher in the log-normal model.

3.4. Natural-exposure infections

K-M plots of exit from exposed in natural-exposure infections of young animals were similar to those for experimentally exposed animals (Figure 6.5a). Exit from early shedding in

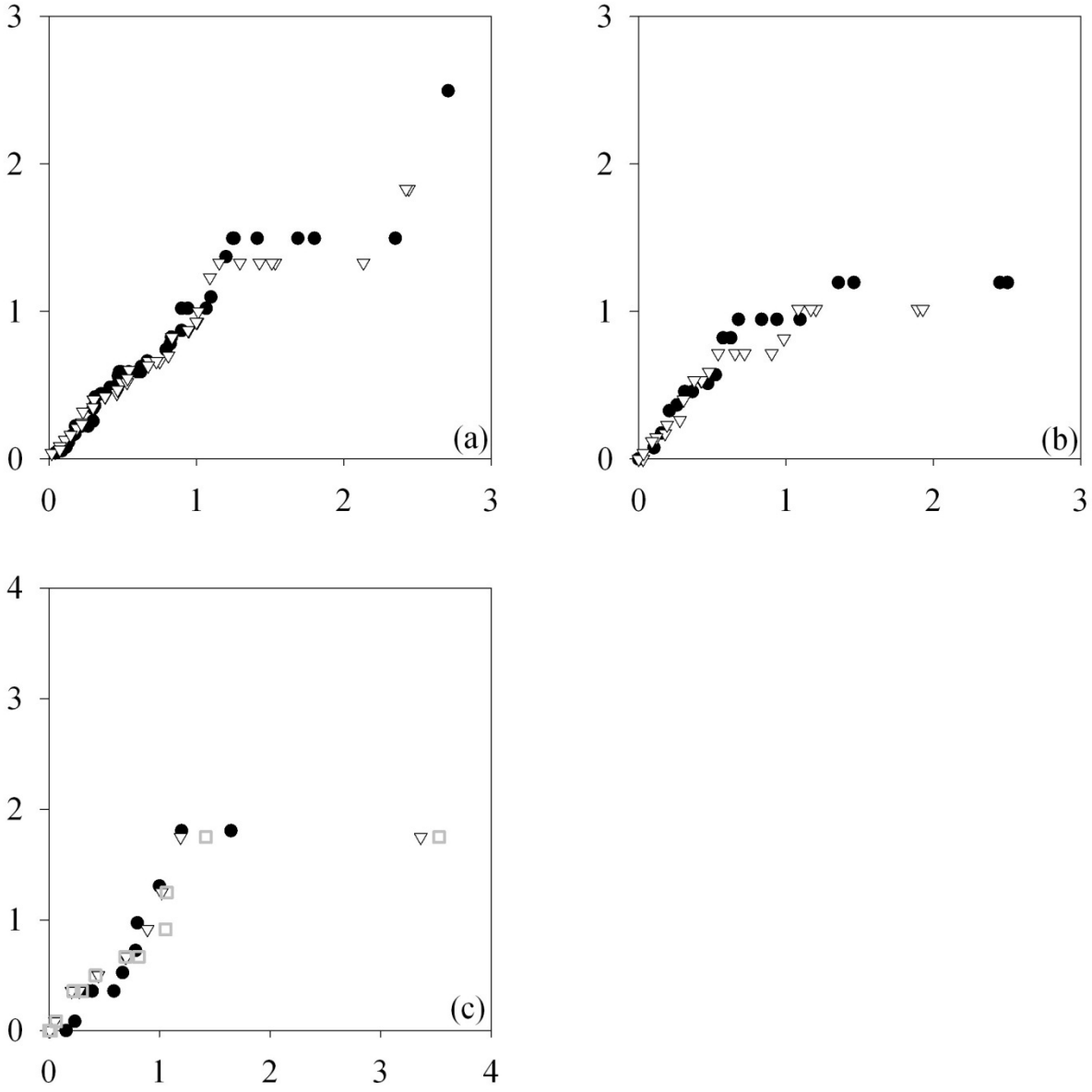

Figure 6.4. Plot of Cox-Snell Residuals (X axis) versus cumulative hazard (Y axis) for exit from early shedding (a), exit from fast-progressing latent (b) and exit from slow-progressing latent (c). Closed circles represent residuals from best fitting exponential models and open triangles represent residuals from best fitting log-normal models. The open grey squares represent the best fitting gamma distribution in exit from slow-progressing latent (c). A model which fit the data well had data points on a 45 degree slope without strong deviations (Dohoo, et al., 2003).

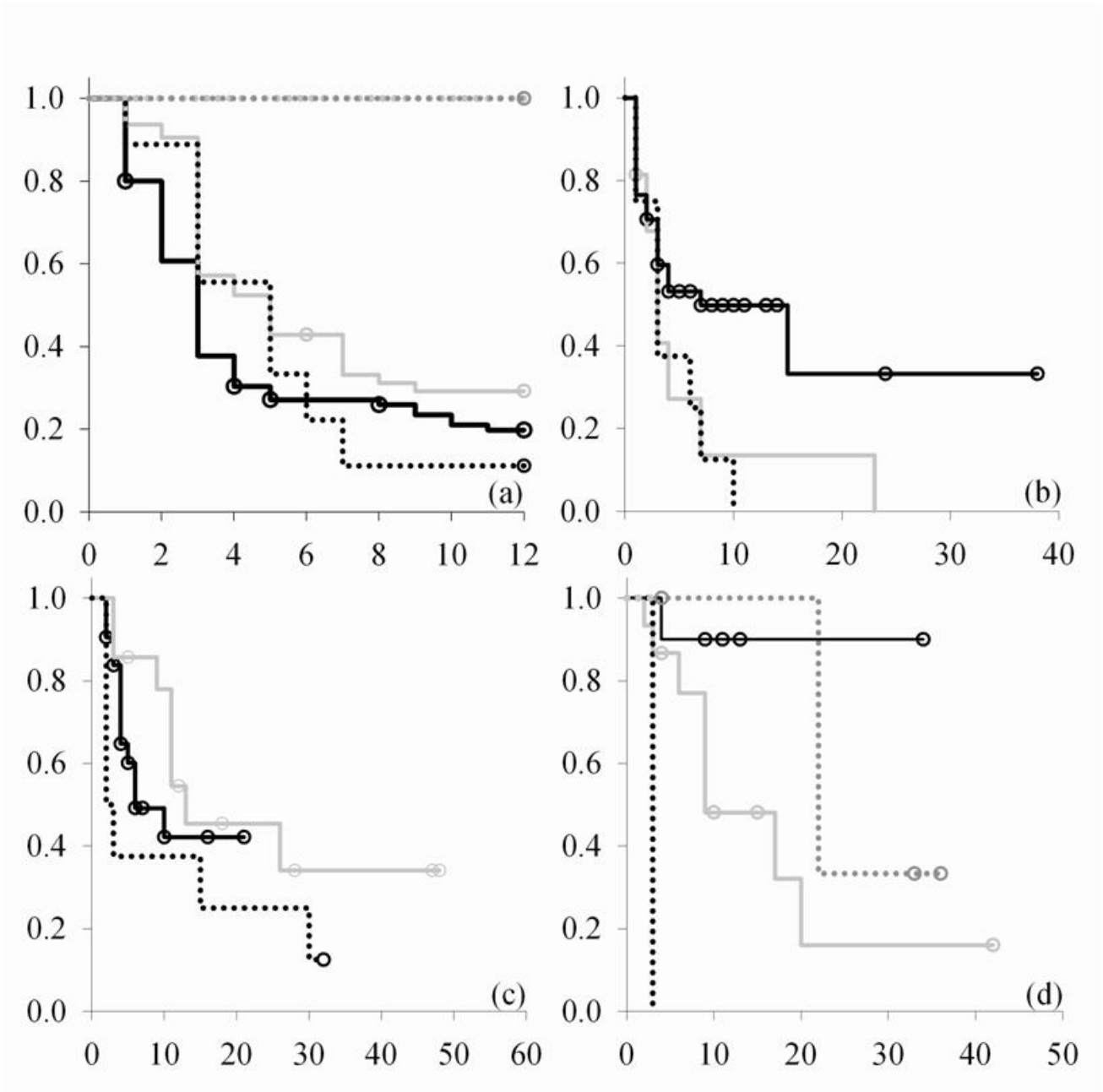

Figure 6.5. Kaplan-Meier survival plots for exit from exposed (a) and exit from early shedding (b), fast-progressing latent (c) and slow-progressing latent (d) by exposure strategy (single or multiple dose). X axis is months and Y axis is proportion of animals still in the infection state. Solid lines indicate different exposure categories: black lines indicate single exposure infections and pale grey lines indicate multiple dose exposures. Dotted lines indicate studies of natural-exposure infections (absolute dose unknown) from three specific studies: black dots (Rankin, 1961b), intermediate grey dots (Rankin, 1962), pale grey dots (McDonald, et al., 1999).

natural-exposure infections of young animals described by Rankin (Rankin, 1961b) was similar to plots of animals exposed multiple times (Figure 6.5b). Exit from fast-progressing latent in these data was more rapid within the first ten months than for both classes of experimentally exposed animals (Figure 6.5c). There were insufficient natural-exposure animals in the slow-progressing latent compartment to assess whether the fit of exit is closer to multiple-exposure or single-exposure experimental animals. The natural exposure of calves appears lower in the study by McDonald (McDonald, et al., 1999) (Figure 6.5) compared to the study by Rankin (Rankin, 1961b). Adults with natural exposure (Rankin, 1962) only shed late following infection, which is consistent with behaviour of adults in experimental dosing regimens.

