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SHEDDING OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN NATURALLY EXPOSED DAIRY CALVES AND ASSOCIATED RISK FACTORS

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Michael William Bolton DVM

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By

Michael William Bolton DVM

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ABSTRACT

SHEDDING OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN NATURALLY EXPOSED DAIRY CALVES AND ASSOCIATED RISK FACTORS

By

Michael William Bolton DVM

With the recent development of liquid culture techniques, and associated higher sensitivity, we conducted a study to detect shedding of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne's disease (JD), in naturally exposed dairy calves from eight Michigan herds. We charted age distribution, MAP status of the dam, MAP prevalence in the herd, sensitivity of two sizes of pooled fecal samples as well as concurrently procuring an ELISA blood sample. We were able to detect MAP in calves with the preponderance of positive animals falling within the seven to fourteen month age group. A higher percentage of infected calves were from positive dams and herds with higher MAP prevalence. There was no apparent association between fecal culture results and ELISA results and fecal pools of five animals showed significantly higher sensitivity than pools of ten. In a separate case study we demonstrated the potential danger in retaining a clinically normal, MAP shedding, cow in a low prevalence herd. The take home message, from the sum of the components in this study, is that close attention has to be paid to the young animal and all risk factors must be considered and controlled to comprehensively manage Johne's disease in an infected dairy herd.

DEDICATION

This work is in memory of my Mom, Jo Bolton. It is a culmination of a process she started with a gift of money, to attend the first Dairy Certificate Program at Michigan State in 1992. That has resulted in many opportunities.

It is also in honor of my Dad, Bill Bolton, a consummate learner and the most unselfish man I have ever met. He has reveled in each of the post-graduate degrees obtained by every one of his seven children.

A multitude of thanks goes to each of my children, Molly, Zach, and Ann. They each have had a hand in the preparation of some portion of this document.

Finally, and above all, my sincerest gratitude goes to my wife Ruthy, for all her patience, encouragement and love.

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Finally, a public thanks to Dr. Jim Lloyd who noticed this graduate position and put me on to it. Jim also was responsible for nominating me to the board of directors of the American Association of Bovine Practitioners for which I am grateful. He is always selflessly surveying the landscape for possibilities for other people. I could not have a finer friend.

I dedicated this manuscript to my entire family who truly deserving it.

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INTRODUCTION

Johne's disease (JD) caused by *Mycobacterium avium* subsp. *paratuberculosis (MAP)* is a chronic, infectious, inflammatory enteric disease of both domestic and non-domestic ruminants. Although JD is over a century old, described in 1895 in Northern Germany and characterized soon after (Twort 1910) as an acid-fast bacillus, it is of increasing international importance in the cattle industry. Very prevalent in the United States, JD is associated with reduced milk production and economic loss (Ott 1999). There is also mounting evidence of a public health risk associating this bacteria to Crohn's disease (Naser 2004). Although widely variable prevalence figures have been published (Adaska 2003; Hirst 2004; Johnson-Ifearulundu 1999; Pillars 2009) it is safe to assume that MAP is present in about half of the US dairy herds and a significant number of beef herds as well. It is widely accepted that calves are often infected before the age of six months (Sweeney 1996) but MAP is a slow-growing bacteria and development of clinical signs may take 2-5 years (Harris 2001). Fecal culture has been an ineffective method to detect low bacterial shedders (Kim 2002). Due to reports of increased sensitivity with the recently developed TREK®ESPII liquid culture system (Stitch 2004) we designed a study to determine if we could detect fecal shedding in naturally infected dairy calves.

HYPOTHESES TESTED

1. Fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis (MAP)* can be detected in calves using liquid culture and this may not be equally distributed across age groups.
2. An association exists between ELISA test results and fecal culture results of these calves.
3. Johne's ELISA test status of the dam is a significant risk factor to her offspring.
4. There is an optimal number of animals contained in a pool of fecal samples when utilizing this method to access MAP shedding in a group of calves.
5. There may be a risk associated with retaining a clinically normal, heavy shedding cow in a dairy herd with low prevalence of Johne's disease.

OBJECTIVES

To test hypothesis one:

Prepare and run fecal cultures using TREK®ESPII liquid culture system on individual calves of four age groups from eight dairy herds. Collect samples at approximately four month intervals for a two year period. The test data from the dam and the herd prevalence is already established.

To test hypothesis two:

Compare positive and negative fecal culture samples of these calves with their ELISA test results to determine if an association exists between them.

To test hypothesis three:

Compare culture or ELISA status of dam with fecal status of various aged calves.

To test hypothesis four:

a) Pool fecal samples (five to ten fecal samples in a pool) and determine ability to detect one positive individual within each of the two pool sizes.

b) Conversely, test that positive pools contain at least one positive sample and negative pools do not, determining relative sensitivity (Se), specificity (Sp), and positive predictive value (PPV) of pools; compare to the MAP shedding status of the calves that comprise the pool.

To test hypothesis five:

Describe a case study whereby a positive heavy MAP shedder is juxtaposed to a group of animals ready to calve. Culture teat ends and other areas that a calf is likely to nuzzle soon after birth for presence of MAP.

OVERVIEW

Chapter 1 is a targeted literature review of Johne's disease focusing on early transmission and detection, utilizing liquid culture methods. Exploration of environmental sampling and sample pooling as well as discussion of various risk factors, management strategies to mitigate these risk factors will be the focus of this literature review. A limited look at various national control programs and the possibility of zoonotic potential will also be explored.

Chapter 2 addresses hypotheses one, two, and three and is a description of a two year prospective, longitudinal, multiple cross-sectional study looking at detection of MAP in calves. Also assessed is if MAP status of the dam is a risk factor to the fecal culture results of the calf. Thirdly, we explored correlation between a calf fecal culture and its ELISA test results.

Chapter 3 addresses hypothesis four comparing pooled fecal samples of two sizes (five and ten samples per pool) looking at comparative sensitivities.

Chapter 4 assesses hypothesis five, a case study to illustrate the potential risk of retaining one clinically normal heavy fecal shedder in a dairy herd.

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CHAPTER 1

RISK FACTORS ASSOCIATED WITH THE TRANSMISSION AND DETECTION OF MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS IN YOUNG DAIRY CATTLE: A REVIEW

1. INTRODUCTION

Given the breadth of literature pertaining to Johne's disease (JD) and the causative bacteria, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), this review targets literature pertinent to the diagnosis of and risk factors related to JD transmission in cattle. In particular, research pertaining to subjects necessary to either understand the pathogenesis of JD, MAP diagnostic testing and risk factors related to management and control of JD on dairy farms is also reviewed. Study of this disease is important because of its' devastating financial impact of nearly one quarter of a billion dollars annually to the US dairy industry (Garry 1998). Furthermore there is interest in this disease beyond the agricultural sector due to potential zoonotic implications (Naser 2004). Understanding risk factors of this complicated disease will assist in the formulation of specific control programs that will be more effective in limiting or extinguishing the impact of JD.

A. SEARCH METHODS

The electronic reference library at Michigan State University was used for obtaining peer-reviewed, relevant articles. These included, but were not restricted to, Science Direct, Pub Med, Medline, CAB abstracts which were used to scan research using key words of interest (e.g., Johne's, calves, immunology, detection, testing methods, environmental sampling, management, risk factors, control, zoonosis and others). Articles were restricted to those from refereed journals, peer-reviewed proceedings, and governmental data including the National Animal Health Monitoring Systems (NAHMS) resources that were relevant to this review.

2. GENERAL DESCRIPTION OF (MAP) AND JOHNE'S DISEASE (JD)

A. BACTERIA

The organism that causes the infection that can ultimately lead to Johne's disease (JD) is *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It is an acid fast, obligate intracellular, aerobic bacillus that needs the host to multiply, (Krieg 1986) but may be viable in the environment for a minimum of one year under proper conditions. Although it is quite hardy, it is susceptible to desiccation in the presence of sunlight (Whittington 2004). Commonly, the dark moist conditions found on farms allow organism stability for an extended period (Whittington 2005).

B. IMMUNOLOGY

A fundamental understanding of the immunological challenges that MAP presents to the host will provide the foundation on which to place the topics of importance, such as risk assessment, testing strategies, environmental and management challenges, and control programs.

MAP is an intracellular bacteria that initiates a cell mediated T Helper (TH) Type I response (Bannantine 2008) by the calf, as it is picked up by M-cells in the gut. The gram positive aerobe is transported from intestinal villi to the endothelium and engulfed by macrophages. This causes a delayed T type IV hypersensitivity reaction (Bannantine 2008). Suppression of pro-inflammatory cytokines and subsequent expression of anti-inflammatory cytokines as the disease progresses (Stabel 2006) is a key to the organisms' survival in the host. As TH 2 responses are usually triggered by extracellular challenges such as parasites, there is an ineffective response to the

intracellular MAP. This transition from the cytotoxic TH 1 response to an ineffective TH 2 response is observed at the onset of clinical signs. The source of the cells that turn off the TH 1 cytokines and inhibit cell-mediated-immunity (CMI) response is thought to occur within the mediastinal lymph nodes draining the infection site (Coussens 2004). This transition to an ineffective TH 2 response allowing progression of JD is not well understood. Additionally, because this does not always happen, not all exposures are pathologic. The recent use of multicolor flow cytometry (Koo 2004) and access to the sequenced (Catanho 2006) genome of these bacteria will allow characterization of these immunologic transformations from start to finish at the cellular level improving our understanding of JD.

C. TRANSMISSION

Fecal-oral transmission of MAP is the primary mode for perpetuation of JD on a dairy operation. However, *in utero* transference is a significant risk to the fetus, especially in dams that are in advanced stages of the disease (Whittington 2009). Other common sources of calf infection at birth include vaginal contamination during the birthing process, teat contamination following birth, feeding of MAP positive colostrums from the dam, and a contaminated calving environment.

In utero transmission becomes a critical issue when other post natal transmission factors are eliminated (Whittington 2009). In the attempt to identify at-risk animals, it has been shown retrospectively that the MAP shedding status of the dam is a major risk factor associated with transmission of JD to her offspring (Aly 2005) due both to congenital transmission as well as periparturient exposure to MAP.

In this situation, removing the infected dam from the herd is the only effective control method. Additionally, recent reports have shown that infected cows housed next to naïve calves can infect these calves and subsequent shedding from these calves can horizontally infect other naïve calves (van Roermund 2007).

