EPIDEMIOLOGIC INVESTIGATIONS OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS INFECTIONS IN OHIO DAIRY HERDS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

The Ohio State University, 2003

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ABSTRACT

The development of effective control programs for *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne’s disease, requires a complete understanding of the epidemiology of this agent in animal populations. The research described herein addresses several aspects of the epidemiology of this infection in dairy cattle.

Fecal culture and ELISA results collected from nine Ohio dairy herds involved in Johne’s disease testing programs are described, and likelihood ratios for multiple levels of ELISA S/P ratios are calculated using these test records. The likelihood ratios calculated here suggest that the quantitative use of ELISA S/P ratios to predict the infection status of an individual cow from a MAP-infected dairy herd provides the most confidence when S/P ratios are considerably higher than the manufacturer’s recommended cut-off value.

A prospective study of 55 Ohio dairy herds evaluated the relationships between farm management practices, milk production parameters and fecal cortisol levels. The influence of herd MAP-infection status on fecal cortisol levels was also assessed. Herd, management groups within herds and time of sample collection appear to
significantly influence fecal cortisol levels; however, few specific management practices or milk production parameters that consistently influence these values were identified.

Production data for individual dairy cows were matched with results for MAP tests in order to compare testday production parameters between cows that were test-positive relative to those that were test-negative for both the ELISA and fecal culture. On a given testday, significant losses in milk production were identified for both fecal culture and ELISA-positive cows relative to test-negative cows. These observations provide additional information for dairy producers to consider as they make culling decisions regarding MAP test-positive cows.

Finally, a mail survey comparing the adoption of management practices recommended for Johne’s disease control between Ohio dairy herds involved in testing programs relative to those herds that were not testing is described. Results of this survey demonstrate that, even if a producer believed his/her herd was not infected, participation in a testing program was associated with the adoption of management practices recommended for Johne’s disease control.
Dedicated to my husband, Tim, and my parents, Carl and Lalah Larew
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# TABLE OF CONTENTS

Abstract ...................................................................................................................................................... ii

Dedication .................................................................................................................................................. iv

Acknowledgments ....................................................................................................................................... v

Vita ............................................................................................................................................................... vii

List of Tables ............................................................................................................................................. xiii

List of Figures ............................................................................................................................................ xvii

List of Abbreviations ................................................................................................................................ xviii

Chapters:

1. Introduction ........................................................................................................................................... 1

2. Literature Review .................................................................................................................................. 6

   2.1 Historical Perspective ......................................................................................................................... 6

   2.2 Bacteriology ..................................................................................................................................... 7

      2.2.1 Taxonomy ................................................................................................................................. 7

      2.2.2 Cultural and Biochemical Characteristics ............................................................................... 7

      2.2.3 Differentiation from other MAC bacteria ............................................................................. 8

      2.2.4 Environmental Survival and Disinfection ............................................................................ 10
2.3 Immunology and Pathophysiology ................................................. 11
  2.3.1 Initial Exposure and Uptake of MAP ................................ 11
  2.3.2 Cell-mediated Immune Response to MAP ....................... 14
  2.3.3 Humoral Immune Response to MAP .............................. 16

2.4 Clinical Disease ............................................................................. 17

2.5 Epidemiology ................................................................................ 19
  2.5.1 Prevalence Estimate .......................................................... 19
    2.5.1.1 International Perspective .................................. 20
    2.5.1.2 United States .................................................. 20
  2.5.2 Transmission ....................................................................... 23
  2.5.3 Risk Factors ....................................................................... 25
    2.5.3.1 Cow-level ........................................................ 25
    2.5.3.2 Herd-level ...................................................... 26
  2.5.4 Host Range ........................................................................ 27
  2.5.5 Economics ......................................................................... 28

2.6 Diagnostic Testing ......................................................................... 28
  2.6.1 Introduction ....................................................................... 28
  2.6.2 Diagnostic Tests Based on MAP Detection ....................... 29
    2.6.2.1 Direct Microscopic Examination ....................... 29
    2.6.2.2 Histopathology and Immunohistochemistry ....... 30
    2.6.2.3 Culture .................................................................. 31
    2.6.2.4 Application of IS900 ......................................... 36
  2.6.3 Diagnostic Tests Based on Detection of Cell-Mediated Immune Response to MAP .............................. 38
    2.6.3.1 Intradermal Skin Testing ................................... 38
    2.6.3.2 Intravenous Johnin Testing ................................ 38
    2.6.3.3 Gamma-Interferon Testing ................................. 39
  2.6.4 Diagnostic Tests Based on Detection of Humoral Immune Response to MAP .............................................. 40
    2.6.4.1 Fluorescent Antibody Test ................................. 40
    2.6.4.2 Complement Fixation Test ................................. 40
    2.6.4.3 Agar Gel Immunodiffusion Test ......................... 41
    2.6.4.4 Enzyme-Linked Immunoabsorbent Assay ......... 42
  2.6.5 Selection of Diagnostic Tests ............................................ 43
  2.6.6 Application of ELISA and Fecal Culture for Herd Screening ............................................. 44
7. Comparison of Management Practices between Ohio Dairy Farms Involved in Johne’s Disease Testing Programs Versus Those Farms Not Involved In Testing ........................................................................................................180