4. Discussion

4.1. Experimental infections with known doses

This paper provides a more robust estimate of duration of stay in infection compartments than was previously available for long-term experimental infection studies of MAP in cattle. Our analysis included all long-term infection studies in cattle from the recent literature reviews by Begg and Whittington (Begg and Whittington, 2008) that fit the study criteria identified in the materials and methods. Many, but not all, of the studies that we used were published in peer-reviewed journals**.** The studies used in this analysis provided a wider range in age of animals at time of challenge and a wider range of challenge dosages than any single study could address.

Early shedding followed by a long duration of non-shedding is common in experimental infections (Figure 6.2) but early shedding is not usually addressed in models of MAP dynamics. Recent epidemiological studies also indicate that the assumption that young animals are not shedding needs to be challenged (Weber, et al., 2010). Not only do young animals shed MAP

following exposure, but they are also capable of transmitting MAP to other calves (van Roermund, et al., 2007). The alternative to considering the independent early-shedding period is to consider all the shedding animals in one population. This means that an animal which sheds in the first 12 months will be considered a shedder throughout its entire life, which is not an adequate reflection of either the experimental or the observational data available. This paper serves to quantify the early shedding phase.

Median time-to-first shedding in animals that shed shortly after infection is three months. Reducing the time period for which animals were eligible to become early shedders to six months would have captured the majority of early shedding animals (Figure 6.3a), but left a population which shed briefly early in life in the late shedding category despite subsequent cessation of shedding. Only a small percentage of infected animals are shedding when they enter the lactating herd, while in the dataset used in this meta-analysis more than half of calves exposed in the youngest age category have an early shedding phase. This population should not be contributing the same amount to infectious transmission as animals which are culture positive and often clinical in the late shedding stage (Whitlock and Buergelt, 1996).

Including studies with a range of dosing methods but allowing for dose method as a parameter in the analysis increased sample size while controlling for artefacts. Infectious progression was influenced by dose method, with oral dosing increasing rate of exit from exposed, but increasing duration of stay in the slow-progressing latent population. If oral dosing presents a risk of transient shedding due to intestinal burden of the infectious dose (Sweeney, et al., 1992), these would both be expected findings. Animals would be more likely to shed early after exposure, and the population of animals which did not shed early (which one could hypothesize received a lower infectious dose than those who did shed early after exposure) would remain non-shedding

longer. The clear difference in infectious progression between subcutaneous dosing and baseline intravenous dosing could be interpreted as decreased efficiency of initial infection due to the regional deposition in an area which is not a reservoir for MAP in infected animals. Despite the extremely large doses of ground intestine given, the cfu delivered could be lower than assumed in this analysis, which placed all ground-intestine dose mechanisms in the high-dose infection category due to sheer volume of infectious material delivered.

Multiple-dose models have a prolonged time of exposure relative to single-dose infections. They might present a model of chronic exposure rather than be equivalent to a single large dose. Duration of the multiple-dose infections in these studies ranged from 3 days to 28 days, which is still a fairly narrow window of exposure relative to the lifetime exposure in a commercial dairy herd. The overall effect of multiple dosing was to decrease the time spent in potentially infectious categories. In KM plots of exit from slow-progressing latent (Figure 6.5d), it appears that multiple-dose exposure causes a slower exit. However, this population includes multiple animals in the oldest age category which is taken into account in the multivariate analysis (Table 6.3). Because all animals infected with ground intestine were in the multiple-dose category, part of the effect of multiple dosing was that of the ground-intestine dosing system. When analyses were re-run excluding the animals dosed with ground intestine (n=7), multiple dosing remained significant in all models (data not shown). Decreased time of shedding in animals which received multiple doses is similar to the pattern of shedding in animals given lower doses of MAP. This could mean that initial dose is the most important factor in infectious progression. Because the longest multiple-exposure period in experimental animals was 28 days, we cannot assess true chronic exposure. However, calves which were naturally exposed to MAP in the Rankin study were in with highly infectious clinical animals for multiple months and had shedding patterns

similar to a moderate or highly exposed animal (Rankin, 1961a). If chronic exposure were to result in rapid infectious progression, we would expect these animals to have moved through the infectious categories faster than highly exposed single-exposure individuals.

Data numbers were not balanced with respect to age of animals at time of infection. Because it is a common belief that most infections happen among young animals, there have been few studies performed historically looking at experimental infection of adult cattle (Rankin, 1961a; Rankin, 1962). These studies are both expensive and offer little value when working under the assumption that adults are resistant to infection. The pattern of shedding is distinct between young animals and adults, with no adult animals experiencing an early shedding period in experimental infections, and rarely entering the late shedding period prior to four years post-infection. We argue that following data from the ten experimentally infected adults in published literature provides substantial information on the trend in this population and that valid conclusions concerning the inverse relationship between age at exposure and likelihood of early shedding can still be drawn from these limited data.

Due to data availability, this analysis is restricted to observation of duration of stay in infection compartments without quantification of bacterial load. We were not able to determine if there was a change in volume of bacteria contributed to the environment while in a shedding phase. Animals that become shedders earlier in adulthood are more likely to become high shedding or clinical during their expected life in the herd (Groenendaal, et al., 2002). It is possible that this population represents a cohort of animals which experience early shedding and moved more rapidly to late shedding than those which entered the slow-progressing latent period initially.

Our exclusion criteria eliminated animals from farms with known MAP-infected animals and animals with non-bovine-specific MAP infections. In addition, the requirement that culture data were published monthly also decreased sample size. However in our opinion this was a necessary step to ensure validity of results. Animals from infected dams or farms with known MAP-positive animals could have been infected any time previous to the initiation of the experimental period. Shedding patterns are influenced by host dependence of MAP strains (Thorel, et al., 1985; Stabel, et al., 2003; Stewart, et al., 2007). Because we are interested in dynamics on farms, we chose to eliminate infections with strains which are not reflective of bovine-specific MAP infection patterns.

We cannot distinguish between false negatives shedding low levels of bacteria and true negatives. It is possible that most animals shed early following infection, but that some animals are below the threshold of detection. In this case, we would have overestimated the rate of exit from early shedding, as well as overestimated time spend in the fast-progressing latent state, both serving to artificially decrease the impact of early shedding on transmission dynamics. Serial testing as performed in these experimental studies increases the sensitivity of the testing system for detecting truly infected individuals. Because animals are tested at least monthly, the sensitivity of these systems to detect any early shedding is much higher than annual testing schedules employed on commercial farms. However, duration of the early shedding period may be artificially truncated.

Because sensitivity of fecal culture increases with bacterial shedding, low shedders that are less likely to test positive are also less important for transmission. If there is truncation of the early shedding period due to test sensitivity, it represents reduction in epidemiologic importance. If degree of bacterial shedding is related to infectiousness then this supports our conjecture in

terms of the importance of high early shedding to herd persistence. Regardless of whether the animals in the slow-progressing latent compartment truly do not experience early shedding or simply shed below the threshold of detection, our data would indicate that animals in this slow-progressing latent compartment represent a distinct population which progresses through infection stages more slowly than animals which shed early. The association between decreased risk of early shedding and prolonged time to late shedding would be important to infection dynamics.

Exponential time-to-event models are compared with the best-fitting log-normal models. Exponential models with constant risk of exit are standard assumptions for mathematical models of infection transmission, but the log-normal distribution is a better alternative to this standard. The distinction between log-normal and exponential exit rates lies in the shape of the duration of the infection states. In exponential models of exit, the rate is constant and infectious individuals have the same risk of exit immediately after entry into the infectious category as they do after substantial time in the infectious category. In log-normal models, individual risk of exit increases with time in category to a peak value and then decreases. This latter scenario is more realistic than constant rate of exit and was previously described for *Salmonella* Cerro infections (Chapagain, et al., 2008). Future MAP models with exponential exit rates might fail to sustain infection in small populations. Mathematical models are sensitive to both the distribution of time in category and mean time in category, with increased probability of elimination in models with a more constant time in each infectious category (Keeling and Grenfell, 1998). Estimates from the exponential models give a frame of reference for future work on disease transmission where we make a choice to either use standard exponential exit assumptions or the log-normal assumptions which better match the disease process.

4.2. Natural exposure experiments

The two natural-exposure studies on young animals likely had different levels of exposure, with most exposed young animals from the Rankin study (Rankin, 1961b) shedding early, but no animals from the McDonald study (McDonald, et al., 1999) shedding early after exposure. This variation is biologically plausible, as level of infection is determined by rate of contact and infectiousness in each situation. The McDonald study focuses on a more pastoral environment (McDonald, et al., 1999) where rate of contact would be likely lower than in a zero-grazing facility. The enclosed animals were group housed with outdoor access in the Rankin study (Rankin, 1961b). It is possible that this housing situation more closely mimics a modern conventional dairy farm than the McDonald study. More studies of this type would be instrumental in determining what level of transmission actually occurs on commercial dairy farms. Adult animals exposed to cows with a clinical MAP infection (Rankin, 1962) presented themselves very similarly to adults that were experimentally infected, with no early shedding period and a long period without late shedding. Because we identified only three long-term studies with naturally-exposed animals that were sampled monthly, we do not expect that they represent the full range of infectious transmission patterns. Our summary data do, however, illustrate the principle that natural infections may have a much shorter time to shedding than generally assumed.

4.3. Application of findings

Age-at-exposure-dependent risk of early shedding might play an important role in transmission dynamics. In contrast to low-shedding adult animals which have only intermittent

contact with young animals, young shedders are intensively mixing with susceptible young animals on most commercial dairies or in heifer-growing facilities (USDA, 2002). If they are shedding at levels equivalent to low-shedding adults as found in experimentally exposed young animals (van Roermund, et al., 2007), young calves potentially contribute more to transmission dynamics than low-shedding adults. In addition to the high rate of contact with other young susceptible calves, our results show an inverse relationship between age at exposure and both duration and likelihood of early shedding. The effect of early shedding is not the only consequence of infection at a younger age. Those animals which enter early shedding progress to late shedding more rapidly than those which do not enter early shedding. Contributions from young high-shedding animals which become late shedders at a young age due to early exposure can perpetuate early infections through direct transmission (e.g. dam-daughter at birth, via colostrum or *in utero*) or indirectly through a grossly contaminated environment (Whitlock, et al., 2005).