Pooled milk from high somatic cell count (indicating intramammary inflammation) cows or feeding bulk tank milk has not been shown to be a significant risk factor for JD transmission to calves, but feeding pooled colostrums was, according to a recent Danish study (Nielsen 2008). The same study showed that the practice of feeding colostrum replacer to calves born to positive cows and feeding colostrum from negative dams only seemed to provide mitigation.

Mycobacterium avium subsp. *paratuberculosis* is nearly ubiquitous on infected dairies. Common cow areas such as alleys and lagoons are the most prevalent areas for this organism to be isolated. Environmental sampling has become a relatively accurate and easy method of detecting positive herds (Lombard 2006; Pillars 2009). These high traffic areas contaminated with MAP are high risk areas for transmission to the unexposed calf. A phenomenon that has been long observed anecdotally is the clustering of cows with JD within a herd which would indicate that several calves in a row, a year or two prior, were infected nearly simultaneously, and could be explained by environmental transmission.

D. CLINICAL PICTURE

The MAP bacteria have worldwide distribution and causes granulomatous enteritis in exotic and domestic ruminants. This bacterium is also harbored in some non-ruminants such as the fox and the badger. There are typically four stages of the infection if allowed to run its course; 1) asymptomatic or "silent", 2) subclinical, 3) manifestation of clinical disease, and, if not culled, 4) advanced clinical disease (Whitlock 1996).

The calf, and animals up to two years of age, may harbor "silent" infection. These animals are asymptomatic, and exhibit only occasional shedding (Bolton 2005). There are no observed clinical signs and no cost-effective confirmatory tests at this time to diagnose JD infection at this stage, even though these animals may be shedding MAP at a low level and be a risk to their herd mates (van Roermund 2007). As the disease progresses to the subclinical phase there may be minor weight loss and increased fecal shedding, especially as the animal approaches the clinical phase (Tiwari 2006).

Early in the clinical portion of JD, vital signs may remain normal, including appetite, though intermittent diarrhea develops and weight loss occurs. Many animals are culled before reaching the clinical stage due to production losses (Abbas 1983). If allowed to progress, the disease enters the advanced clinical stage which is characterized by constant diarrhea, weakness, mandibular edema, extreme weight loss, recumbence and death. During the clinical and advanced clinical phases, the infectious potential of the animal intensifies due to increased shedding of the organism (Manning 2001) in both feces and milk. The common clinical outcome of this severe

granulomatous enteritis, Johne's disease (JD), is easy to visualize with its chronic diarrhea and associated cachexia (Waters 1999). This disease usually is non-responsive to treatment and the results are often fatal or result in culling the animal from the herd for poor performance.

3. DIAGNOSTIC PROCEDURES (DIRECT AND INDIRECT TESTING METHODS)

The value of effective early testing may not only lie in the prediction of outcome of individual animals but also may have the added value of reducing the amount of bacteria in the herd, thus decreasing environmental bacterial load and transmission of this pathogen.

Due to the complexity of the immune response, latency of the disease, and variability of isolation from fecal shedding, (Visser 1999; Kalis 1999) it has been difficult to develop a highly effective test for detecting a MAP infected animal, especially early in the infection process.

A. DIRECT TESTS

Diagnostic tests that detect the presence of the actual MAP organism, such as fecal culture, are direct tests. As it is imperative to identify and cull infected cows, as well as monitor documented management practices and purchasing strategies to control the presence of this disease on the farm, (Whittington 2001) Sensitivity (Se) and Specificity (Sp) are both important qualities of these tests.

The fecal culture using Herrold's Egg Yolk medium (HEYM) has been considered for many years to be the "Gold" standard for direct testing. This egg yolk

emulsion is used to provide a source of iron for MAP and for neutralization of toxins within the sample (Merkal 1974). While this test has high Sp the Se of this method is estimated at only 38% - 50%. Therefore, it has limitations in early detection of MAP when bacterial shedding is the lowest and Se is of the greatest value (Whitlock 2000). Another drawback of the HEYM fecal culture is the length of time required (up to 16 weeks) to demonstrate the organism (Harris 2005). The strength of the recently developed TREK®ESPII[a] liquid culture system is that the time to positive (TTP) is reduced from 16 weeks with HEYM to a maximum of 42 days. This method monitors O_2 consumption by MAP organisms, if present, by sensing the negative pressure in the culture vial via a computerized system. Unlike the HEYM test where colony forming units (CFU) are measured, this test uses TTP as a proxy for the level of MAP shedding. The lower the TTP, the heavier the bacterial shedding (Williams-Bouyer 2000).

Two other liquid culture systems used widely are the MGIT 960 system[b] and the BACTEC 460[c] radiometric system. These systems, with differences in sample handling and preparation, also measure changes in ion levels or pressure changes resulting from shifts in O_2 and CO_2 concentration that occur with growth of the MAP bacillus. With these liquid culture systems the Se is at least equal to HEYM when bacteria are in high concentrations (Sweeney 2007) and they seem to have a slight advantage in Se with subclinical, low shedding animals, with no sacrifice in Sp (Kim 2004). In either case, a critical advantage of liquid culture is a 70% reduction of time

needed to produce the test results with at least equivalent sensitivity and specificity parameters, which is important for the management of positive animals within a herd.

The most recent and one of the most sensitive of the direct tests is an enhanced polymerase chain reaction (PCR) test (Bogli-Stuber 2005). This test reflects improvements of sensitivity (Se 52%) and (Se 59%) over serum enzyme-linked immunosorbent assay (ELISA) and bacterial culture tests, respectively, without sacrificing specificity (Sp 99% - same as the other two methods) (Scott 2007). Unlike direct PCR performed on the fecal sample itself or contents of the culture vial (used in liquid culture), where the test is less sensitive due to contamination, this newer PCR technique utilizes a common extraction and enhancement procedure, which helps to eliminate sample contaminants. This process begins by incubating the fecal samples with magnetic beads coated with rabbit origin polyclonal antibodies (Khare 2004). This is followed by washing, lysing, and precipitation to extract the DNA from the samples. The DNA precipitate is then re-dissolved and IS900 primers are utilized in a real time PCR. Use of this magnetic separation technique to pull in the immuno-captured bacteria has increased the diagnostic Se from less than fecal culture (Collins 1993) to approaching that or higher than fecal culture (Cook 2007). The current challenge with newer diagnostic methods and disease management methods that will have to be rectified is that, as Se increases and the time to obtain results decreases, the price of diagnostic tests also has increased, because newer technologies are expensive. A balance has to be reached as the tests seems to allow for earlier diagnosis of MAP infection, which is critical in the management of Johne's disease as there are no effective treatments.

B. INDIRECT TESTS

Indirect testing for MAP infection measures an immune response to an antigen in contrast to direct testing which detects the presence of the organism. To have a valid indirect test, there has to be a known relationship between the immune response and the presence and shedding of the organism.

The serum ELISA (enzyme linked immunosorbent assay) Idexx Herdchek is a popular test for MAP due to its price (approximately $6/test - 2009), (DCPAH – MSU)[e] quick results reporting, and, most-importantly, its Se. This test uses optical density (OD) to measure IgG antibodies that are capable of binding with MAP, reporting the results as an OD reading. Intra-laboratory variation is corrected by subtracting the OD from a mean negative control value and recording the difference, the OD corrected (ODc). Animals are considered positive when the mean OD minus the plate negative control is greater than 0.100 as recommended by the manufacturer and as reported in other studies (Alvarez 2009). It has been shown that the mean time it took for an ELISA positive cow to commence detectable shedding and be identified via fecal culture was about nine months after being tested, so ELISA antibodies are generally present prior to shedding of the MAP organism. Therefore, serum ELISA was determined to be a good test when used to predict future MAP shedders (Nielson 2008).

A milk ELISA test has been developed in Europe, utilizing the Prionics Parachek[f] test and adapted by Antel Bio[g] in the US. This is a non-invasive test, since the milk of cows is gathered and routinely tested for other components at regular

intervals. This allows for screening large numbers of cows. It has been validated by the United State Department of Agriculture (USDA) and is commercially available in the US at this time. In several studies it has been shown to perform equally as well as the serum ELISA when compared to the HEYM fecal culture (Hendrick 2005; Collins 2005; Lombard 2006). This test is performed on the individual milk sample and is often associated with monthly milk sampling done by the Dairy Herd Improvement Association (DHIA) which is available to all the DHIA's members.

C. DEVELOPMENT OF FUTURE TESTS AND TESTING STRATEGIES

A new version of a serum ELISA test using formaldehyde and sonification (SELISA) holds promise as its Se is higher than traditional ELISA tests without sacrificing Sp (Speer 2006).

In the direct testing arena, use of protein arrays of MAP in a ninety six-well system may be the new frontier. This array may allow us to not only identify organisms but also identify characteristics of the infective organism that may aid in assessing virulence and predicting immune response by host. Another advantage of protein arrays is the lack of any cross-reactivity between antigens. This utilization of genomics, molecular testing and complicated immunological assays may hold the collective key for future testing and control of Johne's disease (Bannantine 2008). Although holding promise, these tests are in the development phase and not available for general use at this time.

Pooled Samples – An example of a new strategy using existing tests involves the pooling of individual fecal samples to detect the existence of one or more positive

MAP shedders within the pool, which has been used as a management tool for assessing whether or not MAP is present within a herd (Kalis 2000; Wells 2002). Pooled fecal sampling for culture has been recommended for some time in Australian sheep flocks (Whittington 2000). The number of individual animals to incorporate in a pool has been debated in the literature with some studies determining the most cost effective pool size is ten individual animals per pool (Tavornpanich 2004; van Schaik 2007). However, in studies that dealt with low shedding cattle (Eamens 2008) or tested herds of various sizes and prevalence levels, it was indicated that pools containing no more than five individual animals were appropriate to maintain adequate Se (van Schaik 2003). The same study showed that pools of five containing at least one low shedding animal had at least a 53% chance of culturing positive for MAP using standard culture methods. Far more variable results were observed using pools of five when using the RT-PCR test (Scott 2007). Pools are a cost effective management tool to utilize in assessing the presence of MAP in an infected herd.

D. UTILIZATION OF TESTS

Because there is no effective therapy for JD, the first requirement to determine which testing method is the most effective is to understand whether it is a desirable goal to cull "infectious (shedding)" animals to prevent JD spread or not. Therefore, for all of these tests, it is important to identify the purpose of the testing strategy to be selected for management purposes (Nielsen 2006). Furthermore, as tests improve over time, it will be possible to focus our decision analysis on how to handle the disease

rather than being diverted by the uncertainty of test results (Smith RD Slenning BD 2000).