7.1 Abstract ..................................................................................................180
7.2 Introduction ...........................................................................................181
7.3 Methods ...............................................................................................184
7.4 Results .................................................................................................186
7.5 Discussion ...........................................................................................192
7.6 Literature Cited ...................................................................................197

8. Conclusions ...........................................................................................208

BIBLIOGRAPHY ...........................................................................................211
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Number of cows tested, percent of positive fecal culture results, and percent of positive ELISA results for whole-herd tests completed for each farm by six-month intervals from 1994-1999</td>
<td>121</td>
</tr>
<tr>
<td>3.2</td>
<td>Likelihood ratios indicating the odds of MAP infection relative to non-infection for multiple ranges of ELISA S/P ratios</td>
<td>123</td>
</tr>
<tr>
<td>4.1</td>
<td>Percent change from baseline in serum and fecal cortisol levels and time of maximum change in fecal cortisol level for individual cows</td>
<td>136</td>
</tr>
<tr>
<td>5.1</td>
<td>Mean fecal cortisol levels, standard deviation and number of samples analyzed by farm</td>
<td>156</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>158</td>
<td></td>
</tr>
<tr>
<td>Mean fecal cortisol levels, standard deviation and number of samples analyzed by location within farm where different subscripts indicate significantly different means.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.3</td>
<td>159</td>
<td></td>
</tr>
<tr>
<td>Mean fecal cortisol levels, standard deviation and number of samples analyzed by visit.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.4</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>Variables identified as significant at the 0.250 level in univariate mixed models and significant at the 0.100 level in management subset models.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>162</td>
<td></td>
</tr>
<tr>
<td>Variables included in the final mixed model for mean herd fecal cortisol levels.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>176</td>
<td></td>
</tr>
<tr>
<td>Mean testday milk, milk fat and milk protein production relative to results of concurrent fecal culture and ELISA testing.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.2</td>
<td>177</td>
<td></td>
</tr>
<tr>
<td>Adjusted means for milk production parameters when controlling for herd, lactation number and days-in-milk relative to results of a concurrent fecal culture.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table Page
6.3 Adjusted means for milk production parameters while controlling for herd, lactation number and days-in-milk relative to results of a concurrent ELISA.................................................................178

6.4 Adjusted means for milk production parameters while controlling for herd, lactation number and days-in-milk relative to S/P ratios of a concurrent ELISA where different superscripts indicate significantly different means using Tukey’s test for multiple comparisons.................................................................179

7.1 Responses to survey questions concerning management practices recommended for Johne’s disease control..................................................199

7.2 Unadjusted odds ratios and 95% confidence intervals representing the odds that TESTING herds reported the specified management practice relative to the odds the same management practice was reported by NON-TESTING herds ..................................................202
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.3</td>
<td>Unadjusted odds ratios and 95% confidence intervals representing the odds that TESTING herds reported the specified change in a management practice relative to the odds that NON-TESTING herds reported the same change</td>
</tr>
<tr>
<td>7.4</td>
<td>Adjusted odds ratios and 95% confidence intervals representing the odds that TESTING herds reported the specified management practice relative to the odds the same management practice was reported by NON-TESTING herds when controlling for producer-perceived infection status</td>
</tr>
<tr>
<td>7.5</td>
<td>Adjusted odds ratios and 95% confidence intervals representing the odds that TESTING herds reported the specified change in a management practice relative to the odds that NON-TESTING herds reported the same change when controlling for producer perceived-infection status</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>134</td>
</tr>
<tr>
<td>4.2</td>
<td>135</td>
</tr>
<tr>
<td>4.3</td>
<td>137</td>
</tr>
<tr>
<td>7.1</td>
<td>205</td>
</tr>
</tbody>
</table>

- **4.1** Serum cortisol levels at baseline and at 1 hour following ACTH administration.
- **4.2** Fecal cortisol values for individual cows over the observation period.
- **4.3** Time of maximum increase in fecal cortisol levels following ACTH administration.
- **7.1** Timing of changes in management practices relative to the initiation of Johne’s disease testing.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADDL</td>
<td>Animal Disease Diagnostic Laboratory</td>
</tr>
<tr>
<td>AGID</td>
<td>Agar gel immunodiffusion</td>
</tr>
<tr>
<td>APHIS</td>
<td>Animal and Plant Health Inspection Service</td>
</tr>
<tr>
<td>CEAH</td>
<td>Center for Epidemiology and Animal Health</td>
</tr>
<tr>
<td>CFR</td>
<td>Code of Federal Regulations</td>
</tr>
<tr>
<td>CFT</td>
<td>Complement fixation test</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>CMI</td>
<td>Cell-mediated immunity</td>
</tr>
<tr>
<td>DHI</td>
<td>Dairy Herd Improvement Cooperative, Inc</td>
</tr>
<tr>
<td>DIM</td>
<td>Days-in-milk</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoabsorbent assay</td>
</tr>
<tr>
<td>EST</td>
<td>Eastern standard time</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocytic-lymphocytic colony stimulating factor</td>
</tr>
<tr>
<td>HEYM</td>
<td>Herrold’s egg yolk media</td>
</tr>
<tr>
<td>HPC</td>
<td>Hexadecylpyridinium chloride</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMS</td>
<td>Immunomagnetic separation</td>
</tr>
<tr>
<td>IS</td>
<td>Insertion sequence</td>
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<tr>
<td>MAC</td>
<td><em>Mycobacterium avium-intracellulare</em> complex</td>
</tr>
<tr>
<td>MAP</td>
<td><em>Mycobacterium avium</em> subsp. <em>paratuberculosis</em></td>
</tr>
<tr>
<td>ME</td>
<td>Mature equivalents</td>
</tr>
<tr>
<td>NADC</td>
<td>National Animal Disease Center</td>
</tr>
<tr>
<td>NAHMS</td>
<td>National Animal Health Monitoring Systems</td>
</tr>
<tr>
<td>NVSL</td>
<td>National Veterinary Service Laboratory</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ODA</td>
<td>Ohio Department of Agriculture</td>
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<tr>
<td>OIE</td>
<td>Office International des Epizooties</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RIA</td>
<td>Radio-immuno assay</td>
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<tr>
<td>S/P ratio</td>
<td>Sample-to-positive ratio</td>
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<tr>
<td>USAHA</td>
<td>United States Animal Health Association</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>VS</td>
<td>Veterinary Services</td>
</tr>
</tbody>
</table>
Mycobacterium avium subsp. paratuberculosis (MAP) is an intracellular pathogen in ruminants. Clinical disease associated with MAP infection is referred to as Johne’s disease or paratuberculosis.