The combination of increased early infectiousness, higher rate of contact among young cattle and a more rapid progression to late shedding could create a forward feedback loop within a herd. Theoretically, the prevalence of infection within the population would then determine what infectious states are reached (Dushoff, 1996). As animals are exposed at a younger age, there is increased risk of calf-to-calf transmission as well as a higher percentage of animals which are transitioning into a late-shedding phase during their time in the herd. Both the increased calf-to-calf transmission and increased number of late shedders will further increase herd prevalence. The high proportion of shedding animals could be self-propagating, so that as a herd reaches a certain prevalence of infectious animals, eradication of infected animals becomes increasingly more difficult. These exposure processes might contribute to the heterogeneity in farm-level

prevalence that is a feature of MAP epidemiology (USDA, 2002). These different exposure experiences may also have an important effect on the success of interventions to control disease, and might explain the persistence of MAP infection on farms undergoing control strategies. If young animals are contributing to exposure of other young animals, we may need intervention strategies that focus on isolation of young animals at risk for shedding from the population of susceptibles in addition to removing shedding adult animals. It should be noted, however, that a recent study failed to detect faecal shedding in naturally exposed calves (Pithua, et al., 2010).

4.4. Impact on transmission dynamics

We have seen in previous transmission models that infectious calves can theoretically sustain infection in populations that would otherwise move towards elimination (Mitchell, et al., 2008). Models of transmission dynamics which lack the early shedding category defined in this analysis do not capture a potentially important transmission cycle within herds (Collins and Morgan, 1992; Groenendaal, et al., 2002). Our previous models which did not distinguish infection progression patterns between animals less than one year old also missed a component of infection dynamics (Mitchell, et al., 2008). Future work will incorporate both early shedding and age-dependence of shedding among animals less than one year old. The positive-feedback relationships that we have shown between age at challenge, dose received and duration in infectious categories might lead to the development of scenarios in which the reproductive number of MAP is bistable depending on the initial prevalence of infection, creating the possibility of multiple endemic states.

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CHAPTER SEVEN

Effect of age- and dose-dependent shedding of *Mycobacterium avium* subsp *paratuberculosis* (MAP) on endemic stability

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Abstract

Maintenance of MAP infection on dairy farms is non-trivial in terms of infection dynamics. Indeed, a large portion of animals do not shed MAP throughout their lifetimes despite being truly MAP infected when their tissues are cultured at slaughter. In this study we incorporate the infection biology of MAP into a mathematical model which takes into account age- and dose-dependent infection progression. The model incorporates a large proportion of truly-infected-but-never-shedding animals which are observed as MAP positive in tissue culture at slaughter but not in fecal shedding during their lifetime. These animals are defined as latent MAP infected.

We modified our previously published models of MAP to incorporate a slow-progressing latent category based on previously published data on dose and age dependent infection probabilities. In this model, animals either shed shortly following exposure and enter an early shedding state, or do not shed early and enter a slow-progressing latent state where they remain for an extended period of time. Animals which shed early have a shorter duration of subsequent latency following this early-shedding state and then progress to late shedding. Progression of infection is modelled differently for animals which receive high or low initial doses of MAP. Animals which receive high doses of MAP spend a longer period of time in the early-shedding state and are therefore more likely to come into contact with susceptible young animals during this time of increased infectiousness.

Model output indicates that endemic MAP infection is possible at transmission rates that would not allow successful entry of MAP in a fully susceptible population indicating that MAP endemicity can be driven by different infection dynamics than the initial establishment of MAP in a dairy herd.

Our model indicates that there are two sustainable MAP infection equilibria at relative contact rates of less than one between adult animals and calves. This structure appears to be unique to dairy cattle, and might explain why endemically MAP infected herds occur frequently and why slaughter data show a dramatically higher prevalence of MAP infection in tissue samples compared to MAP fecal shedding during lifetime.

1. Introduction

Mathematical models of MAP have undergone substantial development recently. We have moved from models which account for only calfhood infection and adult disease to those that introduce transiently shedding animals at a young age (Collins and Morgan, 1991; Groenendaal, et al., 2002; Mitchell, et al., 2008; Marce, et al., 2011). Age at infection influences infection progression in all MAP models. Dose of MAP received influences probability of infection in models where discrete contacts between bacteria and susceptible calves are assumed to occur through milk, colostrum or environment. A recent meta-analysis of experimental MAP infections of cattle indicated that animals can to enter a long-term latent infection category depending on age at infection (Chapter 6). Long-term latent animals contribute to MAP prevalence at slaughter, but are negative on fecal-culture tests, creating a silent (latent) population of infected animals. This population has yet to be incorporated into mathematical models of MAP transmission.

Recent field data on slaughterhouse prevalence of MAP in dairy animals and longitudinal samples of dairy animals indicate that often the low prevalence of shedding animals masks a high within-herd prevalence of infection among adults (McKenna, et al., 2004; McKenna, et al., 2005; Pradhan, et al., 2009; Wells, et al., 2009). Earlier estimates of true infection prevalence assuming ELISA sensitivities of 15-88% using fecal culture as a gold standard (Dargatz, et al., 2001; Dargatz, et al., 2004) or using a Bayesian no-gold-standard approach (van Schaik, et al., 2003) were capturing the population of shedding animals. These studies then ignored the potentially dominant population of non-shedding infected animals. By including the large latently-infected population of animals, the true within-herd prevalence is most likely much

higher than the 10% assumed by the estimation techniques based on only diagnosed shedding animals.

Age-dependent infection progression as introduced in Chapter 6 can cause infection dynamics to be sensitive to within-herd contact structure. Dairy cattle have a fairly consistent contact structure which is segregated by age class (USDA, 2007). Young animals are raised in cohorts in which they mingle freely, and adults are in one big population which is segregated by lactating status and other potential common factors that are farm dependent. Therefore we know that animals, like humans, have age-specific mixing patterns, but we assume homogenous mixing within age classes. However, it is much more difficult to make assumptions on interclass mixing rates. The inter age-class mixing rates vary by farm and management practices. Often heifers are raised completely off the main farm and therefore have very little contact with adult animals. Some farms raise the heifers in the same barn or even penned directly in front of adults, resulting in very high mixing rates. In addition, contact rates between adults and calves are really encompassing both the rate of direct animal to animal contact and the rate of indirect contact between infectious material and susceptible animals. Because MAP survives for at least several weeks in the farm environment (Whittington, et al., 2003; Whittington, et al., 2004), the cow itself does not have to have direct contact with the calves. Shared equipment or clothing and footwear on the animal-care staff that is moving between heifer and cow facilities can result in contact matrices that have nearly equivalent inter- and intra-class contact rates.

A further unknown is the infectious dose needed for an animal to become "highly exposed" to MAP. Doses used in experimental infections are often extremely high (Begg and Whittington, 2008). However, controlled natural infections, where the MAP source is another infected animal, mirror shedding patterns of experimental infections (Chapter 6; van Roermund, et al., 2007),

indicating a biological plausibility for extending findings from experimental infections to models of infection progression.

MAP endemicity is not well understood, despite many theoretical models developed to reflect within-herd infection dynamics. Control strategies based on sub-optimal testing strategies remove the highly infectious individuals with high probability, but leave a large number of infectious animals in the population. Our own research group predicted naively that culling based on testing with a reasonable annual frequency would be sufficient to control and eliminate infection. The low-shedding adults are also removed in these test-and-cull strategies or segregated away from the remainder of the herd. Most models predict eventual elimination of infection under interventions or stochastic fade-out at infection introduction. However, there is the possibility that the contribution of early-shedding animals, which would not be detected in traditional testing strategies, are sustaining MAP infection in the population. These early shedding animals, at no higher risk of exit than an uninfected animal, have a disproportionately high rate of contact with susceptible animals of similar age relative to late shedding animals.

Although the model of MAP transmission is quite complex, the contribution of infectious individuals protected from increased rate of exit has been studied in simpler models of infection (Dushoff, 1996). In these models, there are combinations of parameters for which infection can only be sustained in a population if a large number of infectious animals are introduced simultaneously. The basic reproduction ratio (R_0) in these scenarios is less than 1, so that for each infectious individual introduced in a completely naive population, less than one subsequent infection is realized. Effective reproduction rate (R_e), which is the number of secondary infections from each case in a population that is not entirely susceptible, exceeds the basic reproduction ratio once sufficient population enters the 'protected' infectious compartment.

Highly infectious animals that are not at increased risk of exit create a forward feedback loop by increasing herd prevalence and replenishing their own population at a higher rate than they would be replaced by other infectious individuals. Unlike standard epidemic models for which infection will reach one stable equilibrium for each value of R_0, for those models with forward feedback loops, there are two stable equilibria for values of R_0 surrounding one. Stable equilibrium prevalence bifurcates at $R_0=1$ in a backward direction, with an unstable intermediate threshold determining whether the population will reach the higher or lower stable equilibrium. These backward bifurcation-based transmission patterns are unusually stable, and have been found in both viral (Medley, et al., 2001) and bacterial (Greenhalgh, et al., 2000; Greenhalgh and Griffiths, 2009) endemic pathogens.

Combining our estimates of age- and dose-dependent duration of infection categories with a transiently infectious early-shedding class (that does not run a risk of early exit from the herd based on the absence of clinical disease in young animals) creates the potential for a forward feedback loop in infection dynamics. We therefore hypothesize that high exposure to MAP creates a population of early-shedding animals which remain infectious longer than low-MAP-exposed early-shedding animals. These early-shedding animals can cause more early infections in other animals at the same young age. This would allow the effective reproduction rate in a highly-infected population to exceed the basic reproduction rate in a naive population, increasing probability of infection persistence in the face of elimination attempts. The objective of this study is to evaluate whether these feedback loops can be generated using real-life, observed, values for infection progression and herd turnover.