4. RISK FACTORS: INDIVIDUAL COW LEVEL RISKS AND HERD LEVEL RISKS

Risk of acquiring an infectious dose of MAP resides in whatever surrounds the calf. As described earlier, a herd can maintain infection by many different pathways. For this reason many questionnaires attempting to indentify various risks associated with various management practices on the dairy farm have been developed by investigators. One such survey initially had several hundred questions and after conducting multi-variable statistical analysis it was determined that assessment of only eleven questions resulted in nearly the same accuracy in risk determination (Berghaus 2005).

The risks in this review are divided into two major categories. The first category is individual cow level (inherent) risks, risks that are present in the animal at a point in time and are intrinsic to the cow. Unlike herd level (exposure) risks, inherent risks such as dam status, *in utero* transmission, or variable genetic susceptibility can only be changed over time, by culling or natural attrition. Herd level risks such as environment, housing, colostrum management, among others, are also important but can be altered as quickly as the farm is able to initiate programs to mitigate these risks.

A. INDIVIDUAL COW LEVEL (INHERENT) RISKS

It has been demonstrated that transfer of the MAP infection can occur *in utero* (Sweeney 1992). A recent meta-analysis illustrates that the likelihood of this transfer is related to within herd prevalence. In a herd with a 5% prevalence of infection, the annual incidence of an infected calf at birth is 1%. This is particularly problematic in herds that have managed other risk factors well. Likewise, about 40% of calves born to a cow clinically manifesting symptoms of JD will be born carrying MAP (Whittington 2009). These same high rates of *in utero* transmission of MAP have been shown in sub-clinically infected red deer (*Cervus elaphus*) in New Zealand (Thompson 2007).

MAP status of the dam has always been assumed to be a risk factor. In a recent retrospective study it was found that dairy cows with sero-positive dams were 6.6 times as likely to be sero-positive, compared to cows from sero-negative dams (Aly 2005). A retrospective look at zoo ruminants found nearly the same relationship between the dam and her offspring (odds ratio [OR] = 6.8 $p < 0.01$) (Witte 2009). Although a similar retrospective study in beef herds in Texas did not demonstrate the same relationship (Osterstock 2008) as in the dairy and zoo animal, there was a clearly defined familial relationship in sero-positivity. This may be explained by genetic work that shows susceptibility to MAP infections may be associated with mutations in the captase recruitment domain (CARD 15) gene. Possessing this allelic variant in a case-control study resulted in more than a threefold (OR 3.35) increase in likelihood of infection in beef cows (Pinedo 2009).

B. HERD LEVEL (EXPOSURE) RISKS

There are many ways a susceptible young calf can be exposed to MAP and become infected. Exposure of the young dairy animal to feces laden with MAP is still the primary and most manageable of the exposure risks. A recent retrospective study confirmed this and the authors speculated that once a control program has been implemented, the largest risk factor to calves besides the status of their dam was the recent calving of an infected and MAP shedding cow in the vicinity of birthing (Benedictus 2008).

Another important and early risk factor is the calf suckling a contaminated cow or ingesting colostrum containing MAP. A Danish study showed that calves fed pooled colostrum were 1.24 times as likely to be ELISA positive as calves fed only their own dams colostrum, and if allowed to suckle compared to being fed milk replacer the OR was 2.01 (Nielsen 2008). Previous work has also highlighted this exposure risk (Streeter 1995) by showing that 8/36 of colostrum samples were positive for MAP from subclinical fecal shedding cows.

Environmental contamination due to housing conditions whereby cows shedding the MAP organism are allowed access to the area of the young calf is a major exposure risk. For example housing periparturient cows with pre-weaned calves for more than 24 hours can increase JD prevalence in dairy herds (Wells 2000) as can housing them in crates next to shedding cows (van Roermund 2007). It was also demonstrated that these infected calves became shedders themselves and were able to horizontally infect naïve calves house in the same pen (van Roermund 2007). Another

study showed an increased herd prevalence when calves of less than six weeks of age were housed with positive cows (Obasanjo 1997). Some additional environmental risk factors, including the use of an exercise lot for lactating cows, (Johnson-Ifearulundu 1995) and spreading contaminated manure on pasture (Obasanjo 1997), have been associated with increased prevalence of MAP on dairy operations. The frequency of purchasing of animals and their source is an indirect, but very important risk factor (Wells 2000).

There are other, less common but more regional or farm specific risks of acquiring MAP infection. In a Minnesota study looking at wildlife (rabbits and deer) it was shown that although wildlife prevalence was low (2-4%) the probability of daily contact between cattle and wildlife was 20%, so wildlife reservoirs should be considered to be a potential source of MAP transmission (Raizman 2005). Another area of interest is MAP co-infection with pathogens such as the Bovine Viral Diarrhea Virus (BVDV) and Bovine Leucosis Virus (BLV). Recent work showed proper vaccination of calves with BVDV vaccine was associated with fewer MAP sero-positive cows (Tiwari 2009). Although there are many avenues for this agent to infect the young animal, many of these risks can be managed to decrease within-herd prevalence.

5. HERD CONTROL

A. CONTROL WITHIN HERD (Management Practices)

Due to the ubiquitous nature of MAP and its presence in a high percentage of dairy herds, control of Johne's disease and the reduction of within-herd prevalence has been the focus of the USDA, multi-state Johne's disease Demonstration Project. Their focus has been producer education as well as risk assessment and mitigation. In Michigan, for example, using slightly different sampling protocols, the percentage of herds with at least one MAP culture positive animal has decreased from 66% to 48% in the past ten years (Johnson-Ifearulundu 1999; Pillars 2009). This may, or may not, be a result of this educational effort and increased awareness by producer, but seems that there may be an association.

Even in smaller countries, such as Germany, where the pathogen was first identified, eradication does not seem to be on the near horizon. The reason is that due to recent work, MAP is considered an environmental pathogen with reservoirs in a variety of animals, (Stratmann 2005) thus the German focus continues to be on decreasing spread of the disease by identifying heavily infected herds with milk ELISA and removing strong shedders in those herds. All herd control strategies should strive to decrease new infections (i.e. decrease exposure of the calf to MAP) with adoption of best management practices as well as to decrease the prevalence of shedding animals in the herd (Tiwari 2006; McKenna 2006). There have been methodologies developed to identify positive herds by environmental sampling of targeted areas on the dairy. These areas include the lagoon, alleyways, fresh cow pens, and other "common adult cow areas." When culturing these environmental

samples, rather than individual cows in the herd, the culture of pooled environmental samples identified positive herds with relative sensitivity of 70% compared to individual animal culture of all samples within pools (Raizman 2005). This is inexpensive and requires no individual animal handling or restraint. A USDA study validated this earlier work and found that greater than 70% of herds with either a positive ELISA or positive fecal sample were identified by this method (Lombard 2006).

A study completed in California (Tavornpanich 2008) compared particular management practices with herd seroprevalence, correcting for other variables. Not surprisingly, this study found utilizing feeding equipment for manure handling, exposing young calves (less than six months of age) to manure of adult cattle, exposure of cattle to lagoon water, and feeding unsalable milk to calves held the highest predictive values for MAP infected herds. Management practices that were not significant included the use of individual calving areas, time before separation of calf from cow at birth and frequency of bedding changes.

There have been protocols installed on dairy operations addressing their particular risk profile. Some things, such as using separate machines for feeding and manure handling, or moving calves from infected cows, are intuitively obvious. Other changes to mitigate risk to young stock are somewhat more intensive and creative. An example of this is the feeding of unsalable milk after running it through a high-temperature short-time (HTST) pasteurizer. This became an important risk mitigation practice after the 2002 NAHMS Dairy Survey showed that almost 90% of US dairy operations fed unsalable milk to neonates (NAHMS 2002 Dairy Survey). Ancillary

benefits were removal of other pathogens such as Salmonella and Mycoplasma which resulted in a better product to feed calves (Stabel 2004). These are just some of the tools to reduce exposure to the most susceptible animal on the dairy—the calf.

Two other specific management measures are available to help reduce the shedding of MAP in dairy herds. The first, vaccination, is only allowed in a few states for calves <35 days of age. At present there is only one vaccine, Mycopar® (Fort Dodge)[h], licensed in the US. It is a whole-cell killed suspension of MAP suspended in oil. It is tissue reactive and has some human health risks with accidental injection (Solvay MSDS *Mycobacterium paratuberculosis bacterium*—1990). This vaccine seems to have an impact on shedding reduction but not on overall colonization (Uzonna 2003). With the advent of genomic unraveling of both the cow and the MAP pathogen, an entire new foray into vaccine development has begun. Due to less antigenic diversity within MAP, compared to some other Mycobacterium, (Wu 2006), a recent vaccine containing recombinant MAP proteins has been developed and successfully tested (Kathperumal 2008). Although there was occasional colonization of a single tissue site in vaccinates at <10 colony forming units (CFU), the controls had several tissues found to be culture positive at >250 CFU.

The second herd level MAP management measure to control shedding, which has been used in Canada for sometime, is the addition of monensin to the ration. Monensin use was legalized in US for dairy herds effective January 4, 2006 by FDA (FDA – 2006 (http://www.fsis.usda.gove/News_&_Events/Agenda_NACMCF_Mar2006/index.asp

Last Accessed July 31, 2009)). It has been shown to be protective in a murine model against hepatic granulomas in susceptible mice (Brumbaugh 2004). Two studies designed to test efficacy of this use for monensin were conducted in Canada. It was found that herd sero-positivity was reduced with monensin use (Hendrick 2006) but that it only marginally reduced the level of MAP shedding (Hendrick 2006). The authors concluded that monensin or any other drug would never be a replacement for good management practices but could aid in prevention and control of Johne's disease.

B. US CONTROL PROGRAM

In the US the Johne's disease control programs are specific to each state and are primarily voluntary. They concentrate on managing risk and reducing within-herd prevalence over time through testing, culling, and management changes rather than testing and culling only. To increase national uniformity the US Voluntary Johne's Control Program was established. Under this umbrella the Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program (VBJDCP) were developed with input from the National Johne's Working Group, Johne's Committee from US Animal Health Association, state veterinarians, and representatives from the cattle industry. It was approved by USDA Animal and Plant Health Inspection Service (APHIS) Veterinary Services, effective as of June 1, 2006. Part (1) focuses on Education, Part (2) on Assessment of Risk and Management, and Part (3) deals with Testing and Classification. (USDA – APHIS, 2006 http://www.aphis.usda.gov/animal_health/animal_diseases/johnes/downloads/johnes-umr.pdf (Last accessed July 31, 2009))

Some of the best evidence shows that control, rather than eradication is a sensible approach to JD management results from complex statistical modeling, which has shown that vertical transmission and calf-to-calf transmission will allow persistence of MAP even in well managed herds with low-prevalence and therefore be difficult to eradicate (Mitchell 2008). In fighting JD, utilizing all the tools in our knowledge base will be necessary to control the effects of this pathogen in US dairy herds. A combination of increased producer knowledge and awareness through education, as well as efficient use of testing, and decreasing exposure risks to the young diary animal through better management will result in a decrease of JD prevalence in the US dairy herd.