The Office International des Epizooties (OIE) classifies paratuberculosis as a List B Disease. Thus, Johne’s disease is of socio-economic and/or public health importance within countries and is significant in the international trade of animals and animal products.1 Johne’s disease has a world-wide distribution. Most countries are considered infected.

In the United States, the prevalence of MAP infection has been estimated to be 3.4% of individual dairy cattle and 21.6% of dairy herds.2 Additionally, production losses associated with Johne’s disease for the United States dairy industry are estimated to be between $200 and $250 million annually.3

The primary means of transmission for MAP is via the feco-oral route. It is generally accepted that calves less than six months of age are at the greatest risk of
infection with MAP.4-8 Calves are believed to be exposed to MAP through the ingestion of contaminated milk or colostrum.4,5,9,10 Because environmental contamination with MAP can be significant on infected farms, the tendency for calves to lick surfaces while exploring their environment can serve as another means of exposure to MAP.4,6,8,9,11

MAP infection does not progress to clinical disease in all animals. In fact, most animals are able to eliminate or control the progression of MAP infections.8 The development of clinical signs represents the culmination of pathologic and immunologic changes that occur as a result of this infection and signify terminal events in the chronic progression of a fatal disease process.

The majority of infected animals within a herd are subclinically infected. These apparently healthy individuals serve as an important source of environmental contamination because fecal shedding of MAP begins during this stage of infection. Identification of subclinically infected animals requires the use of diagnostic tests.

In addition to the implementation of management practices designed to limit transmission to susceptible animals, an important aspect of control of MAP infection is the identification and removal of infected animals from a herd. Removal of infected animals immediately reduces the within herd prevalence of the infection, and is considered to produce the quickest, most effective impact on Johne’s disease control.11 However, the identification of animals infected with MAP, especially those in subclinical stages of the infection, continues to present a diagnostic challenge despite nearly a century of development of diagnostic testing methods.
The possibility that MAP may be a zoonotic pathogen is also an important consideration regarding this agent. This question has generated considerable speculation by the scientific community for nearly a century. In 1984, a series of reports re-initiated the controversy regarding the role of mycobacteria, specifically MAP, in the etiology of Crohn’s disease.\textsuperscript{12-15} Although multiple, independent reviews of the entire body of scientific literature have determined that the evidence regarding this proposed link is inconclusive, controversy regarding this issue remains.\textsuperscript{16-19}

Several aspects of the epidemiology of MAP infection and Johne’s disease in dairy cattle require continued scientific investigation. The research described here addresses several of these issues.

\textbf{Literature Cited}


18. Rubery E. A review of the evidence for a link between exposure to *Mycobacterium paratuberculosis* (MAP) and Crohn's disease (CD) in humans. Food Standards Agency, United Kingdom.
CHAPTER 2
LITERATURE REVIEW

2.1 Historical Perspective

Although reports of enteritis associated with chronic diarrhea and death in cattle appeared as early as 1826, the first formal description of the clinical disease, known today as paratuberculosis or Johne’s disease, appeared in 1895 as a case report, ‘Ein eigenthumlicher Fall von Tuberculose beim Rind’ (‘A singular case of bovine tuberculosis’).\(^1,2\) Johne and Frothingham believed the disease to be an atypical form of bovine tuberculosis.\(^3\) However, Bang successfully distinguished the infection from tuberculosis in 1906, and proposed the condition be termed pseudotuberculous enteritis.\(^4\) Twort first isolated the causative agent in 1910 and provided the name \textit{Mycobacterium enteriditis chronicae pseudotuberculosa bovis johne}.\(^3,5\)

Following its earliest description, Johne’s disease was reported to exist throughout northern and western Europe, and was first reported in North America in 1908.\(^2,3\) Today, it is considered to be one of the most economically significant diseases in dairy cattle.\(^2,5\) In the United States, economic losses associated with this infection in the dairy industry are estimated to be $200 to $250 million annually.\(^6\)
2.2 Bacteriology

2.2.1 Taxonomy

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a member of the *M. avium-intracellulare* complex (MAC). In addition to the other *M. avium* subspecies, *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* (wood pigeon bacillus), the MAC also includes the species *M. intracellulare*. MAC organisms represent a common non-tuberculous mycobacterial cause of disease in many species, including humans, and are considered to be ubiquitous in the environment.7,8

The genus *Mycobacterium* is the sole member of the family *Mycobacteriaceae*. Related genera from the *Actinomycetales* order include *Nocardia*, *Rhodococcus* and *Corynebacterium*.