2. Materials and Methods

2.1. Model structure

The framework for MAP transmission models was taken from Chapter 6, with the addition of low- and high-shedding late compartments (Figure 1, ODEs are in Appendix 1). Susceptible animals move through age categories (0-3 months, 4-12 months, >12 months) in which they become progressively more resistant to infection. All animals are assumed to be born into the $Susceptible_{age\ category\ 1}$ compartment. Infection rates (λ) depend on both age class of the susceptible animals and infectious dose received (age=i, dose=j). For each age class there are two exposed compartments, high MAP exposure and low MAP exposure. In this model only low and high exposure categories are modelled. Once an animal is infected it follows age-specific infection progression rates. In the current model parameterization, only young and intermediate aged animals are able to become infected after exposure to MAP ($\lambda_{1j}=\lambda_{2j}> \lambda_{3j}=0$). Early shedding animals are considered equally infectious compared to late low-shedding animals. Late high-shedding animals are evaluated as either equally infectious to other shedding animals, or twice or ten times as infectious (Table 1). When categories do not need to be separated by age or dose (all late shedders will be adults, and exit from all Latent compartments is independent of dose received), they are collapsed back into one category.

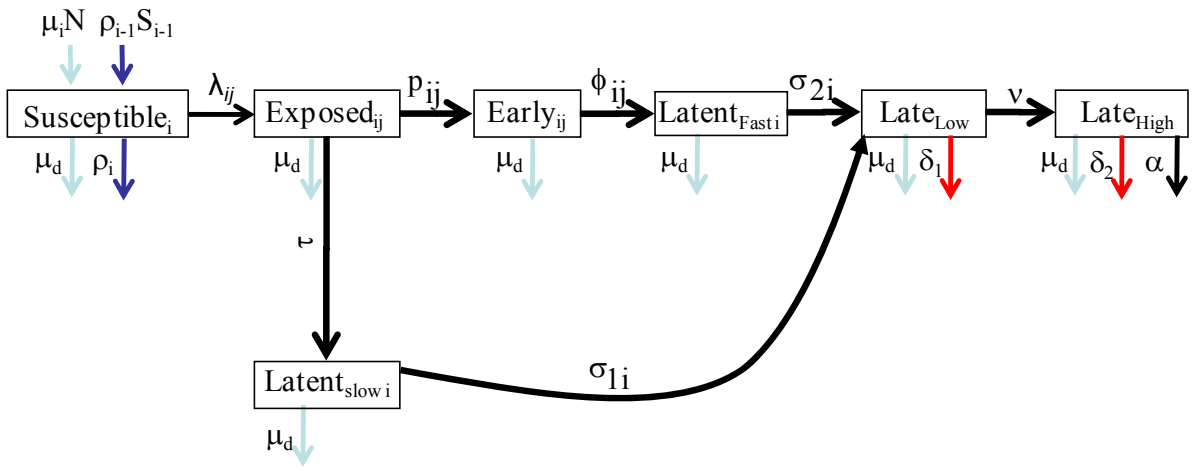

Figure 1: MAP infectious progression state-transition model. Transition rates can be dependent on age at exposure (i) or dose received (j). Black arrows represent rates inherent to infection dynamics. Blue arrows represent herd turnover (pale blue = births/deaths, dark blue= aging). Red arrows represent exit related to intervention-driven culling.

2.2 Input parameters

Parameters for infectious progression were based on a previously published meta-analysis by Mitchell and colleagues, 2011 (Figure 7.1, Table 7.1). Constant rate of exit from each compartment was assumed, and thus values from exponential survival curves were used. Contact structure was modelled using a Who-Acquires-Infection-From-Whom (WAIFW) matrix as in Chapter 2, with all intra-class contact rates assumed to be identical (Table 7.1). Interventions introduced represent stringent test-and-cull strategies which remove all high shedders at first positive test: on average six months after the start of shedding given an annual testing scheme. Low shedders are removed at on average two years after initiation of shedding to account for low test sensitivity. Three unknowns were modified in these simulations: the underlying beta, WAIFW matrix values and the relationship between exposure and likelihood of becoming a high or low shedder.

2.2.1. Beta

Input values of beta were selected to reflect reasonable intervention outcomes: (1) infection needed to persist both with and without intervention, and (2) there was a decrease in observed test-positive animals when interventions were used. Because average within-herd test-positive prevalence of MAP is <10%, we restricted our analysis to values of beta which sustain infection under intervention, but with a maximum prevalence of test-positive animals that was less than ten percent. As in previous work, test-positive animals were assumed to be either late high shedders with a test sensitivity of 100% or late low shedders with a test sensitivity of 25% (Mitchell, et al., 2008).

2.2.2. Contact structure (WAIFW matrix)

As in previous models (Chapter 2) we used a symmetric contact matrix with highest rate of contact within the same class of animals (Table 1). We assumed that the interclass contact rate was equal among all classes and varied from 0 to 1.0 of intra-class contacts.

2.2.3. Dose dependence

Dose-dependent transition from high-to-low exposure depended on (Eq1) overall infectious contacts or (Eq2) proportion of infectious contacts from high-shedding adult animals (Table 1). We assigned transitions that were either linear with exposure or on a logistic curve, so that as number of infectious contacts reached maximum saturation there was an abrupt increase in number of highly-exposed individuals. The linear transition rates were bounded at 1, so that proportion of animals entering the high-exposure categories in each age class was the minimum of value of the transition equation and one.

Table 7.1: Parameter values for mathematical model

Symbol	Definition	Estimate	Source
β_i	infectiousness	$[\beta \quad \beta \quad 0]$	Defined (4-25)
τ	Exit exposed→latent$_{slow}$	0.083/animal/month	Chapter 6
p_{ij}	rate entering early	$\begin{bmatrix} 0.40(1-k_i) & 0.40k_i \\ 0.20(1-k_i) & 0.20k_i \\ 0 & 0 \end{bmatrix}$/animal/month	Chapter 6
$\varphi_{(i,j)}$	exit from early	$\begin{bmatrix} 0.30 & 0.04 \\ 1.22 & 0.18 \end{bmatrix}$ /animal/month	Chapter 6
σ_{1i}	exit from latent$_{slow}$	$[0.06 \quad 0 \quad 0]$/animal/month	Chapter 6
σ_{2i}	exit from latent$_{fast}$	0.05/animal/month	Chapter 6
ν	exit from late$_{low}$	1/36/animal/month	Chapter 2
RC_{ij}	inter-class contact matrix	$\begin{bmatrix} 1 & w & w \\ w & 1 & w \\ w & w & 1 \end{bmatrix}$	Chapter 2
w	relative interclass contact	(0,1)	Chapter 2
ρ_i	rate of aging from category/year	$[0.028 \quad 0.111 \quad 0]$/animal/month	Chapter 6
μ_i	introduction rate	$[0.0167 \quad 0 \quad 0]$/animal/month	Chapter 2
α	disease related death in late$_{low}$	0.083/animal/month	Chapter 2
δ_1	control-related culling late$_{low}$	0.042/animal/month	Chapter 2
δ_2	additional culling late$_{high}$	0.167/animal/month	Chapter 2
ε	relative contribution of late$_{high}$	2	Defined
κ	slope of transition into highly exposed class (0 to 50)		Defined
λ_{ij}	force of infection	$\lambda_{ij} = \beta_i RC_{ij}\left(Early_j + Late_{low\,j} + \varepsilon Late_{high\,j}\right)$	Calculated
μ_d	death rate adjusted for infection	$\mu_d = \mu - \delta_1 \frac{Late_{low}}{N} - (\delta_2 + \alpha)\frac{Late_{high}}{N}$	Calculated
k_{ij}	proportion entering exposure class	$[max\{0,1-Eq\}, \quad min\{Eq,1\}]$	Calculated
	Eq(1): based on summed exposure	$\kappa \sum_{j=1}^{j=2} \lambda_{ij}$	
	Eq(2): based on proportion late$_{high}$	$\kappa\beta_i \frac{RC_{i3}\varepsilon Late_{high\,3}}{\sum_{j=1}^{j=2} RC_{i,j}(Early_j + Late_{low\,j} + \varepsilon Late_{high\,j})}$	

2.3. Simulations

All models were constructed and run in Matlab R2010b v7.11.0 (Mathworks, Inc, Natick, MA, USA). Differential equations were solved numerically using a variable order Adams-Bashforth-Moulton PECE solver.

The initial population was introduced into the model in the youngest susceptible age category and the model was run for 200 months to reach a final age distribution of naive animals. To assess whether models had more than one stable equilibrium at any given combination of input parameters, two initial MAP population prevalences were entered: 1% of the adult population infected in the low-shedding compartment, or 99% of the adult population infected and low shedding. For models with one stable equilibrium point, these two values converged slowly to one final infection prevalence. For models with two stable equilibria (0 and >0), final infected prevalence depended on initial infection prevalence. Models were run for 5000 months to ensure a stable final prevalence.