C. ZOONOTIC POTENTIAL

No literature review on the topic of MAP would be complete without touching on the zoonotic potential of this pathogen. First it should be noted that live MAP has been cultured from retail pasteurized milk in the US. Of the 22 brands of milk tested in the US, 12 yielded at least one sample of viable MAP (Ellingson 2005) with 20/702 of the total pints tested from three top producing milk states (CA, MN, WI) yielding viable MAP. Thus, a common source of human exposure has been established, but the association between the MAP organism and inflammatory bowel disease (Crohn's disease) is a less clear. A study finding a disproportionately higher number of MAP positive blood cultures in people with Inflammatory Bowel Syndrome has triggered many studies with variable results (Naser 2004).

A systematic review of the literature in 2008 concluded that evidence of zoonotic potential is not strong but should not be ignored. The review cited several conflicting studies almost equally split between association and no association of MAP with Crohn's disease. They also noted the absence of experimental design consistency that would be needed to confirm such an association (Waddell 2008). Thus, while the negative impact of MAP on the US dairy cattle industry has been well documented, another impetus for reducing the shedding of this organism, in both milk and the environment, is the zoonotic unknown.

6. SUMMARY

During the past three decades there has been an international effort to reduce the impact of MAP in dairy herds. It is important that we have a better understanding of MAP transmission and its immunological processes so that it can be better diagnosed and prevented. The knowledge of management of risk factors to reduce transmission of organism is a key element to decreasing herd prevalence. The improvement in testing techniques as well as refinement in testing strategies have allowed us to establish a comprehensive framework to assist producers in their effort to mitigate the effects of this disease. As test sensitivities improve, the ability to detect infected animals earlier and to better characterize prevalence through targeted environmental sampling may allow for more timely intervention opportunities that were not previously possible. Implementation of these developments should be further explored as we strive to decrease the prevalence of MAP in the US cattle herd. With the unlocking of the genome of both the cow and the bacterium, further work in the

areas of genetic predisposition of the cow as well as the variable virulence of the pathogen hold promise in understanding the disease. This may be the key to developing an efficacious vaccine against Johne's disease. Furthermore, the impetus for future research may lie in the results of intense ongoing investigations into the zoonotic potential of the MAP organism.

FOOTNOTES

[a] TREK Diagnostic Systems, Inc.
982 Keynote Circle, Suite 6
Cleveland, OH 44131
USA

[b] MIGIT 960
Becton-Dickinson Biosciences
1 Becton Drive
Franklin Lakes, NJ
USA

[c] BACTEC 460
Johnson Laboratories
Towson, MD
USA

[d] Idexx Laboratories Inc.
Westbrook, Maine
USA

[e] (DCPAH –MSU) Price Guide – 2007
Diagnostic Center for Population Medicine and Animal Health
Michigan State University
East Lansing, MI 48824
USA

[f] Prionics Paracheck
Prionics AG
Schlieren
SWITZERLAND

[g] Antel Bio
North Star Cooperative
East Lansing MI 48823
USA

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CHAPTER 2

DETECTION OF *MYCOBATERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN NATURALLY EXPOSED DAIRY CALVES, RELATIONSHIP TO DAM STATUS, AND OTHER RISK FACTORS

Objectives—Determine 1) if fecal shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) can be detected in naturally exposed dairy calves, 2) if there is an association between fecal shedding of MAP in calves and their ELISA test status, and 3) if a relationship exists between MAP ELISA test positive cows and fecal shedding in their offspring.

Design—28 month-longitudinal study.

Sample Population-Heifer calves from eight dairy herds in Michigan participating in the Michigan Johne's Disease Control Demonstration Project.

Procedures—Fecal and blood samples were obtained from calves at 4-month intervals for 28 months. Liquid culture was used on fecal samples and serum ELISA testing on blood samples. Multivariable mixed logistic regression was utilized to evaluate the relationship between herd and dam risk factors and the MAP test status of calves.

Results—A total of 27/1088 (2.51 %) calves were MAP fecal test positive. A total of 26/1036 calves (2.50%) were MAP ELISA test positive. Positive serum ELISA samples from calves showed no significant association with their concomitant fecal status, ($r^2 = 0.16$, $p < 0.0001$). Calves born to ELISA positive dams were 11.5 times more likely to become a fecal shedder of MAP than calves born to ELISA negative dams (Odds Ratio = 11.5 [95% CI: 4.7 – 28.2]; $p<0.0001$).

Conclusions and Clinical Relevance—Calves born to ELISA positive cows are at high risk for shedding MAP, thus management of ELISA positive cows is important. Given the relatively high likelihood of a calf shedding MAP when their

dam is ELISA positive, consideration must be given to identifying ELISA positive dams and housing their calves separately.

ABBREVIATIONS

MAP	*Mycobacterium avium* subspecies *paratuberculosis*
JD	Johne's disease
OR	Odds ratio
CI	Confidence interval
DCPAH	Diagnostic Center for Population and Animal Health

Introduction

Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic granulomatous enteric disease of both domestic and non-domestic ruminants. It was first described in Germany and its etiologic agent was characterized as an acid fast bacillus by Twort (Twort 1910). Recent studies placed the percentage of infected herds in Michigan at 64% (Johnson-Ifearulundu 1999) and 48% (Pillars 2009) respectively. Nationally the published prevalence figures are widely variable (Adaska 2003; Hirst 2004). The cost to the US dairy producer is more than $250 million annually (Ott 1999). Although JD has been recognized for over a century, it has emerged as a major economic factor in the US dairy industry in the past three decades.

Calves are most often infected before the age of six months via the ingestion of MAP contaminated feces, colostrum, or waste milk (Sweeney 1996). Transplacental infection (Kopecky 1967; Seitz 1989; Sweeney 1992) also occurs. Because MAP is a slow-growing bacterium, development of clinical signs of JD may take 2-5 years yet transmission can occur on farms via unapparent carriers (Harris 2001).

Attempts to identify naturally infected calves at a young age have been unsatisfactory (Ayele 2004; McDonald 1999), presumably due to low levels of bacterial shedding present in the feces of young animals. Furthermore, immunological assays looking for antibodies are not as diagnostic with Mycobacteria, as with some organisms (Bannantine 2008) due to a cell-mediated, rather than humoral, response to infection. Also compromising detection efforts in calves are intermittent shedding of MAP (Whitlock 1996) and reproducibility deficits within the same fecal sample

(Visser 1999). Thus, standard fecal culture has not been effective at detecting low-level bacterial shedding (Kim 2004) and overall sensitivity may be as low as 33% (Whitlock 2000).

Early identification of calves that may be at risk of MAP infection or that may be shedding MAP into the environment is important for ensuring that effective JD prevention and control strategies exist in dairy herds therefore, the purpose of this study was to determine 1) if fecal shedding of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) can be detected in naturally exposed calves using the TREK®ESPII[a] liquid culture system, 2) if there is an association between fecal shedding of MAP in the calf and their ELISA test status, and 3) if there is an association between MAP status of the dam and the MAP status of her calf.

Materials and Methods

Study design and criteria for inclusion— This was a longitudinal study spanning 28 months. The study population included calves from eight commercial dairy herds located in Michigan that were enrolled in the Michigan Johne's Disease Control Demonstration Program. Herds were enrolled in the JD demonstration project based on known infection with MAP, herd size, geographic location, and willingness to cooperate in the study. This study met the Michigan State University guidelines for animal research administered by the Animal Use Committee and owner's permission was received to conduct the study. Ten heifer calves from each of four age groups: 0-3 months, 4-6 months, 7-14 months, and 15-24 months were selected for fecal culture and serum ELISA testing during each herd visit. Age group composition was based on

distinct housing groups including pre-weaning (hutch calves), post-weaning (small pens), growth phase (large pens) and breeding group. Calves from fecal or ELISA positive dams were preferentially targeted for testing and the remainder of each age cohort were chosen randomly. Throughout the study period, samples were collected at approximately four-month intervals with a maximum of eight visits over 28 months.

Fecal samples were collected from each calf by digital exculpation using individual latex gloves and sterile water for lubrication. At least eight grams of fecal material was collected, labeled, refrigerated at 5°C, and submitted to the laboratory within 1-2 days of collection.

A total of five milliliters (5 ml) of blood was collected from the jugular vein or caudal tail vein. Blood samples were labeled, placed on ice, centrifuged within six hours, and serum submitted to the laboratory within 1-2 days of collection.

Laboratory testing— Both fecal and serum samples were analyzed at the Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University. The DCPAH is an accredited laboratory and has been certified by USDA National Veterinary Science Laboratory to conduct TREK®ESPII[a] liquid fecal culture for MAP, as well as Parachek®[b] serum ELISA antibody testing.

The Cornell method (Stabel 1997) was used to prepare fecal samples for culture. The samples were placed in the TREK®ESPII[a] culture system incubator. Positive and negative controls were used on each batch of forty fecal samples. Because this is a semi-quantitative test, a positive sample was described as a high shedder if it took 7-21 days to turn positive, moderate shedder if it took 22-28 days to

turn positive and low shedder if it took 29-42 days to turn positive. If a culture was not positive by day 42 it was stained with acid fast Kenyon stain and the supernatant tested with ISP900 real time PCR[c] (Kim 2002). If negative to this follow-up it was classified as not shedding (negative). All positive samples from liquid culture were also confirmed with both acid fast Kenyon stain and IS900 real time PCR[c]. This method was also used on the dams on an annual basis to determine their fecal MAP status.

The blood samples were centrifuged and the serum tested for MAP antibodies using the Parachek®[b] ELISA test following the manufacturers recommended procedures. A corrected optical density (OD_c) ≥ 1.0 was considered ELISA positive while an $OD_c < 1.0$ was considered ELISA negative for this study. This same procedure was also used to assess the dam status on an annual basis.

Risk factors— The primary outcome of interest was the presence, or lack thereof, of MAP in the calf fecal sample. Additionally, the results of the calf serum ELISA test were evaluated.

Several risk factors were evaluated to determine their relationship with the MAP test status of calves. The primary risk factor of interest was the MAP ELISA test status of the dam. Dam fecal culture status was also evaluated.