2.2.2 Cultural and Biochemical Characteristics

MAP is an aerobic, non-spore forming, non-motile, acid-fast bacillus that is an intracellular pathogen in ruminants. In addition to conferring acid-fastness, the high lipid content of the cell wall also results in the formation of clumps containing multiple bacilli.8

MAP is a fastidious organism with specific requirements for growth in culture. MAP is the only *Mycobacterium* species that requires supplemental mycobactin, a cell wall-associated iron chelator produced by some mycobacterial species during iron-limited conditions, for growth in culture.8,9 Mycobactin dependence, acid-fastness and very slow growth are key characteristics used to identify MAP.
Although colorless to white, rough colonies can appear following 6 to 8 weeks of culture at 37°C, generally 16 weeks of incubation are allowed for primary isolation from clinical samples. As a result of this slow growth, MAP generally lacks reactivity to many of the standard biochemical tests utilized for organism identification.  

Considerable strain variation exists within the MAP species. Strain differences both within and between wild type and laboratory strains of MAP have been noted. Additionally, differences between strains isolated from different host species have been well documented. For example, sheep strains of MAP are extremely difficult, if not impossible, to culture using traditional culture methods while cattle strains are generally easily cultured. A yellow or orange pigmentation in intestinal lesions and colonies isolated in culture has been observed with sheep strains of MAP, but not strains isolated from cattle.  

More recently, polymorphisms in the DNA insertion sequence IS1311 have distinguished between cattle and sheep strains of MAP. This example demonstrates that strain variation in MAP may have clinical, diagnostic and epidemiologic consequences. Further exploration of the significance of strain variations may be warranted.

2.2.3 Differentiation from other MAC mycobacteria

The advent of molecular techniques has facilitated the differentiation of MAP from other genetically similar mycobacteria. Even then, DNA homology between MAC species is high and can approach 100%. Restriction fragment length polymorphism (RFLP) analysis of mycobacterial DNA permits differentiation between closely related species.
In 1989, Green et al identified a 1451 base pair insertion sequence, IS900, that repeated 15 to 20 times within the MAP genome. Subsequent analysis appeared to confirm the specificity of this sequence for MAP. Because IS900 is believed to be highly specific for MAP, portions of this DNA sequence have been incorporated into confirmatory tests for the presence of MAP in both diagnostic settings and epidemiologic investigations.

Recently, the specificity of IS900 for MAP has been challenged. Workers in Australia identified the presence of IS900-like sequences in M. scrofulaceum isolates from four clinically normal ruminants. Another report identified IS900 in 15 of 26 M. avium subsp. avium isolates obtained from AIDS patients. However, it has been suggested that studies demonstrating imperfect specificity of IS900 utilized inappropriate primer sequences contained within the IS900 sequence, and that IS900 in its entirety is specific for MAP. The identification of IS900 remains an important confirmatory test; however, the sole use of IS900 detection to identify MAP should be approached with caution.

Recently, considerable research has focused on identifying other genomic components that would allow for differentiation of MAP from other mycobacterial species. In November 2002, the genomic sequencing of MAP was completed. Preliminary analysis has identified 21 coding sequences unique to MAP that will be further investigated for their utility in diagnostic techniques and possibly vaccine development.
2.2.4 Environmental Survival and Disinfection

Generally, MAP is considered to be a very hardy organism in the environment.2,23 The high mycolic acid content of the cell wall imparts hydrophobicity and a tendency to form clumps.8,23 Both characteristics may enhance survival in the environment. Environmental conditions such as temperature, acidity, humidity, ultraviolet light and salt content may also influence the ability of MAP to survive in the environment.1,2,8,23-25

An early review evaluating the environmental survival of MAP documents survival greater than 1 year in soil and on pasture, at least 100 days in liquid manure, less than 30 days in bovine urine, greater than 240 days in tap water and pond water exposed to light and over 420 days in tap water maintained in darkness.26 However, because these studies were performed from 1940-1960, prior to recent advances in cultural methods, Collins et al suggest that extrapolation of this experimental data to natural conditions may be inappropriate, and indicate scientific data regarding the survival of MAP under various environmental conditions continues to be generally lacking.24

Research concerning the ability of MAP to survive pasteurization indicates that the organism may be more resistant to heat than both M. bovis and Coxiella burnetti, the current targets of pasteurization.27-34 Freezing at −70°C for three weeks resulted in a significant reduction in MAP viability.35 MAP may also be relatively resistant to acidic environments.36
Disinfection and sanitizing agents aimed at reducing quantities of MAP on environmental surfaces are chosen based on their demonstrated activity against *M. tuberculosis* or *M. bovis* rather than their activity specifically against MAP.\textsuperscript{24} In general, phenol-based disinfectants are favored for on-farm environments because of their ability to be effective in the presence of limited quantities of organic material.\textsuperscript{37} Commercially available phenol-based disinfectants include One Stroke Environ®, Osyl®, and Amphyl®.\textsuperscript{37} Chlorine-based disinfectants are unlikely to be effective against MAP.\textsuperscript{23} A recent report demonstrated that water contaminated with more than 10⁶ cfu/ml of MAP was not adequately disinfected after 30 minutes of contact time with 2 µg/ml of chlorine.\textsuperscript{38} Additionally, chlorine-based disinfectants have limited activity in situations where organic debris exists.