3. Results

3.1 Range of acceptable beta value x WAIFW matrix pairs is narrow

Initially it is assumed that there is no dose-dependence in rate of exit from early shedding and simulations allowed either all early shedders in the low-dose category (Figure 7.2, panel A) or all early shedders in the high-dose category (Figure 7.2, panel B). Without intervention, prevalence of fecal-positive animals plateaus at between 15 and 20% at the highest rates of contact for both models. The range of values of beta that produce true prevalence in the range of expected tissue-positives (>40%) and simultaneously fecal-positives less than ten percent are narrow. The inflection point at which infection persists is related to both WAIFW and beta

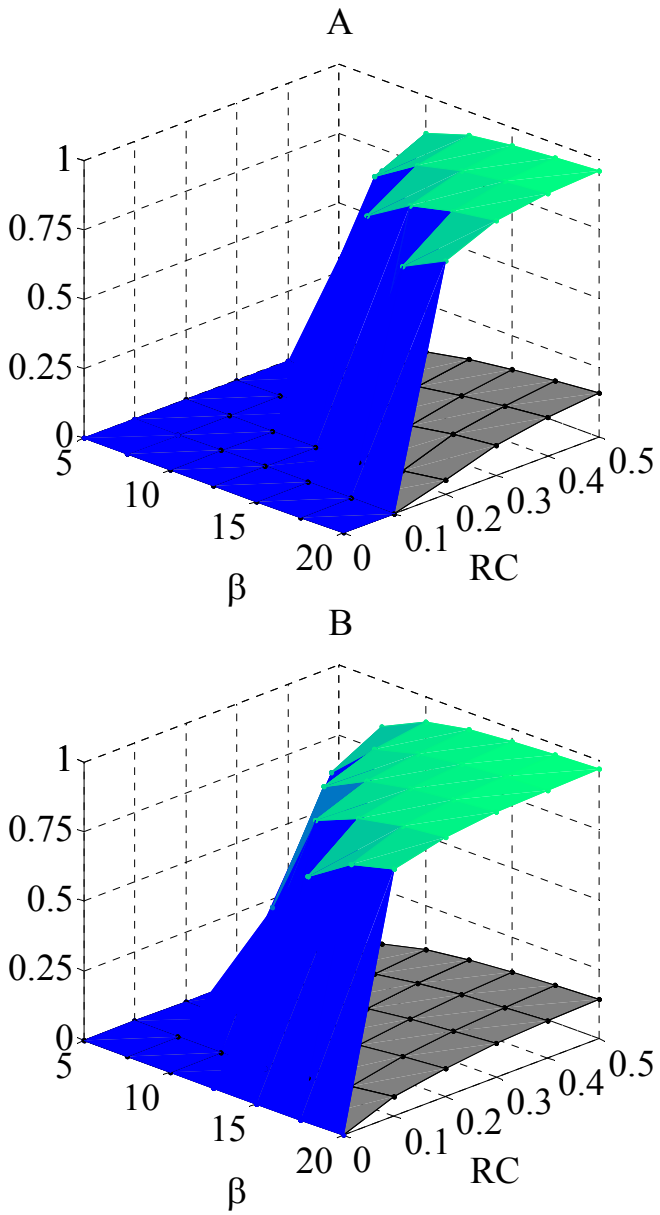

Figure 7.2: Prevalence of MAP infected animals without intervention run for 5000 months. Relative rate of contact between age classes (RC 0 to 0.5 on the bottom plane), β(0 to 20 on the bottom planes). Either 1% (solid planes) or 99% (mesh planes) of adult animals are infected at the onset of the simulation. Colored planes are total prevalence (all infected animals regardless of test status) and grayscale planes are test prevalence (25% of low shedding adult animals and 100% of high-shedding adult animals). In this figure, mesh and solid planes are completely superimposed. In Panel A, all animals enter the low-exposure pathway (with a short duration of early shedding), and in Panel B, all animals enter the high-exposure pathway (with long duration of early shedding).

values, and differs depending on whether or not there is a substantial population in the early-shedding high-dose category. After introducing culling into the simulation, populations are unable to sustain infection at low-beta, high-WAIFW combinations (Figure 7.3). This finding was independent of the assumption of early shedding. Longer duration of early shedding does decrease the effect of the intervention on overall infection prevalence, and for both models the maximum test-prevalence of MAP is less than ten percent of all animals.

3.2 Shift between dose categories

We focus on combinations of beta and WAIFW that allow MAP infection to persist under intervention and produce reasonable prevalence estimates without intervention. Shift between dose categories could be a function of total exposure (Eq1, Table 7.1) or due to exposure specifically from high-shedding animals (Eq2, Table 7.1). If total exposure within an age class (λ_i) determines transition between high and low dose categories (Figure 7.3, panel A and B) then introducing an intervention into an infected population may not eliminate infection.

When high-shedding adults spur the transition between high- and low-exposure categories, then there is no backward bifurcation created because intervention increases the exit rate of high-shedding adults which contribute to high-shedding calves. There are very few high-shedding adults present on a farm so this is always a very small proportion of the infectious animals, even if fast-progression infections are common.

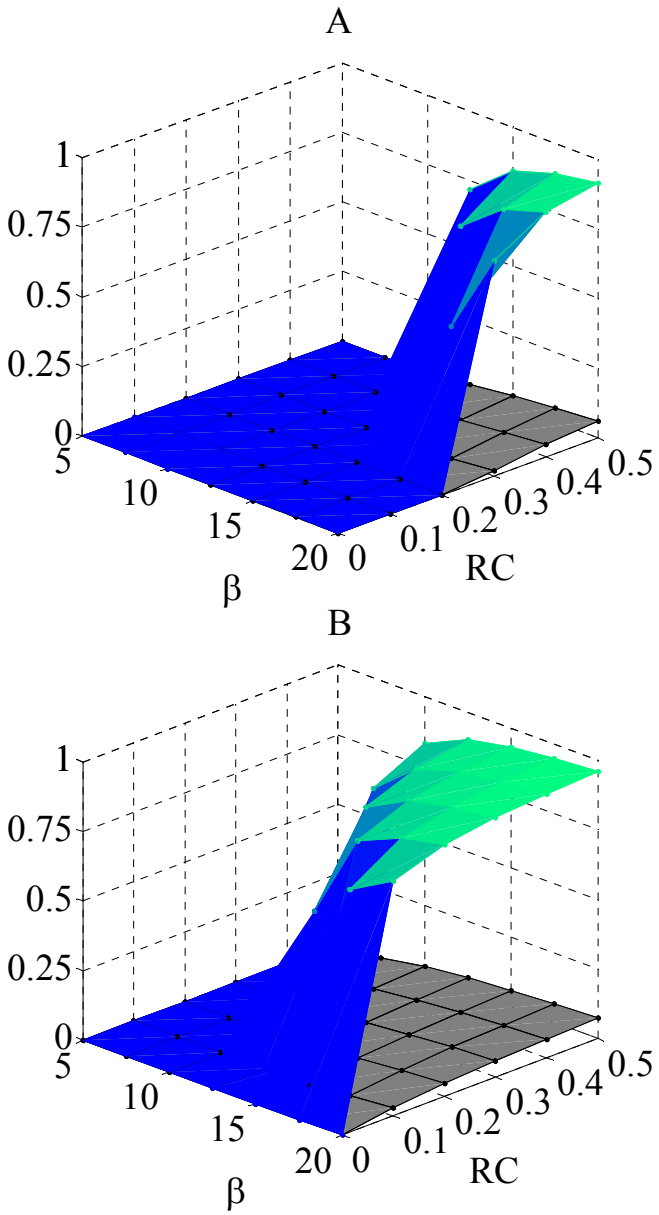

Figure 7.3: Prevalence of MAP infected animals (with intervention) run for 5000 months (Y axis). Intervention was modelled as removal of low-shedding adults (late shedders) at a rate of 0.25/animal/year and high-shedding adults at a rate of 1/animal/year. Relative rate of contact between age classes is (RC 0 to 0.5 on the bottom right axis), β (0 to 20 on the bottom plane left axis). Either 1% (solid planes) or 99% (mesh planes) of adult animals are infected at the onset of the simulation. In this figure, mesh and solid planes are completely superimposed. Colored planes are total prevalence (all infected animals regardless of test status) and grayscale planes are test prevalence (25% of low-shedding adult animals and 100% of high - adult animals). In Panel A, all animals enter the low-exposure pathway (with a short duration of early infection), and in Panel B, all animals enter the high-exposure pathway.