Because the age of the calf may be associated with whether or not it is shedding MAP (Weber 2005) the relationship between the age group when tested and the calf MAP status was also evaluated. Age at testing was categorized as follows: 0-3 months, 4-6 months, 7-14 months, and 15-24 months.

The relationship between calf MAP status and average herd size was evaluated by calculating the population size recorded closest to the midpoint of calf sample collection for each herd. Average herd size was categorized as small (80–130 cows [three herds]), medium (140–165 cows [two herds]), and large (330–600 cows [three herds]).

In addition, herd test prevalence of MAP, using serum ELISA, was established annually. This was evaluated by using the herd MAP prevalence recorded closest to the midpoint of calf sample collection for each herd. Prevalence was categorized as low (0.5-5% [three herds]), moderate (6.5-8% [three herds]), and high (10–15 % [two herds]).

Statistical analysis—Summary statistics were computed to identify relationships between the outcome (calf MAP test status) and the risk factors of interest (Proc FREQ[d]). Spearman correlation coefficients were computed to identify potential collinearity between the risk factors of interest (Proc CORR[e]). Results were considered statistically significant at $p \leq 0.05$.

Multivariable mixed logistic regression models were created to describe the association between the outcome (calf fecal MAP) and the risk factors of interest (herd size, herd prevalence, age group) (Proc GLIMMIX[f]). The random effect term incorporated herd and observation visit to ensure the model contained adequate variance and degrees of freedom relative to the study design. All risk factors with $p<0.05$ in the univariable model were considered for inclusion in the multivariable model. Herd size served as a proxy for undefined management factors as there were no

herds that grazed entirely or were totally confined. Herd prevalence was explored but since it was reflected by dam ELISA, it was not included as a variable to avoid model over-specification.

Results

Calf Fecal Culture—Calf MAP fecal culture test results, positive calves and MAP shedding level, positive calves and herd prevalence, and ELISA test results by calf age group distribution were evaluated (Table 2.1). Overall, results showed that 27/1088 (2.48%) individual calves cultured positive for MAP. A total of 7 /27 (25.92%) positive calves were positive on two or more serial cultures.

Calf ELISA—The distribution of samples collected and results of the ELISA testing by age of calf and their dam ELISA test results were evaluated (Table 2.2). Overall, results showed that 26/1038 (2.50%) calves were ELISA positive for MAP and 20/26 (77.0%) were < 6 months of age; 8/26 (30.77%) ELISA positive calves tested positive on two or more serial cultures.

Dam MAP Status–There were significantly more MAP fecal culture positive calves born to MAP serum ELISA positive dams and fecal culture positive dams compared to their test negative cohorts (Table 2.2).

There also were significantly more serum ELISA positive calves born to MAP serum ELISA positive dams and fecal culture positive dams compared to their test negative cohorts (Table 2.3).

Statistical analysis—With respect to age, there was no significant difference in positive fecal cultures between age group 1 (0-3 months) compared to age group 2

(4-6 months), and age group 3 (7-14 months) compared to age group 4 (15-24 months) (Table 2.4). However, there was a significant difference when comparing younger calves (age groups 1 and 2 combined [0-6 months]) to older calves (age groups 3 and 4 combined [7-24 months]). Furthermore, significantly more positive fecal cultures were found in age group 3 (7-14 months) compared to age group 2 (4-6 months).

No correlation was demonstrated between the fecal culture status of a calf and their concomitant serum ELISA results ($r^2 = 0.16$; $p < .0001$).

A multivariable mixed logistic regression model was constructed (Table 2.5). The exposure variable of interest was dam serum ELISA test status. Calves born to ELISA positive dams were 11.5 times more likely to become a fecal shedder of MAP than calves born to ELISA negative dams. Using Least Squares Means it was determined that the probability of a calf shedding MAP from an ELISA positive dam was 6.9% while the probability was < 1% (0.0064) when the dam was ELISA negative ($p < .0001$).

Discussion

In this study we were able to detect MAP in young dairy calves using TREK®ESPII[a] liquid fecal culture, a relatively new diagnostic technique. One of the goals of Johne's disease management is early detection of MAP. The response variable of interest was the calf fecal culture status, as this is considered the best indicator of MAP infection (Stich 2004; Wells 2006). We evaluated four age groups of calves to determine if there was a group with a higher likelihood of shedding MAP (positive fecal culture). In our prospective study, in a natural setting, not only did the older

animals have significantly more fecal culture positives than younger calves but, more specifically, age group 3 (7-14 months) had significantly more fecal positives than age group 2 (4-6 months). Retrospectively, it has been shown that, starting at seven months of age, there has been detection of MAP shedding, which then wanes before recurring at two years of age, or older (Weber 2005). This may allow for development of JD prevention and control strategies, targeting this younger age group, that utilize fecal culture for early detection of MAP in dairy herds.

The majority (76.9%) of serum ELISA positive calves were less than 6 months of age. However, there was virtually no correlation between their ELISA and fecal culture results. Since many of these positive calves were less than two months of age, most of the ELISA positive samples may be attributed to the presence of maternal antibodies which have been shown to persist in calves for 200 days (Menanteau-Horta 1985). Additionally, Mycobacteria tend to stimulate more of a cell-mediated, rather than humoral, response early on in the infection (Bannantine 2008; Kalis 2003). Most studies report that ELISA test results are a better indicator of infection in the older animal (over two years of age) (McDonald 1999). Therefore, ELISA results in young calves are probably not a good indicator of infection at this age (Antongnoli 2007).

Although herd size was not found to be significant in this study it was maintained in the final model to account for potential confounding factors. It has been reported to have a significant impact on JD occurrence in another study (Crossley 2005). The difference could be due to the fact that, in the former study, they measured shedding level differences in adults of various herd sizes, while we were looking at calves and thus concentrated on number of shedding animals. Also, with n=8, we had

fewer comparative herds. Though herd prevalence was not assessed as a risk factor for calf MAP status in this study, the majority of positive fecal cultures were in calves from high prevalence herds which does agree with earlier work study (Crossley 2005).

In a natural setting, we found that a calf born to an ELISA positive dam was 11.5 times more likely to become fecal culture positive than a calf born to an ELISA negative dam. The probability of a calf with an ELISA positive dam becoming fecal culture positive was 6.8 % compared to only 0.6 % when the dam was ELISA negative. It has been reported in retrospective studies that the MAP status of the dam significantly increases the risk of positive MAP fecal cultures in the offspring of both dairy cows (Aly 2005) and non-domestic ruminants from zoos (Witte 2009). This may be important knowledge in developing a target testing model utilizing this piece of information while targeting the 7-14 month age group.

In conclusion, results of this study suggest that there is a target age (7-14 months) to start testing for fecal shedding of MAP in the naturally exposed dairy calf. Additionally, dam ELISA status may be an important predictor of MAP fecal shedding in the dairy calf. Even if MAP shedding in the young calf does not progress to JD later in life, it is, at the very least, a potential risk factor for transmission to nearby calves through environmental contamination (van Roermund 2007). Given the relatively high likelihood of a calf shedding MAP when their dam is ELISA positive, consideration must be given to identifying ELISA positive dams and housing their calves separately.

Further investigation needs to be done using serial testing of 7-14 month old calves with positive MAP fecal cultures, over time, to follow their production

parameters and clinical outcomes, especially in high prevalence herds. Given recent work with pooled fecal samples, (Tavornpanich 2004; Eamens 2008) it may also be interesting to investigate implementation of a fecal culture pooling strategy in this age group of calves to obtain an early, more cost effective, assessment of management changes.

Footnotes

[a]TREK®ESPII
TREK Diagnostic Systems Inc.
Cleveland, OH 44131
USA

[b]Parachek®
Prionics AG
Wagistrasse 27a
CH-8952
Schlieren-Zurich
SWITZERLAND

[c]IS900 PCR
Applied Biosystems
Foster City, CA 94404
USA

[d]Proc FREQ,
SAS Institute
Cary, NC
USA

[e]Proc CORR
SAS Institute
Cary, NC
USA

[f]Proc GLIMMIX
SAS Institute
Cary, NC
USA

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Table 2.1: *Mycobacterium avian* subspecies *paratuberculosis* calf fecal culture test results, positive calves and MAP shedding level, positive calves and herd prevalence, and ELISA test results by calf age group distribution: Michigan 2005-2007

Test Result	Age Group 1 (0-3 months)	Age Group 2 (4-6 months)	Age Group 3 (7-14 months)	Age Group 4 (15-24 months)	Total # (%)
Fecal Culture					
# Tests	424	438	438	437	1737
# Positive Tests	2	3	26	10	41(2.4)[a]
# Calves Tested	395	317	181	195	1088
# Positive Calves	2	2	15	8	27(2.5)[b]
Positive Calves and MAP Shedding Level					
Low	2	2	12	7	23
Moderate	0	0	1	0	1
High	0	0	2	1	3
Positive Calves and Herd Prevalence					
Low	1	0	2	0	3
Moderate	1	1	3	4	9
High	0	1	10	4	15
ELISA Serum					
# Tests	402	418	415	424	1659
# Positive Tests	17	9	4	6	36(2.2)[a]
# Calves Tested	378	304	166	190	1038
# Positive Calves	14	6	2	4	26(2.5)[b]

[a] *% positive of total fecal or ELISA tests* [b] *% positive of total individual calves tested*

Table 2.2 –*Mycobacterium avium* subspecies *paratuberculosis* fecal culture results of calves in relation to serum ELISA and fecal culture status of their dam: Michigan 2005 – 2007

Dam Status	Calves (+)	Calves (-)	Total	% Positive	X^2_p
ELISA +	17	174	191	8.9	
ELISA -	10	887	897	1.1	<.0001
Total	27	1061	1088	2.5	
Fecal +	10	125	135	7.4	
Fecal -	4	683	687	0.6	<.0001
Total	14	808	822	1.7	

* X^2p – Chi Square P– value significantly different between groups (p< 0.05)

Table 2.3–*Mycobacterium avium* subspecies *paratuberculosis* serum ELISA results of calves in relation to serum ELISA and fecal culture status of their dam: Michigan 2005 –2007

Dam Status	Calves (+)	Calves (-)	Total	% Positive	X^2_p
ELISA +	15	152	167	9.0	
ELISA -	11	843	854	1.3	<.0001
Total	26	995	1021	2.6	
Fecal +	11	124	135	8.1	
Fecal -	7	681	688	1.0	<.0001
Total	18	805	823	2.2	

* X^2p – Chi Square P– value significantly different between groups (p< 0.05)