### 2.3 Immunology and Pathophysiology

#### 2.3.1 Initial Exposure and Uptake of MAP

It is generally accepted that calves less than six months of age are at the greatest risk for infection with MAP.\textsuperscript{2,11,23,39,40} Calves ingest viable MAP as a result of fecal or hematogenous contamination of colostrum/milk or as a result of suckling on contaminated surfaces.\textsuperscript{2,40} The esophageal groove reflex in neonatal ruminants allows for colostrum and milk to bypass the rumino-reticulum and reach the omasum directly from the esophagus. It is suggested that this prevents ingested MAP from exposure to innate, physiological defenses such as pH, dilution, enzymes, mucus and bacterial competition normally encountered in the rumen.\textsuperscript{11}
Once in the ileum, M cells play a pivotal role in uptake of MAP. M cells endocytize and transport the infectious agent to the submucosa. Calves have a higher proportion of M cells in their intestinal epithelium than adults. This anatomic difference may partially explain the predisposition of young calves to infection with MAP.

Macrophages scour the submucosa for foreign material, serving as both an antigen presenting cell and an effector cell in the immune response against MAP. *In vitro* studies have demonstrated enhanced MAP uptake in the presence of immune serum. Zurbrick and Czuprynski have suggested that opsonization may be required for optimal phagocytosis of MAP by macrophages. These observations could further explain the susceptibility of calves to infection with MAP. Colostrum contains large quantities of immunoglobulins that are easily absorbed by neonatal intestines and are present in high quantities in the submucosa, perhaps enhancing MAP ingestion by macrophages.

MAP is now within its target cell, the macrophage. Theoretically, phagocytized material is now exposed to lysosomal enzymes or biochemical pathways that should degrade the bacteria and afford protection to the host. However, neither macrophages nor monocytes, the precursor cell of the macrophage, are efficient at eliminating intracellular MAP, and both cell types supported intracellular growth in *in vitro* experiments.

The ability of mycobacterial cells to survive within the harsh environment of the macrophage is the key to their ultimate ability to cause disease. Evasive
mechanisms of MAP against macrophage activity are expected to be similar to those of *M. tuberculosis*, although these assumptions have not been fully explored with respect to MAP. Several survival mechanisms have been proposed: production of sulphatides which prevent phagosome-lysosome fusion, escape into the cytoplasm of the macrophage, production of glycolipids which inhibit nitric oxide and presence of superoxide dismutase and glycolipid which inhibits the respiratory burst system.\(^{11}\) Saha et al propose that mycobacteria utilize a prostaglandin-mediated pathway resulting in selective regulation of co-stimulatory molecules on infected macrophages, ultimately suppressing the CMI response against the pathogen.\(^{43}\) More recently, up-regulation of certain genes within MAP following phagocytosis has been suggested to play a critical role in intracellular survival.\(^{44}\)

Considerable research has focused on the relationship between MAP and the macrophage and monocyte. However, several authors suggest that the use of peripheral blood monocytes in *in vitro* experiments may not reflect the local activities of macrophages in the lamina propria of the gastrointestinal tract.\(^{45}\)

Macrophages transport MAP to deeper areas of the lamina propria and throughout the lymphatic system.\(^{41}\) In addition to repeated exogenous exposures, the host must also continually mount an immune response against endogenous exposures associated with circulation of the agent within macrophages. This series of events is occurring simultaneously in many areas of the submucosa.\(^{2,40}\)
2.3.2 The Cell-Mediated Immune Response to MAP

As an intracellular pathogen, the primary immune response that limits or eliminates MAP infection is a cell-mediated response (CMI).\textsuperscript{11,40} Uptake of MAP by macrophages initiates a focal CMI response with multiple foci active simultaneously.

An important cell type involved with CMI is the $T_{\text{H}1}$ cell. It has been suggested that if the host can eliminate an MAP infection, it is likely the result of $T_{\text{H}1}$ cell activity.\textsuperscript{40}

$T_{\text{H}1}$ cells are activated in response to the presentation of mycobacterial antigen by antigen presenting cells, such as the macrophage. $T_{\text{H}1}$ cell activation generates cytokines that include TNF-$\alpha$, IFN-$\gamma$, GM-CSF, IL-1, IL-2 and IL-6.\textsuperscript{46} Adams and Czuprynski demonstrated that bovine monocytes secreted TNF-$\alpha$, IL-1 and IL-6 when exposed to MAP \textit{in vivo}.\textsuperscript{47}

The function of specific cytokines in the immune response to MAP is somewhat ambiguous. TNF-$\alpha$ which is associated with granuloma formation and cachexia, has both stimulatory and inhibitory effects on MAP \textit{in vivo}, depending upon its concentration as well as the timing of its presence.\textsuperscript{48} Zhao et al demonstrated that, although IFN-$\gamma$ did activate monocytes, it was not capable of maintaining prolonged inhibition of intracellular MAP growth.\textsuperscript{45} IFN-$\gamma$ gene expression was higher in the ileal and cecal lymph nodes of subclinically infected cows than in clinically infected cows.\textsuperscript{49} This suggests that low IFN-$\gamma$ levels are associated with the presence of clinical disease; however, the temporality of IFN-$\gamma$ in this disease process is not known –
are cows with inherently low levels of IFN-γ more susceptible to disease or is there a decrease in IFN-γ levels that is related to the development of disease?