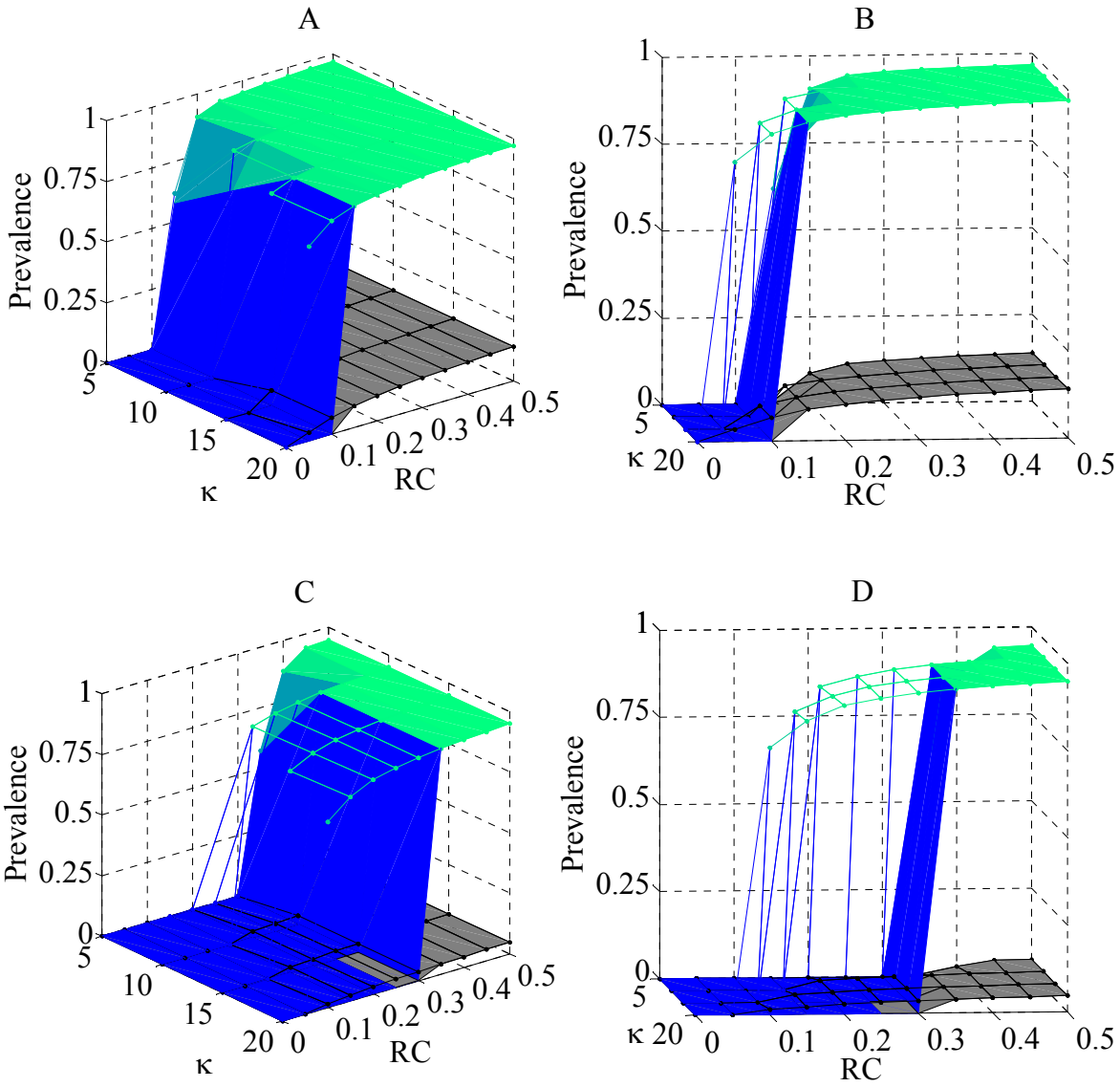

Figure 4: Prevalence of MAP infection in a model which allows calves to enter either low- or high-exposure pathways with a rate of transition k dependent on total infectious contacts (Eq1). ε=2, β=20). Pairs of panels (A&B, C&D) are the same model output, rotated to highlight the two final output prevalence estimates. In panels A and B, no intervention is employed. In panels C and D, interventions are introduced which remove low-shedding adults (late shedders) at a rate of 0.5/animal/year and high-shedding adults at a rate of 2/animal/year.

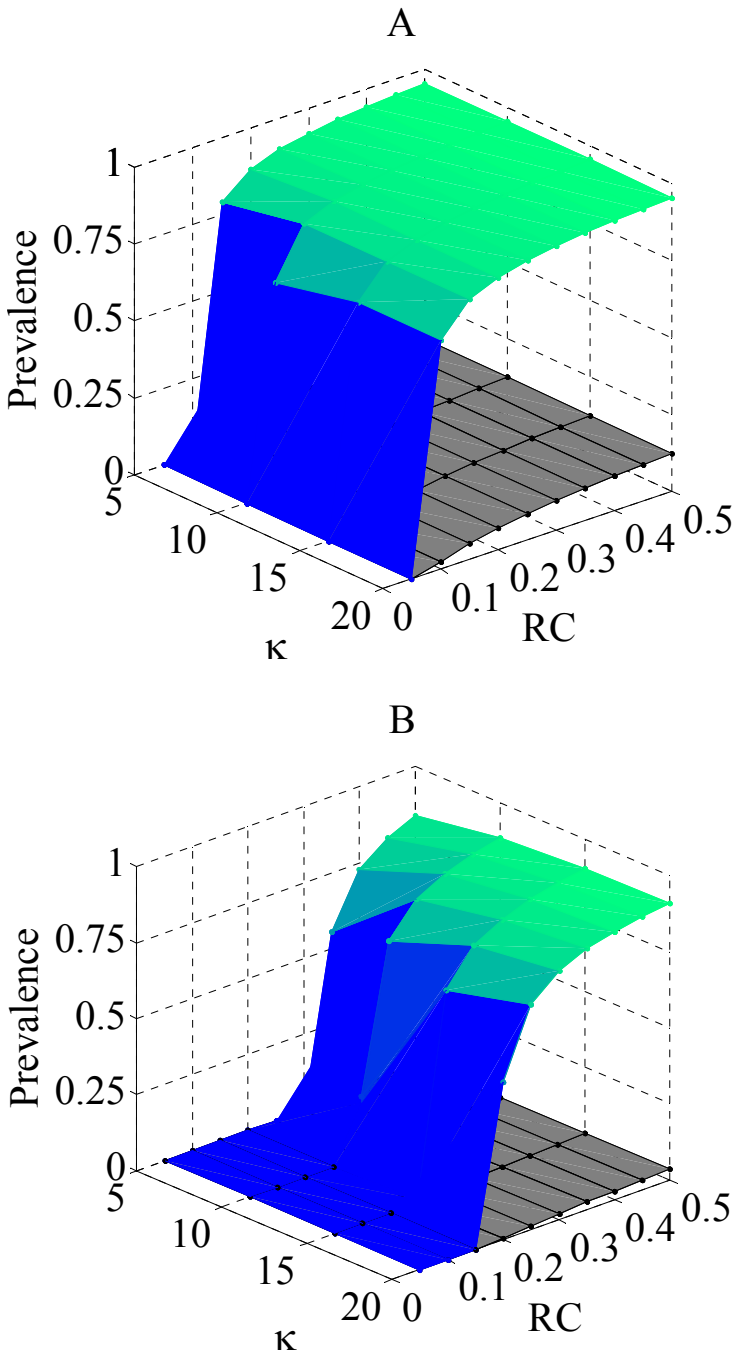

Figure 7.5: Prevalence of MAP infection in a model which allows calves to enter either low- or high-exposure pathways with a rate of transition κ dependent on ratio of infectious contacts from high-shedding animals (Eq2, ε=2, β=20). RC is the inter-class rate of contact in the WAIFW matrix. In panel A, no intervention is used. In panel B, intervention was modelled as removal of low-shedding adults (late shedders) at a rate of 0.5/animal/year and high-shedding adults at a rate of 2/animal/year.

4. Discussion

The most effective interventions for MAP involve management changes as well as removal of test-positive animals. In this case, not only is the proportion of highly infectious animals decreasing, but in intervention strategies which focus on preventing contact between cows and calves, the rate of contact between age classes is decreasing.

The presence of backward bifurcation in this model of MAP transmission using total MAP exposure as a trigger for high-dose infections offers an explanation of MAP persistence that was not previously considered in our control strategies. The feedback loop created is only possible if early shedding animals are able to transmit infection. Interclass mixing must remain low relative to intra-class mixing for infection to be sustained if only the test-positive (shedding) categories are assumed to transmit infection. Once the interclass contact rate approaches the intra-class contact rate, the overwhelming contribution from late-shedding animals that can be detected in test-and-cull control strategies makes it difficult to sustain non-zero prevalence under interventions.

MAP endemicity is a real life problem with probably over 70% of US herds permanently infected (USDA:APHIS:VS, 2008). The observed backward bifurcation that was shown in our simulations may partly explain the difficulty in eliminating MAP from farms.

Even when control procedures are in place that would not allow MAP introduction, MAP would still be able to persist in an endemically infected herd. These concepts are important to communicate to dairy farmers and their herd health consultants, as they may explain the often unsuccessful efforts that are currently practised. If backward bifurcation is indeed a contributing factor to endemicity, then a pulse of very strict control procedures may be necessary to truly eliminate MAP infection from the herd. The effectiveness of such focused intense control

procedures will need to be evaluated under field conditions.

The large population of latent animals is currently considered as not contributing to transmission of MAP. However, based on the work in Chapter 6 and the associated work by Benedictus *et al* (2008), we see increased risk of transmission from dams that are latent to their daughters. Whether this represents true vertical transmission or a pseudovertical transmission remains unknown. If this increased risk of MAP transmission is a result of intermittent shedding of latent animals, then these animals would also contribute to transmission in the rest of the herd.

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Appendix

$$\frac{dSusceptible_1}{dt} = \mu_1 N - \left(\left(\sum_{j=1}^{2} \lambda_{1j}\right) + \rho_1 + \mu_d\right) Susceptible_1$$

$$\frac{dSusceptible_i}{dt} = \rho_{i-1} Susceptible_{i-1} - \left(\left(\sum_{j=1}^{2} \lambda_{ij}\right) + \rho_i + \mu_d\right) Susceptible_i$$

$$\frac{dExposed_{ij}}{dt} = \lambda_{ij} k_{ij} Susceptible_i - (\mu_d + p_{ij} + \tau) Exposed_{ij}$$

$$\frac{dEarly_{ij}}{dt} = p_{ij} Exposed_{ij} - (\varphi_{ij} + \mu_d) Early_{ij}$$

$$\frac{dLatent_{fast\,i}}{dt} = \left(\sum_{j=1}^{2} \varphi_{ij} Early_{ij}\right) - (\sigma_{2i} + \mu_d) Latent_{fast\,i}$$

$$\frac{dLatent_{slow\,i}}{dt} = \tau\left(\sum_{j=1}^{2} Exposed_{ij}\right) - (\sigma_{1i} + \mu_d) Latent_{slow\,i}$$

$$\frac{dLate_{low}}{dt} = \left(\sum_{i=1}^{i=3} \sigma_{2i} Latent_{slow\,i} + \sigma_{1i} Latent_{fast\,i}\right) - (\nu + \delta_1 + \mu_d) Late_{low}$$

$$\frac{dLate_{high}}{dt} = \nu Late_{slow} - (\alpha + \delta_2 + \mu_d) Late_{high}$$

$$\frac{dN}{dt} = 0$$

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CHAPTER EIGHT

Discussion of findings

In this body of research, our goal was to improve understanding of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) transmission and endemicity. We combined knowledge gained from mathematical modeling, molecular epidemiology and field studies to determine which aspects of transmission dynamics might influence maintenance of endemic infection.