Table 2.4–Comparisons between age groups of fecal culture positive calves shedding *Mycobacterium avium* subspecies *paratuberculosis:* Michigan 2005–2007

Age Group	df	F Value	Pr > F
Age group 1 vs. age group 2	165	0.01	0.913
Age group 3 vs. age group 4	165	0.25	0.618
Older vs. younger age groups (1&2) vs. (3&4)	165	10.890	0.001
Age group 3 vs. all others 3 vs. (1, 2, & 4)	165	3.130	0.079
Age group 2 vs. age group 3	165	4.38	0.038

Table 2.5–Multivariable mixed logistic regression of risk factors associated with *Mycobacterium avium* subspecies *paratuberculosis* positive calf fecal cultures: Michigan 2005 –2007

Risk Factor	t-value	P value[a]	df[b]	OR[c]	95% CI[d]
Dam ELISA +	5.33	<.0001	911	11.498	4.680, 28.246
Dam ELISA -	-	-	-		
Age group 1(0-3 mos.)	- 2.61	0.01	165	0.166	0.043, 0.648
Age group 2 (4-6 mos.)	- 2.50	0.0135	165	0.152	0.034, 0.674
Age group 3 (7-14 mos.)	- 0.50	0.618	165	0.743	0.230, 2.404
Age group 4 (15-24 mos.)	-	-	-	1.000	-, -
Herd size 1 (80-130 cows)	-	-	-	1.000	-, -
Herd size 2 (140-165 cows)	1.27	0.203	911	0.315	0.053, 1.871
Herd size 3 (330-600 cows)	1.03	0.301	911	2.009	0.535, 7.546

[a]P-value-level of significance for t-value
[b]df-degrees of freedom
[c]OR-Odds Ratio
[d]CI-95% Confidence Interval

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CHAPTER 3

USE OF POOLED FECAL CULTURES TO DETECT MYCOBACTERIUM *AVIUM* SUBSP. *PARATUBERCULOSIS* IN NATURALLY EXPOSED DAIRY CALVES: COMPARISON OF RELATIVE SENSITIVITY AND SPECIFICITY OF POOLS OF FIVE AND TEN INDIVIDUAL SAMPLES

ABSTRACT

Objectives—This study was conducted using *Mycobacterium avium* subsp. *Paratuberculosis* (MAP) liquid fecal culture to determine if pooled sample of five or ten individual fecal samples from dairy calves in a natural field setting from eight known Johne's disease infected herds could be used to detect a single positive individual sample within their respective pool. If so, then to determine if there was any difference between the sensitivity of pools containing one positive calf sample in a pool of five individual samples compared to one positive sample in a pool of ten individual samples.

Design— 28-month longitudinal study.

Sample Population—Heifer calves categorized into four age groups from eight dairy herds in Michigan participating in the Michigan Johne's Disease Control Demonstration Project.

Procedure— At each herd visit an eight gram fecal sample was collected from ten individual calves within each of four age groups: 0-3 months, 4-6 months, 7-14 months, and 15-24 months. From each group of individual fecal samples, two pools of five and one pool of ten. The pools, as well as the individual fecal samples, were cultured using the rapid liquid culture (TREK®ESPII) system and positive samples were confirmed with acid fast staining and IS900 real time PCR. Sensitivity (Se) and Specificity (Sp) of the two sizes of pooled samples were calculated and compared to the individual calf fecal culture result as the gold standard.

Results—Pools containing five compared to ten calves were more sensitive (Se) (79% and 67%, respectively) and had higher positive predictive value (PPV) (52%

compared to 18%). These pools represented 2405 individual samples of which 32 were culture positive. No pool contained more than one MAP positive calf sample and all positive calves were classified as light shedders. Using Chi Square, test status results from pools of ten as well as pools of five showed a relationship with the fecal status of the individual calves that comprised these pools ($p = 0.0353$ and $p > 0.00001$ respectively). Pooling of feces from individual calves for MAP culturing was ineffective in animals less than seven months of age but showed no difference in results between age group three (7-14 months) and age group four (15-24 months) in both pools of ten ($p = 0.401$) and pools of five ($p = 0.860$).

Conclusions and Clinical Relevance Pooling individual fecal samples from dairy calves naturally exposed to MAP may be used as a tool to determine if a population of calves is shedding this organism. Pools of five are more sensitive than pools of ten and have a higher positive predictive value making it a more optimal pool size. Pooling was shown to be ineffective in the detection of MAP in calves less than seven months of age. Pooling may offer the opportunity to utilize samples from calves greater than six months old to get an earlier assessment of any management changes instituted to mitigate MAP transmission.

Introduction

Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic granulomatous enteric disease of both domestic and non-domestic ruminants. It was first described in Germany and its etiologic agent

was characterized as an acid fast bacillus by Twort (Twort 1910). Recent studies placed the percent of infected herds in Michigan at 64% (Johnson-Ifearulundu 1999) and 48% (Pillars 2009) respectively. Nationally the published prevalence figures are widely variable (Adaska 2003; Hirst 2004). The cost to the US dairy producer is more than $250 million annually (Ott 1999). Although JD has been recognized for over a century, it has emerged as a major economic factor in the US dairy industry in the past three decades.

Calves are most often infected with MAP before the age of six months via the ingestion of MAP feces, colostrum or waste milk contaminated with this organism (Sweeney 1996). Transplacental infection also occurs (Kopecky 1967; Seitz 1989; Sweeney 1992). Because MAP is a slow-growing bacterium, development of clinical signs of JD may take 2-5 years yet transmission can occur on farms via unapparent carriers (Harris 2001).

Attempts to identify naturally infected calves at a young age using fecal culture have been unsatisfactory (Ayele 2004; McDonald 1999), presumably due to low levels of bacterial shedding present in the feces of young animals and intermittent shedding of MAP (Whitlock 1996). Reasons for this include the fact that standard fecal culture has not been effective at detecting low-level bacterial shedding (Kim 2004) and overall sensitivity may be as low as 33% (Whitlock 2000). Immunological assays looking for antibodies are not as diagnostic with Mycobacteria, as with some organisms (Bannantine 2008), due to a cell-mediated, rather than humoral, response infection. Also compromising detection efforts in calves is reproducibility deficits within the same fecal sample (Visser 1999) with low levels of bacterial shedders.

Thus, standard fecal culture has not been effective at detecting this low-level bacterial shedding (Kim 2004).

Early identification of calves that may be at risk of MAP infection or that may be shedding MAP into the environment is important for ensuring that effective JD prevention and control strategies exist in dairy herds. An example of a recent strategy using existing tests involves the pooling of individual fecal samples to detect the existence of one or more positive adult MAP shedders within the pool, which has been used as a management tool for assessing whether or not MAP is present within a dairy herd (Kalis 2000; Wells 2002). Pooled fecal sampling for JD culture has been recommended for some time in Australian sheep flocks (Whittington 2000).

The number of individual adult cattle to incorporate in a pool has been debated in the literature. Some studies report that the most cost effective pool size is ten individual animals per pool (Tavornpanich 2004). However, in studies that dealt with low shedding cattle (Eamens 2008) or tested herds of various sizes and prevalence levels were cultured, (van Schaik 2003) pools containing no more than five individual animals were appropriate to maintain adequate sensitivity. Pools of five containing at least one low shedding animal had at least a 53% chance of culturing positive for MAP using standard culture methods (van Schaik 2003). In contrast far more variable results were observed using pools of five with the direct qRT(real time)-PCR test (Scott 2007).

In general it has been shown that, in adult dairy cattle, the culture of pooled fecal samples may be a cost effective management tool to utilize in assessing the presences of MAP in an infected herd. The advantage of pooled samples is that more

individual animals can be represented per test which may increase overall herd Se of the test although it will decrease the Se of detecting a specific individual (especially low shedding) animal (van Schaik 2003). Thus it is important to have a strategy for the use of this method, and an *a priori* sense of overall prevalence. Although most of the studies were prospective and done in a natural field setting few have utilized liquid culture diagnostic testing and more focused on the adult animal, although on study grouped animals by age (Kalis 2000).

The purpose of this study was to determine 1) if pooled fecal samples could be utilized to detect a single MAP positive animal within the pool using the TREK®ESPII[a] liquid culture system in a population of naturally exposed dairy calves 2) to determine if there was a difference in the sensitivity (Se) and specificity (Sp) of the test when comparing pools of ten to pools of five and their respective positive predictive value (PPV) and 3) to determine if age of the dairy calves tested affected MAP detection in pooled fecal samples.

Materials and Methods

Study design and criteria for inclusion— This was a longitudinal study spanning 28 months. The study population included calves from eight commercial dairy herds located in Michigan that were enrolled in the Michigan Johne's Disease Control Demonstration Program. Herds were enrolled in the JD demonstration project based on known infection with MAP, herd size, geographic location, and willingness to cooperate in the study. This study met the Michigan State University guidelines for

animal research administered by the Animal Use Committee and owner's permission was received to conduct study.

Ten heifer calves from each of four age groups: 0-3 months, 4-6 months, 7-14 months, and 15-24 months were selected for fecal culture. Age group composition was based on distinct housing groups including pre-weaning (hutch calves), post-weaning (small pens), growth phase (large pens) and breeding group. Calves from fecal or ELISA positive dams were preferentially targeted for testing and the remainder of each age cohort were chosen randomly. Throughout the study period, samples were collected at approximately four-month intervals with a maximum of eight visits over 28 months.

Fecal samples were collected from each calf by digital exculpation using individual latex gloves and sterile water for lubrication. At least eight grams of fecal material was collected and the individual samples from each of the four age groups were identified. Two grams from each individual calf sample were used to assemble a pool of ten and also another two grams were used to make two pools of five across each of the four age groups. The pools were complied without knowing *a priori* the status of an individual calf. When a pool was complete, a standard tongue depressor was used to mechanically stir the pool for one minute to homogenize the contents within the container. From these pools a total of four grams of feces, gathered from four sites within pool, was placed in a sample vial. The pooled samples as well as the individual samples from each of the age groups were refrigerated at 5°C, and submitted simultaneously to the laboratory for MAP culture within one to two days of collection. Although the majority of pools of ten were comprised of the same

individual calves that were in the two pools of five in a particular age group, there were pools of ten that stood alone as well as pools of five tested that were not included in pools of ten. Thus we did not always directly compare the ability of pools of ten to pools of five to detect the same positive individual, although this was the most common scenario, but rather we compared the difference between pools of ten to pools of five to detect a positive MAP sample across a particular age group.