The exact function of IL-2, IL-4 and IL-6, in the immune response to MAP infection remains unclear. These cytokines serve to recruit and activate other immune cells, initiate inflammatory pathways and contribute to the development of clinical signs.49,50

It is clear that most animals are able to eliminate or control progression of MAP infections.40 Establishment of an infection requires that large numbers of bacteria gain access into macrophages and evade the mechanisms designed to eliminate the bacteria. The factors that facilitate the establishment of a MAP infection are not fully understood; however, dose, route of infection, strain variations, environmental factors, local and systemic immune status and the age of host appear to influence development of the disease.11

The inability to eliminate intracellular MAP results in prolonged secretion of cytokines and pro-inflammatory factors. The chronic stimulation continuously recruits lymphocytic cells to locations with mycobacteria. These areas of intestine eventually exhibit histologic changes associated with chronic immune stimulation and infiltration: chronic granulomatous enteritis and regional lymphadenitis.11

Initially, focal areas of granulocytic inflammation with granuloma formation effectively limit the MAP infection. Lesions initially occur in the ileum or ileo-cecal junction. Hematogenous spread and continued ingestion of MAP is proposed to initiate multiple lesions that occur simultaneously, each with their own individual CMI
response. Lesions continue to expand and eventually involve luminal surfaces throughout the ileum and large intestine. As the balance between CMI and MAP begins to favor MAP, these lesions increase in size. Eventually, lesions become adjacent to the intestinal lumen resulting in fecal shedding of MAP.

The balance between the CMI and MAP waxes and wanes. Animals may not be persistently shedding the bacteria since lesions are constantly progressing and regressing in a dynamic state. The multiple lesions coalesce into larger and larger lesions, eventually resulting in a single lesion. The role of type III hypersensitivity reaction in the development of pathologic lesions in Johne’s disease has also been suggested.

2.3.3 The Humoral Immune Response to MAP

The specific factors that initiate the humoral response are not well established. However, a shift from $T_{H1}$ to $T_{H2}$-mediated immune processes appear to result in the development of the humoral immune response. In cattle, IgG and IgM responses predominate. Development of a detectable humoral immune response has been correlated with increased fecal shedding of the organism, but less strongly associated with severity of pathology. It has been suggested that as CMI responses diminish, humoral responses increase.

Because of its intracellular nature, humoral immune responses are largely ineffective at eliminating MAP. In fact, some refer to the presence of antibodies as "an ominous sign" because it signifies failure of cell-mediated immunity to control the infection. Recent work suggests that as the humoral response progresses, peripheral
B cell responsiveness decreases.\textsuperscript{54} If the animal survives long enough, it is believed that it will eventually become completely anergic, with no evidence of CMI or humoral response.\textsuperscript{40,53}

\subsection*{2.4 Clinical Disease}

Clinical disease associated with MAP infection is referred to as Johne’s disease or paratuberculosis. Clinical signs represent the culmination of pathologic and immunologic changes that occur as a result of MAP infection and signify terminal events in the chronic progression of a fatal disease process.

In cattle, Johne’s disease is manifested as progressive weight loss despite a normal appetite and chronic, possibly intermittent, diarrhea that is unresponsive to medical therapy. General unthriftiness, including a poor hair coat, is also observed. In end-stage cases, hypoproteinemia may occur resulting in submandibular edema commonly referred to as “bottle jaw”. Typically, cattle develop clinical signs between 3 to 5 years of age that progress over a 3 to 6 month period. Small ruminant species, such as sheep and goats, generally do not exhibit diarrhea although the feces may be less formed than normal. The progression of disease is generally more rapid in these species than in cattle, with weight loss being the predominant sign.\textsuperscript{1,55}

In addition to the classic clinical signs attributed to Johne’s disease, other secondary clinical effects have been reported in dairy cattle. Decreased milk production is considered a major economic consequence of this infection and has been documented for both subclinical and clinical MAP infection.\textsuperscript{6,56-59} Increased mortality rates have been identified in infected dairy herds.\textsuperscript{57,60} Other reports have identified
reduced reproductive performance in infected cows.\textsuperscript{61} Several anecdotal reports have suggested that MAP infection may predispose cows to the development of mastitis; however, in one epidemiologic study, MAP infection was associated with decreased incidence of mastitis.\textsuperscript{56}

MAP infection does not progress to clinical disease in all animals. Whitlock and Buergelt propose that MAP infection be compartmentalized into four “stages of disease” relative to “the severity of clinical signs, the potential for shedding organisms into the environment and the ease with which the disease may be detected using current laboratory methods.” These stages include “silent” infection, subclinical disease, clinical disease and advanced clinical disease. In a dairy herd, for every animal with advanced clinical disease there may be as many as 25 additional animals infected at earlier stages.\textsuperscript{55}