Impact of high-shedding adults

Super-shedding MAP-infected adults are believed to 1) cause transient shedding among other adults (termed passive shedding or 'pass-through' infections) (Sweeney, et al., 1992; Fecteau, et al., 2010) as well as 2) cause high levels of MAP contamination throughout the farm, including a high exposure to susceptible young animals (Whitlock, et al., 2005). Super-shedders have been implicated as important sources of infection in other cattle diseases including *E. coli* in feedlot cattle (Chase-Topping, et al., 2008; Matthews, et al., 2009). However, low environmental burden of strains from known super-shedders on Regional Dairy Quality Manangement Alliance (RDQMA) farms, which would indicate low probability of infection via indirect contacts, leads one to wonder about the impact of pass-through infections in MAP dynamics.

Passive shedding

In work concurrent to this dissertation, MAP isolates were selected from fecal samples and tissues at slaughter from all animals that were culture-positive at the same time that super-shedders were present in the RDQMA herds (Pradhan, *et al.*, 2011). Shedding level (MAP cfu/g)

of animals at each culture-positive occasion was determined. Tissues from 82% of cows with at least one positive fecal culture (other than super-shedders) were culture-positive, indicating true MAP infection rather than bacterial pass-through. At least 50% of low-shedders which began shedding at times contemporaneous with shedding of a known super-shedder had the same MAP strain as the super-shedder, suggesting that low-shedders might indeed have been infected as adults by these super-shedders. These data suggest a role of high-shedders in adult-to-adult MAP infection transmission.

Infection prevalence

Our initial simulation studies showed the importance of super-shedders on prevalence of shedding adults but also indicated that relatively simple test-and-cull control programs would substantially reduce the impact of these super-shedders on MAP transmission (Chapter 2). In ODE-based models, if we assume the infectiousness of super-shedders is ten-fold that of normal animals (but assume they maintain the same contact structure as low shedders) we find that super-shedders quickly overwhelm infection dynamics. Assuming high relative transmission rates from super-shedders (relative infectiousness, $\varepsilon=10$) and removing super-shedders under standard intervention schemes decreases the prevalence of MAP into the controllable range on farms undergoing interventions (Chapter 2), but this does not match with real-world data where MAP proves robust to elimination attempts.

Age and dose-dependent MAP shedding

Our objective in the meta-analysis (Chapter 6) was to quantify the impact of experimental MAP infection (delivery method, multiple or single dose, CFUs given) on likelihood of shedding

in young animals and progression of infection. The association between dose received and duration of early shedding could prove important in preventing calf-to-calf transmission on farms. In addition, delaying exposure to greater than three months can virtually eliminate the risk of a long period of early shedding. We explored the potential contribution of an age- and dose-related infectious progression to within-herd transmission dynamics. There had not yet been a data-based (vs. theoretical) paper published on veterinary pathogens which illustrates backward bifurcation in both age- and dose-dependent fashion. Backward bifurcations are one method by which we can explain sustained infectious transmission at a low prevalence or where aggressive intervention strategies are hypothesized to bring basic reproduction ratio less than one, but MAP persists. We used deterministic mathematical models to simulate farms undergoing active interventions.

This work could help us understand the potential impact of a MAP vaccine which may or may not reduce the early shedding period but delays infectious progression. The larger the proportion of animals which enter the slow-progressing latent category rather than the fast-progression track, the fewer the animals that will become clinical later in life. Moving most animals onto the slow-progression track could allow MAP control with minimum impact on herd economics.

Infectious contribution from calves

Calves shed MAP after experimental infection (Chapter 6); however, until recently this shedding has not been considered as a potential source of MAP transmission on the farm (Bolton, et al., 2005; Weber, et al., 2005; van Roermund, et al., 2007). Although MAP shedding by calves is detected on farms, it might be infrequent or at a low level. Recent modelling work sought to quantify the relative importance of calf-to-calf transmission in French dairy herds,

where animals have frequent access to pasture and found only a minor contribution of transiently shedding animals to transmission in this context (Marce, et al., 2011). Differences between these two models of MAP transmission indicate further research is needed on the importance of these infectious young animals.

Vertical Transmission

MAP-infected calves born to infected dams might have been infected as fetuses, but face two additional risk factors for MAP infection: increased genetic susceptibility and increased exposure to MAP immediately following birth. Dam-to-daughter transmission prior to birth is a very low risk barring disseminated high bacterial loads in the dam (Whittington and Windsor, 2009); however, daughters are at high risk of pseudovertical transmission due to close contact during and immediately after birth. We have previously shown that even under a strict intervention scheme designed to limit dam-daughter contact, there is an association between dam infection status and daughter infection status (Benedictus, et al., 2008). Calves born to latent dams (dams which are fecal-culture negative at the time of a calf's birth, but progress to fecal shedding later in life) have increased risk of becoming MAP-positive as adults relative to calves from consistently fecal-culture-negative dams (Benedictus, et al., 2008). In this previous work, the risk of MAP-positive calves was not different between latent and low-shedding dams. Vertical or pseudo-vertical transmission from latent dams has not been addressed in previous models of within-herd transmission.

Infected daughters from low-risk MAP-infected dams could represent a subset of the population which is genetically more susceptible to MAP than other animals (Settles, et al., 2009; Zanella, et al., 2010). Based on molecular epidemiology of MAP transmission in the

RDQMA herds (Chapter 5), daughters of infected dams are at increased risk of picking up any MAP strain (increased genetic susceptibility) as well as the dam-specific MAP strain (increased exposure or intra-uterine infection). In many cases, the other strains present on the farm at the time of birth were of the same type as the strain identified in the daughter, so horizontal transmission cannot be excluded. Also, at least 50% of daughters carried a MAP strain in addition to their dam's straing, suggesting infection from sources other than the dam itself. We therefore concluded that vertical transmission appears to play a role in MAP transmission but does not appear to be a dominant phenomenon.

Planned work looking at molecular typing of MAP strains using the RDQMA database includes: (1) evaluation of birth cohorts for clusters of MAP infection by strain, (2) evaluation of relationship between burden of MAP strain in the first year of life and probability of becoming infected with that strain and (3) impact of presence of concurrent supershedders on likelihood of shedding the same strain of MAP.

Contribution of latent animals to MAP transmission

Because vertical transmission of MAP from non-shedding dams to daughters is common to both Chapter 5 and in the accompanying work by Benedictus and colleagues (2008), we hypothesize that latent animals might play an important role in transmission dynamics. If we integrate the age- and dose-dependent infectious progression from Chapter 6 with the assumption that latent animals contribute to infection dynamics in a deterministic model of within-herd transmission, MAP persists under intervention (Figure 8.1) with a more realistic within-herd MAP prevalence than those in Chapter 7.

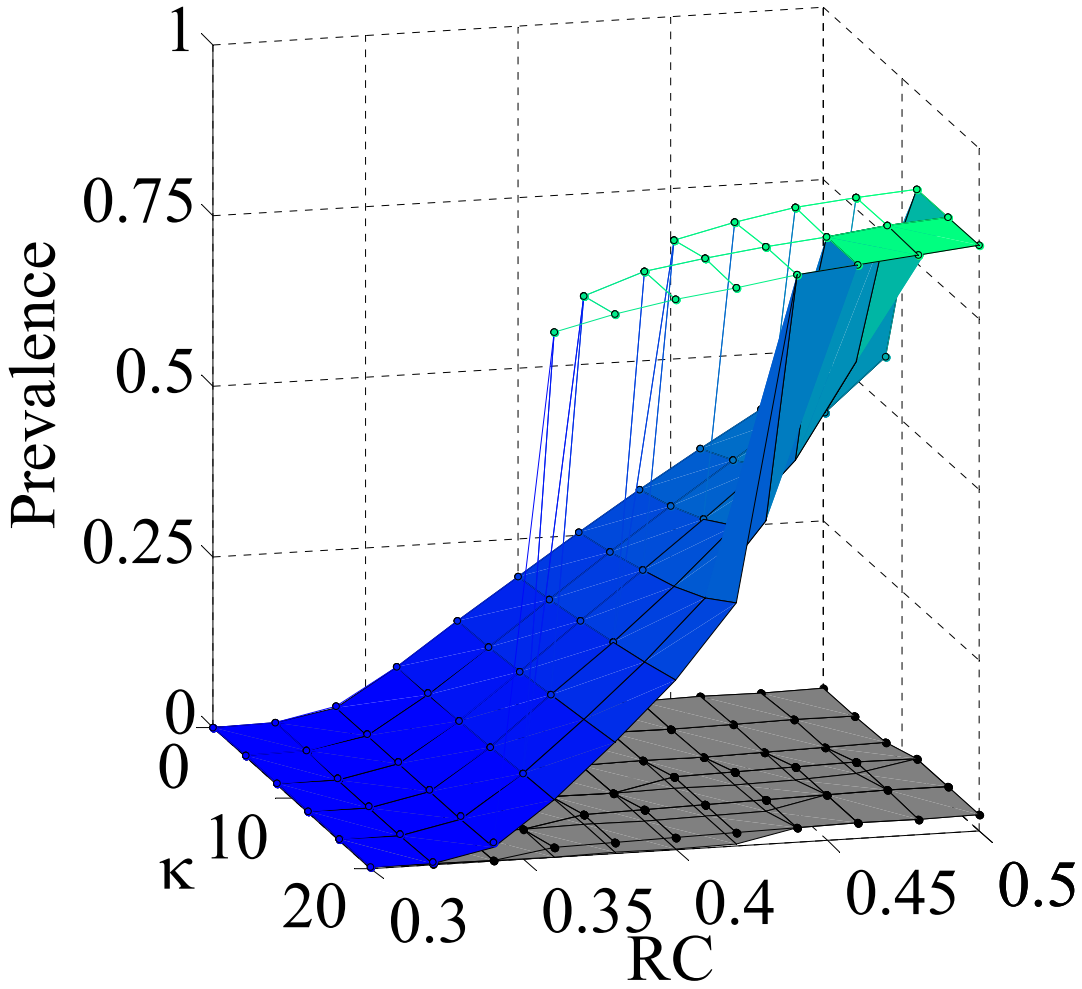

Figure 8.1. Simulation output for the model specified in the Appendix to Chapter 7 with an additional infectious contribution from both fast and slow latent animals. In this simulation, animals in latent categories contribute to infectious burden from their respective age categories at a rate $1/100^{th}$ that of animals that are shedding MAP at a level detectable by culture (low shedders). κ is slope of rate of transition from low to high exposure categories, RC is inter-class rate of contact in the WAIFW matrix defined in Chapter 7. Strict intervention was employed in this simulation (δ_1=0.5/animal/year, δ_2=2/animal/year). All additional parameter values not specified are as listed in Chapter 7, Table 7.1. (β=12, ε=2)