Laboratory testing—Fecal samples were analyzed at the Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University. The DCPAH is an accredited laboratory and has been certified by USDA National Veterinary Science Laboratory to conduct TREK®ESPII[a] liquid fecal culture for MAP.

The Cornell method (Stabel JR 1997) was used to prepare fecal samples for culture. The samples were placed in the TREK®ESPII[a] culture system incubator. Positive and negative controls were used on each batch of 40 fecal samples from individual calves and their respective pools. Because this is a semi-quantitative test, a positive sample and/or pool was described as a high shedder (or in the case of a pool, contained high concentration of MAP) if it took 7-21 days to turn positive, moderate shedder (moderate concentration of MAP) if it took 22-28 days to turn positive and low shedder (low concentration of MAP) if it took 29-42 days to turn positive. If a culture was not positive by day 42, it was stained with acid fast Kenyon stain and the supernatant tested with ISP900 real time PCR[c] (Kim 2002). If negative to this follow-up it was classified as not shedding (negative). All positive samples, individual animal

as well as pool, from liquid culture were also confirmed with both acid fast Kenyon stain and IS900 real time PCR[c].

Statistical Analysis – Descriptive statistics were used, including frequencies, Mantel- Haenszel Chi-Square table, as well as Fisher's Exact Test - where appropriate.

Results – During the course of this study 940 individual samples were combined in pools of ten grouped by age and 1465 individual samples comprised the pools of five. Of these individual samples, 32 cultured MAP positive. (Table 3.1)

Table 3.1–Distribution and fecal culture results for *Mycobacterium avium* subspecies *paratuberculosis* of individual samples across the four age groups as well as combined age groups of older compared to younger in pools containing five individual samples and pools containing ten individual samples per pool: Michigan 2005 – 2007

	Pools of Ten		**Pools of Five**	
Age Group	# Individual Samples	# Positive Samples	# Individual Samples	# Positive Samples
Age Group 1	240	1	365	1
Age Group 2	270	1	380	2
Younger (Age Group 1 and 2)	510	2	745	3
Age Group 3	210	6	380	9
Age Group 4	220	3	340	9
Older (Age Group 3 and 4)	430	9	720	18
Total	**940**	**11**	**1465**	**21**

Six of 94 pools of ten and 14 of 291 pools of five were culture positive. Distribution of the total pools tested and the positive pools is shown in Table 3.2. The

distribution of positive pools across age groups for both pools of ten and pools of five were similar, ($p = 0.827$) and ($p = 0.887$) respectively. There was no difference in positive MAP test status between age group one and age group two ($p = 1.0$) and age group three compared to four ($p = 1.0$) regardless of pool size. However, there was a significant difference in MAP positive results when comparing pools from age groups one and two (younger) with age groups three and four (older) across both pool sizes ($p = 0.0143$ (pools of ten) and $p = 0.002$ (pools of five)), Table 3.2 with nearly equal sample distribution between groups across both pool sizes ($p = 0.401$ and $p = 0.860$ respectively((Table 3.2).

Table 3.2–Distribution of pools containing five calves compared to pools containing ten calves across individual age groups as well as younger compared to older calves and their subsequent MAP culture status: Michigan 2005 – 2007

	Pools of Ten		**Pools of Five**	
Age Group	# Pools	# Positive pools	# Pools	# Positive pools
Age Group 1	24	0	71	0
Age Group 2	27	0	76	0
Younger (Age Group 1 and 2)	51	0	147	0
Age Group 3	21	3	76	7
Age Group 4	22	3	68	7
Older (Age Group 3 and 4)	43	6	144	14
Total	**94**	**6**	**291**	**14**

Although we did not know the individual calf MAP test status prior to assignment to a pool, the prevalence of MAP detected in the individual samples was

low (2.3%) such that any positive pool that happened to contain a MAP positive calf, contained only one MAP positive calf. Also, all of the individual MAP positive samples contained in the pools were low shedders. Both of these factors helped standardize the comparison of positive pools. Furthermore, it was found that there were a higher proportion of false negative pools in pools containing ten calves when compared to pools containing five calves indicating lower Se (Table 3.3).

Table 3.3 - *Mycobacterium avium* subspecies *paratuberculosis* fecal culture results when comparing pools looking at false negatives and false positives distributed by age group: Michigan 2005 – 2007

Age Group	+ Pools of 10 with no + calves	(-) Pools of 10 with + calves	+ Pools of 5 with no + calves	(-) Pools of 5 with + calves)
Younger[a]	0	2	0	3
Older[b]	1	7	3	7
Total	**1**	**9**	**3**	**10**

[a] Age Groups 1 and 3 (0-6 months) [b] Age Groups 3 and 4 (7-24 months)

The relative Se of the fecal culture from pools containing ten calves was 67% and the relative Sp was 90% (Table 3.4). The positive predictive value (PPV) was 18%. In comparison, pools containing five individual calves had a Se of 79%, Sp of 96% and a PPV of 52%.

Table 3.4 Relative sensitivity, specificity, and positive predictive value comparing pools of ten with pools of five in detecting *Mycobacterium avium* subsp. *Paratuberculosis* in relationship to individual fecal samples from dairy calves across all age groups: Michigan 2005-2007

	Pools of Ten		**Pools of Five**	
	Positive Pools	Negative Pools	Positive Pools	Negative Pools
Pools containing 1 positive calf	2	9	11	10
Pools containing no positive calves	1	82	3	267
Sensitivity (Se)	67% (95% CI 21-94%)		79% (95% CI 56-92%)	
Specificity (Sp)	90% (95% CI 89-91%)		96% (95% CI 95-97%)	
Positive Predictive Value (PPV)	18% (95% CI 6-26%)		52% (95% CI 37-61%)	
Mantel-Haenszel (Yates Corrected)	$p = 0.036$		$p > 0.0001$	

Discussion

Pooling fecal samples in calves for the fecal culture of MAP, using liquid culture, from targeted age groups may be useful in the overall management of Johne's disease on a dairy. Pooling allows the testing of multiple animals with one test which significantly decreases cost (Kalis 2000; van Schaik 2007). Although detection of shedding in the individual animal may be decreased by pooling, shown previously in adult dairy cattle, (van Schaik 2003) overall herd Se may be increased because you use

many more animals per test. This increases the odds of detecting MAP in the herd, especially in a herd with low prevalence (van Schaik 2003). Calves that are infected tend to be in the early phase of bacterial shedding and shed in low numbers (Weber 2005; Bolton - Chapter 2 of this thesis) so pooling is a tool that increases odds of MAP detection in a low prevalence population.

In this study, there was more success in detecting a positive animal in a pool of five compared to pools of ten. Our positive predictive value of 52% with pools of five was similar to a previous report using pools of five in low shedding adult dairy cows (van Schaik 2003). In this study, pooling was found to be inefficient in calves less than seven months of age as zero positive pools were detected with a corresponding low number of calves actually shedding MAP within these groups. Interestingly, in our study, there was no difference between the older two age groups (7-14 months of age and 15-24 moths of age) in Se, Sp, or PPV of pooled cultures. This break point of seven to nine months before onset of MAP shedding has been shown in a prior study (van Roermund 2005). However, the sensitivity of pools of five was greater than pools of ten.

Given these findings, it may be sensible to target the age group 7-14 months in a strategic pooling strategy, using pools of five to gain an early assessment of the effectiveness of any management changes in the herd. Early detection of shedding in this age group and subsequent housing changes may also decrease the shedding threat of horizontal spread of MAP to young herd mates (van Roermund 2005 However with a 53% PPV for pools of five, this would be an inappropriate test for finding individual shedders, as pools of three were far better for this application in a study that focused

on low prevalence adult cattle (van Schaik 2003). There is a need for future research in this area to explore the practical applications of these findings. In lieu of repeating a comprehensive field study such as this, a more prudent approach may be to examine herds of various prevalence levels, and, utilizing pools of five in 7-14 month old calves, sample these animals to determine if a change in prevalence of this age group within a herd is a predictor of future herd prevalence. This procedure may produce an "early report card" of the efficacy of prior management changes.

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CHAPTER 4

POTENTIAL FOR ENVIRONMENTAL TRANSMISSION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* FROM NON-SHEDDING DAIRY COWS TO THEIR CALVES: A CASE REPORT

ABSTRACT

Objectives—This study was conducted to determine if skin contamination of dairy cows with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) could be detected on areas that a suckling calf would nuzzle during teat seeking. Fecal and ELISA testing of cows was also conducted to identify the potential source of MAP contamination.

Design—A case report.

Sample Population—Seven adult dairy cows, nearly due to calve and residing in the "close-up" pen (n=6) and maternity pen (n=1), were selected for testing from a 120 cow dairy herd in Michigan (USA) with a MAP fecal test herd prevalence of 3%.

Procedure—The base of the left front teat and 5cm X 5cm areas of the lateral left tarsus (hock) and the left lateral brisket posterior to the olecranon were swabbed using sterile technique. Blood for MAP serum antibody ELISA testing and feces for MAP culture were also collected. Skin swabs and fecal samples were analyzed using the rapid liquid culture (TREK®ESPII) system and positive samples were confirmed with acid fast staining and IS900 real time PCR. Serum MAP antibody levels were determined using the Parachek® ELISA assay (Prionics).

Results— Six of the seven (86%) cows tested had at least one positive skin swab. The hock (71%) and the udder (57%) were the most common sites where MAP was isolated. MAP was isolated from the skin of five of the six (83%) cows residing in the "close-up" pen, yet each of the 5 was fecal and ELISA test negative. The seventh cow, residing in the maternity pen was fecal positive (a heavy shedder 100-300cfu), ELISA positive, and MAP was isolated from all three skin sites.

Conclusions and Clinical Relevance—MAP was isolated from the skin of dairy cows despite their negative MAP serum antibody or fecal culture status. This suggests that 1) environmental contamination with MAP may occur even in low prevalence dairy herds, 2) the immediate removal of calf from dam at birth is an important element in prevention of MAP transmission, and 3) removal of MAP fecal positive cows, especially heavy shedders, from dairy herds may be critical in eliminating an important source of environmental MAP contamination, and subsequent risk of MAP transmission to newborn calves.

BACKGROUND

Johne's disease (JD), caused by the acid-fast bacteria *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is of international importance in the cattle industry. Prevalent in the US, it is known to cause reduced production and increased culling rates on dairy farms, (Ott 1999) and there is mounting evidence of a potential public health risk (Naser 2004).