Several pathologic processes likely contribute to the development of clinical signs: cachexic factors such as TNF-\(\alpha\), hypersensitivity to MAP, anergy to MAP and progressive ileal and large intestinal thickening resulting in a malabsorptive diarrhea.\textsuperscript{11} However, severe histological lesions do not necessarily result in severe clinical disease.\textsuperscript{3}

Individual animal characteristics also appear to be important determinants in the development of clinical signs. Age and dose of MAP at time of initial exposure have been reported to influence the development of clinical signs.\textsuperscript{2} There is some suggestion that hormonal fluctuations or “stressful” events may facilitate the onset of clinical disease.\textsuperscript{2-3,62-64}
Symptomatic treatment of the clinical signs or antimicrobial therapy directed at the MAP infection in ruminants is not routine. Disease can recur following the cessation of antimicrobial therapy. Additionally, there are no anti-tuberculoid drugs currently approved for the treatment of MAP infections in dairy cattle. Avoidance of chemical residues in meat or milk products from treated animals limits the extralabel use of antimycobacterial drugs.

2.5 Epidemiology

2.5.1 Prevalence Estimates

Early attempts to estimate the prevalence of MAP infection in various geographic areas relied primarily on the culture of mesenteric lymph node or ileal tissue obtained from cattle presented at slaughter. Although this approach was the best available at the time, the cost of culture, labor requirements for sample collection and location of slaughter facilities limited sample size. Additionally, animals presented at slaughterhouses represented a convenience sample of the cattle population rather than a random sample. Thus, prevalence estimates obtained from these early studies were potentially biased, and their extrapolation to different geographic areas was limited.

The advent of ELISA technology has facilitated the implementation of large-scale studies based on random sampling to estimate the prevalence of MAP infection. Advantages of the ELISA in these studies include decreased cost, ease of sample collection and shipment and more rapid results. However, the imperfect diagnostic sensitivity and specificity intrinsic to the ELISA requires adjustment of apparent
prevalence to estimate the true prevalence in an area.\textsuperscript{66} In an effort to improve the herd-level specificity of the ELISA, classification as an infected herd generally requires the detection of multiple ELISA-positive animals or incorporation of historical information about the herd.\textsuperscript{57,67}

2.5.1.1 International Perspectives

Johne’s disease has a world-wide distribution. Most countries are considered infected. Only Sweden and certain areas of Australia have conducted the extensive testing required to establish freedom from the infection.\textsuperscript{1,23,25} Several countries have attempted to estimate the national prevalence of Johne’s disease in various animal populations, including dairy cattle.\textsuperscript{68,69}

The Office International des Epizooties (OIE) classifies paratuberculosis as a List B Disease. This indicates that it is considered to be of socio-economic and/or public health importance within countries and significant in the international trade of animals and animal products.\textsuperscript{70} Reports of List B diseases are normally submitted to the OIE by official government sources yearly. Because prevalence estimation at a national level is difficult and expensive, these reports generally indicate that confirmation of infection has occurred rather than providing prevalence estimates for a given country. In addition to the infection status, information on paratuberculosis control measures in place in various countries is also maintained by the OIE.\textsuperscript{25,70}

2.5.1.2 United States

Several studies have estimated the prevalence of MAP infections in individual states or geographic regions in the United States. Early studies relied on culture of
tissue samples collected from slaughterhouses. Using this approach, prevalence in the northeastern US was estimated to be 7.2% in 1985. A second study conducted at a similar time estimated 18% prevalence of MAP infection in New England.

More recently, the ELISA has been used to estimate prevalence of MAP infection in the dairy cattle populations of various states. The prevalence of MAP infection in Wisconsin was estimated to be 4.79% of dairy cattle and 34% of dairy herds. In Michigan, 6.9% of dairy cattle were seropositive and 55% of herds had two or more seropositive cattle. Antibodies against MAP were detected in 8% of dairy cattle and 74% of dairy herds in Missouri. A prevalence survey in Florida identified 17.1% of dairy cattle as seropositive.

In 1996, the Centers for Epidemiology and Animal Health (CEAH) of the United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services (USDA: APHIS:VS) conducted Dairy ‘96 as part of its National Animal Health Monitoring System (NAHMS). Dairy ’96 represented the first comprehensive attempt to estimate the national prevalence of MAP infection in the United States dairy cattle population. However, collecting epidemiological information about Johne’s disease was not the sole intent of Dairy ’96. The following aims were identified with regard to Johne’s disease: assess producer awareness, estimate national and regional herd-level prevalence of MAP infection, estimate economic losses in dairy cattle and evaluate associations between specific management practices and herd-level MAP prevalence.
Data collection for Dairy ’96 included two phases. Initially, a random sample of dairy herds, stratified by geographical region, was selected from 20 states. The dairy cow inventory in these states represented 83.1% of the total US inventory. A baseline questionnaire regarding management practices was administered to 2542 herd operators that agreed to participate in the study. Based on the information obtained from the baseline survey, dairy herds with greater than 30 cows were invited to participate in the second phase of data collection that included administration of a more comprehensive health management questionnaire and serum sampling of a randomly selected subset of herd members for ELISA testing. The number of cows to be sampled in each herd was predefined to achieve a 90% confidence that an infected herd with a prevalence of 10% or greater would be detected. In total, 1219 dairy producers voluntarily responded to the health management questionnaire, and 1004 of these permitted biological sampling of a total of 32,622 cows.57