Pathogen heterogeneity

In our work, strain-specific survival of MAP was similar among strains from the same farm (which might represent evolutionarily similar strains), but was not necessarily similar among

bovine isolates (Chapter 3 and 4). Different *in vitro* survival patterns of bovine-specific MAP strains in both studies could contribute to understanding MAP shedding patterns found on dairy farms. Strains which do not stimulate the pro-inflammatory immune response could survive longer in the host without causing disease and result in more subclinical infections than those which cause a rapid and strong pro-inflammatory immune response, triggering clinical disease. Based on the RDQMA dataset presented in Chapter 5, we have the tools to evaluate whether there are different responses to dominant MAP strains on Farm A. This will be the first evaluation of strain-specific disease progression in MAP. If all strains are found to have equal infectiousness (β) and infectious progression rates, infection can be managed as an absolute (farms are infected or uninfected). If strain type strongly relates to infectiousness or progression, there could be need to determine the strain profile of farms to best target control strategies.

Putting it all together

Our goal in this work was to understand the mystery of persistent MAP infection with very low prevalence of apparent (test-positive) MAP-infected animals. Our original modelling work created a problem that had not previously been addressed: if MAP is endemic even with conventional test-and-cull strategies applied, then our models cannot predict successful control and still be considered accurate. Using our assumptions that calves are infectious and that high-shedding adults are not that much more infectious than low-shedding adults, we produce models that can reflect the actual prevalence of MAP in farms.

Overall, all of our models agree with the observation that super-shedders don't drive MAP persistence by just serving as the dominant contributors to cow-to-calf infection. Indeed, assuming that the contribution to horizontal transmission from latent animals was only 1% of

that of low-shedders in an environment where the contribution from low- and high-shedders is assumed to be very similar allowed us to produce models which are close to observed prevalence data. Vertical transmission from latent dams may play an important role in infection maintenance as well, although we showed in our field data that vertical transmission is happening in only a small proportion of animals.

When we implement test-and-cull strategies, we are removing animals deemed 'more likely to influence infection dynamics' relative to all other animals. However, if calf-to-calf transmission is really driving a positive feedback loop as suggested in Chapter 7, then to prevent new MAP infections we would also need to target calf-to-calf transmission rather than only cow-to-calf transmission. Testing and culling high-shedding adults would also remove the calves of these high-shedders. The calves of high-shedding adults have a high risk of being infected at birth and remain transient infectious for a longer period of time. Hence, high shedders have an indirect effect on infection dynamics through calves and their direct contribution to infection dynamics appears to be similar compared to low shedding animals.

Our most accurate models appear to indicate that, in populations with low prevalence of MAP-shedders, infection is maintained by a population of animals that is not consistently positive on tests. The animals that are not consistently positive on test are either transiently infected calves, resulting in calf-to-calf transmission or latent animals that contribute MAP transmission to either calves or adult animals. Given that these calves and latently infected individuals are currently undetected in our testing strategies, one does question the value of continued testing in populations with low MAP prevalence and good management practices. It is possible that in such herds strategies for control that do not use diagnostic testing at all but focus on preventative management practices might be most economically efficient. Actual prevalence

of MAP infection on endemic farms is high, but the proportion of MAP-infected animals that is detected during their life time is low. It also appears from our simulations that non-detected but truly infected animals play an important role in the observed endemicity of MAP on farms.

In survey data we appear to observe two populations of farms, most farms have a prevalence of MAP between 3 and 10% whereas a small proportion of farms has a prevalence that is much higher than 10%. The difference between these two groups of farms might be in the rate of contact between animals on the farm. A small proportion of farms that have extremely high test-prevalence on herd surveys would be characterized by a high rate of contact between cows and calves. The majority of farms with a low MAP prevalence would be characterized by a low rate of contact. In farms with high inter-class rate of contact and a large population of adults with clinical Johne's disease, decreasing rate of contact between calves is going to have a minor impact. The first step in controlling MAP infection (and controlling clinical disease by increasing the proportion of slow-progression infections) is to decrease rate of contact everywhere possible. Reducing contact rate will be executed by preventing distribution of infectious fecal material. Once inter-class contact is decreased below a threshold where everyone is guaranteed to be highly exposed to MAP, then targeted interventions are going to start playing an observable role in transmission dynamics. Vaccination might play an important role in this transition. Vaccination might allow more animals to enter the slow progression pathway of infection by decreasing the number of infectious contacts or the response to infection once an animal becomes infected. Through both mechanisms there would be a decrease in the number of early shedders on the farm.

Future work

Develop an optimal testing strategy

I would like to look at the value of a positive test in predicting true infection status and infectious progression using tissue culture as a gold standard. We have many farms that institute a policy of following up ELISA-positive cows with a fecal culture. Culling is then based on a fecal-positive test. If we truly believe that two populations of latent animals exist, then intermittent shedding in the slow-progressing latent would result in unnecessary culling. It is possible that multiple positive tests in rapid succession are better predictors of clinical disease or future high shedding than single positive tests or non-consecutive positive tests. Optimizing testing strategies based on reducing the incidence of high shedding and clinical animals would reduce both the testing costs and unnecessary culling in herds attempting to control but not eliminate infection.

Importance of environmental contamination (indirect transmission)

The current screening methods are likely to identify the vast majority of high-shedders correctly. Using annual fecal-culture will identify all high-shedders within an average of 6 months since the start of high shedding. On our closely-monitored RDQMA farms we described that even when there is a substantial population of high- or super-shedding animals on a farm, there is not a massive environmental contamination (RL Smith, 2011 Thesis Cornell University). The calf area never cultured positive on any of the RDQMA farms (preliminary data). Although there is an association between super-shedders being in a pen and environmental MAP-positive samples from that pen (Smith, *et al*., 2011 *in press*), in preliminary work looking at strain distribution of MAP-positive environmental strains, dominant environmental strains do not

match the supershedder strain in the majority of sampling periods on one of the farms (Farm A). A better understanding of the contribution of indirect transmission is an important consideration in parameterizing future models.

Strain competition within closed herds

Within-host heterogeneity of MAP strains is not well characterized at this point. Based on the strain-typing data from chapter 5, in the RDQMA dataset, there are many animals which are infected with multiple strains of MAP throughout their lifespan (18 of 37 animals with two or more samples in a preliminary analysis carried two or more distinct strains). Through continued exposure to MAP throughout life in contaminated barn environments, most animals are likely exposed to MAP multiple times in their lifetime. Preliminary work identifying multiple MAP strains on RDQMA farms provides the option to move toward evaluating strain-specific infection dynamics and within farm or within host strain competition. The evaluation of potential strain competition in combination with the multiple-strain infections in MAP infected hosts would be an exciting avenue for future work.

Stochastic model objectives

Our existing series of mathematical models of MAP transmission in US dairy herds does not take the unique infection history of individual cattle into account but rather models population averages (Mitchell, et al., 2008). We have the tools to build stochastic models of MAP transmission using updated model structure and parameter estimates calculated in Chapter 6. Stochastic models also allow a much more precise evaluation of infection fade-out. The apparent absence of MAP fade-out is precisely what drove the primary interest in the research undertaken

in this thesis. A more precise understanding of how MAP escapes stochastic fade-out on US dairies will help shape future control efforts.

Conclusions

We set out to solve the mystery of MAP endemicity in low-prevalence populations. We solved the mystery in the sense that it is really low-apparent-prevalence endemicity in high-prevalence populations, with a large proportion of infected animals that are never test-positive during life.

The insights from this work based on longitudinal field data and novel within-herd MAP strain diversity studies, is changing the way we should approach MAP on farms. MAP is much more pervasive within herds than was understood before we started this research project. Our work, which indicates that despite long-term follow-up the majority of infected cows may remain undetected because they are on a separate infection progression pathway, changes the way we should approach control of MAP. There is very little value to removing the low-shedding animals in a herd when there are nearly ten-fold as many low-shedding or latent animals present as detectable in the herd at any given point. The vast majority of these low-shedding or latent infected animals are not detectable during the life of animals at this point in time. The long-term follow-up of the MAP-infected animals with the use of MAP strain typing also showed that there are many different strains of MAP on closed farms. The finding of bistable equilibria where farms which reach a certain threshold of infection are refractory towards elimination attempts reinforces the idea of managing MAP as an endemic pathogen. Testing for MAP using the current test-and-cull policies might not be an economically beneficial control strategy, whereas the strict implementation of MAP preventive management practices would be. It is expected that

vaccination might be an important avenue for bringing MAP infection under control to minimize economic losses on farms with endemic infection.

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