This case study is focused on a 120 lactating cow dairy herd which is participating in the Michigan Johne's Disease Control Demonstration Project. In 2002 this herd had a MAP culture positive test prevalence of 12% and six JD clinical cases. After the 2005 annual whole herd test, there were 4 MAP test positive cows (MAP prevalence: approximately 3%) and only one clinical case. This change was attributed primarily to intense culling and feeding of milk replacer instead of unpasteurized whole milk to newborn calves. Environmental sampling of this herd also demonstrated a concomitant reduction in the number of contaminated areas on the farm, with only

the manure storage lagoon testing positive in 2005 compared to multiple positive sites in 2003 (*e.g.*, common alleyways, maternity pen) (Grooms 2003-2007).

With this historical backdrop, and the knowledge that most infections with MAP occur in the neonate (Hendrick 2005) the purpose of this study was to determine if MAP could be isolated from areas on the exterior of dairy cows that are commonly nuzzled in "teat seeking" behavior by the newborn calf (Ventorp 1992). The presence of MAP on these areas could have implications for transmitting MAP to calves. Understanding this risk will provide additional information to support development of management strategies for effective Johne's disease prevention and control (Zdanowicz 2004).

PROBLEM STATEMENT

The first objective of this study was to determine if skin contamination of dairy cows with MAP could be detected on areas that a suckling calf would nuzzle during teat seeking resulting in possible exposure to the pathogen.

The second objective of this study was to determine the MAP fecal culture and ELISA antibody status of cows to identify potential sources of MAP skin contamination.

MATERIALS AND METHODS

Population Description—The source population for this study was a Michigan Johne's Disease Control Demonstration Project herd of 120 Holstein cows with a MAP culture positive test prevalence of nearly 12% in 2002 and approximately 3% in 2005 (Grooms 2003-2007). The study population included all cows due to calve within the three weeks following the test date (based on a 283-day gestation period and their artificial insemination date), which occurred on a single herd visit in 2005. This population was chosen as they present a risk to the newborn calf (Whittington 2009).

On the day of sample collection, six of the seven cows sampled were housed in a single row of sand-bedded free stalls adjacent to a maternity pen that was referred to as the "close-up" pen, our target area. The seventh cow swabbed had recently been moved to the maternity pen. She had spent the prior four weeks in the "close-up" pen.

Sample Collection—Three areas known to be commonly nuzzled by newborn calves during teat seeking behavior (Ventorp 1992) were swabbed including the base of the left front teat, and a 5cm X 5cm area on the lateral left tarsus (hock) and the left lateral brisket area posterior to the olecranon. Using a scrubbing method described in an Ohio study (Shulaw 2005), sterile 2in X 2in gauze pads saturated in 0.9% saline were used. Each area was scrubbed briskly for ten seconds and the gauze pad deposited in labeled sterile conical tubes. Serum for MAP antibody ELISA testing and feces for MAP culture were collected from each cow.

Testing—All skin swabs and fecal samples were analyzed at the Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University

using the rapid liquid culture system; TREK®ESPII, and processing using the Cornell method (Stabel 1997). All positive samples were confirmed with acid fast staining and IS900 real time PCR (Kim 2004). Serum MAP antibody levels in each cow were determined using the Parachek® ELISA assay (Prionics).

RESULTS

Six of the seven (86%) cows tested had at least one MAP positive skin swab (Table 4.1). Three of the swab positive cows (43%) had more than one positive site. The hock (71%) and the udder (57%) were the most common site of MAP isolation. Each of these six cows was fecal and ELISA test negative. The seventh cow (#7) was MAP positive on all three swabbed skin sites and was also MAP fecal culture and serum ELISA positive. She was classified as a "heavy shedder" based on the days to positive in the TREK®ESPII culture system (Shin 2001; van Schaik 2003).

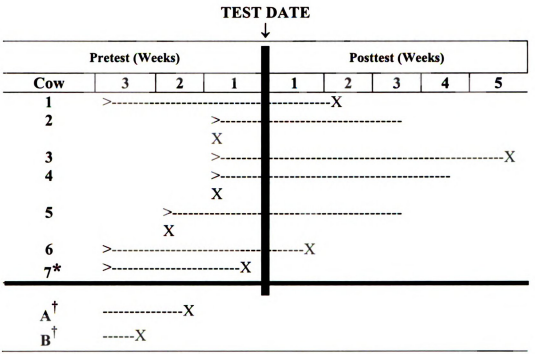
Table 4.1 - Presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) by site swabbed on seven dairy cows and their respective serum ELISA and fecal culture status: Michigan 2005

Cow Number	Brisket	Hock	Udder	Total # Sites (+)	Serum ELISA	Fecal Culture
1	+	+	+	3	-	-
2	-	-	-	0	-	-
3	-	+	-	1	-	-
4	-	+	+	2	-	-
5	-	-	+	1	-	-
6	-	+	-	1	-	-
7	+	+	+	3	+	+++*
Total	**2**	**5**	**4**	**11/21†**		

*Cow #7 was characterized as a MAP "Heavy Shedder" on this fecal test.
†Total number of positive swab samples from total number of collected samples.

Additionally, during post-test investigation, it was discovered that, of the 3 other fecal culture positive cows in the herd, 2 of them (Cows A and B) had also recently been housed in the "close-up" pen, calved and vacated the maternity pen (Table 4.2). Table 4.2 depicts the timeline of cows housed in the "close-up" pen for all seven of the cows swabbed and the two additional MAP fecal culture positive cows in the herd.

Table 4.2 - Timeline illustrating the presence of dairy cows (seven swabbed for MAP, two known MAP fecal culture positive) in the "close-up" pen relative to the test date (> enter pen, X exit pen, ---weeks in pen): Michigan 2005.



*MAP "Heavy Shedder"

†Fecal positive cow in herd but not part of swab study.

DISCUSSION

In this case study, it was demonstrated that MAP skin contamination of cows near calving can be common. This may be a significant risk factor for MAP transmission to the newborn calves. Although the two maternity pens evaluated were relatively small (8ft X 12ft), acceptable maternity management practices were being used. These included one cow in a maternity pen at a time, frequent cleaning and bedding of the maternity pen, and removing calf from cow and maternity pen as soon as possible after birth. There have been many studies describing the importance of maternity pen management in controlling JD (Johnson-Ifearulundu 1999; Tiwari 2009), and no reports were identified that addressed the potential risk to the environment or to newborn calves of MAP-contaminated animals entering the maternity area. Isolating MAP on the exterior of these cows, especially areas where newborn calves suckle, suggests that the potential for MAP transmission to the newborn calf exists.

Another factor of interest in this study was the impact of a heavy shedding animal on environmental contamination and subsequent contamination of non-infected cows. There is evidence that many animals classified as "heavy shedders" actually shed at even higher rates than once thought (Whitlock 2005). The concept of a "super shedder" has been used in characterizing these animals in dairy herds. This "super shedder" concept has also been modeled in other disease risk assessments, such as *E. coli* O157 (Matthews 2006). These animals can shed a hundred fold more of MAP than the parameters of a heavy shedder (100-300) but are classified as heavy shedders due to limitations in quantifying the standard fecal culture. As cow #7 was a "heavy

shedder" of MAP, the potential existed for her to be a "super shedder". Furthermore, although MAP is an obligate intracellular pathogen, it is hardy and can live in the environment for more than a year (Whittington 2004). This highlights the potential risk to newborn calves for MAP transmission when retaining a heavy shedder of MAP in a herd.

Most of the high-risk swabbed areas on the cows tested were positive for MAP contamination. Therefore, there is evidence, through our MAP fecal culture results, that #7, a heavy shedder, along with her two known fecal positive herd mates, had the opportunity to contaminate the environment of the fecal culture negative cows, as they had passed through the same pen only days earlier.

In summary, MAP was isolated from multiple skin sites on cows that were not themselves shedding MAP. This suggests that 1) a reduced herd test prevalence of MAP does not free a dairy herd from environmental MAP contamination, 2) that immediate removal of calf from dam, as suggested by USDA Johne's group (Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program Effective June 1, 2006), is an important element in prevention of MAP transmission, and 3) removal of "heavy shedders" may be critical in eliminating an important source of environmental MAP contamination and subsequent risk of transmission to newborn calves. Furthermore, attention to the cleanliness of the cows themselves as they enter the maternity pen may be important. Future prospective studies should be conducted to examine the efficiency of MAP transmission to the calf as a result of contaminated skin on cows and the subsequent development of clinical Johne's disease in these calves as they mature.

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OVERALL SUMMARY

Nearly one hundred years after *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was characterized as the causative agent of Johne's disease in cattle the battle to control this disease continues world wide. The difficulties are many, as MAP infection is characterized by latent signs, lives up to a year in the environment, resides in wildlife reservoirs, and is refractory to treatment. Also, to date, there are no satisfactory vaccines commercially available, and present testing methods are helpful but continue to need improvement.

However, substantial money and time have been invested in the past three decades and our understanding of this pathogen has increased many-fold. Researchers have shown that the calf is most susceptible, can shed bacteria to others, and there may be some heritability involved in successful transmission. We have shown, with our work that one of the greatest risks for a dairy calf is the MAP status of her dam. A calf from a MAP positive dam is eleven times more likely to have a calf that will also shed MAP. Colostrum and waste milk have to be monitored and the environment kept clean, as they are also risk factors. Besides early MAP detection in calves greater than six months of age utilizing liquid culture, we also found that we were able to pool fecal samples of these low shedding calves and detect a single calf in the pool. Small pools (pools of five calves) had higher sensitivity than pools of ten in detecting a single positive sample. This parallels work that has been done in pooling samples of low shedding adult dairy cattle.

A demonstrative case study completed during the course of this research pointed to the ability that one heavy shedding cow could contaminate the environment

in such a fashion as to cause the organism to reside on the teats of several other "non-infected" cows. This also highlights the need to remove all calves from their dams quickly after birth, so as to minimize likelihood of suckling. As the zoonotic potential of MAP continues to be explored by researchers, it is clear that for the health of the dairy industry and the consuming public, Johne's disease needs to be controlled.

Recommendations-

From our work several factors emerged:

1) The status of the dam should be considered when deciding to retain or cull a newborn calf. If retaining calf, identification as a calf from MAP infected cow and relegation to separate housing may be indicated.
2) MAP fecal culture status of the calves may indicate future prevalence of Johne's disease in the herd. More work should be conducted in this area, especially in calves greater than seven months of age.
3) Pooling of calf fecal samples should be explored as a less expensive method to monitor status of calves in a herd.
4) Indications are that, no matter how low the prevalence of MAP is within a herd, it is extremely important to remove calf before it has a chance to suckle. To mitigate this, concentration on pre-calving udder hygiene should be emphasized.

These are some of the management recommendations that were illustrated or augmented by this study.