Based on Dairy ’96, the true prevalence of MAP infection in the United States is estimated to be 3.4% of individual dairy cattle and 21.6% of dairy herds. However, these may be underestimates. At the individual cow-level, the apparent prevalence was weighted to account for the sampling design and adjusted with a calculation assuming a sensitivity of 45% and a specificity of 99% of the ELISA. Because ELISA sensitivity may be less than the assumed 45%, it is possible that truly infected cows may not have been identified as ELISA-positive. At the herd-level, infected herds were defined as those with two or more positive ELISA results or those with one positive ELISA result and clinical signs in 5% or more of cows culled during
the previous year. Because the number of animals sampled per herd was designed to detect herds with relatively high infection rates (10% or greater), detection of herds with low levels of infection was unlikely. Despite these potential problems, Dairy ’96 is still considered to provide the most accurate prevalence estimate available to date for Johne’s disease in the United States dairy population.

In 2002, NAHMS again focused its attention on the dairy industry. A primary focus of this most recent study was to identify changes that have occurred with regard to Johne’s disease since the completion of Dairy’ 96. Specifically, the current state of producer knowledge about Johne’s disease, participation in control or certification programs and preventive management strategies utilized to facilitate prevention or control will be assessed and compared to results from Dairy ’96.

2.5.2 Transmission

The primary route of transmission for MAP is via ingestion of the organism. The exposure of calves to MAP through ingestion of contaminated milk or colostrum is considered to be an important route of transmission. Fecal contamination of the udder and teat surfaces can result in the contamination of milk or colostrum with MAP. Additionally, the excretion of MAP into both colostrum and milk has been documented to occur in both subclinically and clinically infected cows. The potential for MAP to survive pasteurization further emphasizes the importance of transmission of MAP through milk and/or colostrum.

Environmental contamination with MAP can be significant on infected farms. The tendency for calves to lick surfaces while exploring their
environment can serve as another means of feco-oral transmission of MAP.\textsuperscript{2,40} Additionally, the contamination of food or water sources with MAP is of concern.

Several studies have documented the potential for \textit{in utero} transmission of MAP.\textsuperscript{83,84} In one, 26.4\% (9/34) of fetuses from cows with positive mesenteric lymph node cultures were infected.\textsuperscript{83} In subclinically infected cows, 17.8\% (5/58) of fetuses were infected.\textsuperscript{84} The authors of this study further suggest that cows that are either clinically ill or those that are classified as “heavy shedders” appear to be more likely to transmit MAP \textit{in utero} than animals that are shedding low numbers of MAP.\textsuperscript{84}

The role of semen in the transmission of MAP is unclear.\textsuperscript{64,78} Although MAP has been cultured from the reproductive tissue and seminal fluid of infected bulls, rigorous screening of bulls used for artificial insemination greatly limits the possibility that MAP transmission would occur.\textsuperscript{78} The ability for MAP transmission to occur during natural service and the significance of this potential route of transmission is unknown.

Two possible sources of infection exist during embryo transfer. MAP has been recovered from uterine fluids and washed embryos.\textsuperscript{85,86} Thus, infection may be transmitted from an infected donor cow to its embryo. The practical significance of this is unknown, although one source indicates this rarely occurs.\textsuperscript{78} Secondly, an infected recipient cow can infect the embryo during gestation. This scenario has been indicated to be a more likely means of infection, although scientific evidence to support this claim is lacking.\textsuperscript{78}
2.5.3 Risk Factors

2.5.3.1 Cow-level

At the individual-animal level, the best characterized risk factor for MAP infection is age. Animals less than 6 months of age are generally regarded to be at the highest risk for the development of MAP infection.\textsuperscript{2,11,23,39,40} Several explanations have been proposed for this increased susceptibility of young animals: the presence of the esophageal groove allowing the agent to bypass the rumeno-reticulum, an increased proportion of M cells in the ileum increasing the phagocytic capacity, immunoglobulins serving as opsonizing agents and “leaky bowel” facilitating the uptake of MAP.\textsuperscript{2,11,40-42}

Several early reports suggested that Channel Island breeds, such as the Jersey and Guernsey, were at increased risk for MAP infection and subsequent Johne’s disease.\textsuperscript{23,39} However, today all breeds are believed to be susceptible to infection.

Stressful events are frequently reported to induce the onset of clinical Johne’s disease in cattle. Parturition, heavy milk production, low plane of nutrition, transport and immunosuppression secondary to other infectious diseases have been identified as factors possibly contributing to the onset of clinical signs.\textsuperscript{1-3,23,39,62}

However, the evidence supporting the role of stress as a predisposing factor for either the initial MAP infection or the onset of clinical disease is primarily anecdotal. Two experiments have indicated that glucocorticoids may influence MAP bacterial loads and the immune response to MAP in animals. Hamsters treated with dexamethsone, a glucocorticoid, exhibited higher bacterial counts in both the spleen
and mesenteric lymph nodes following exposure to MAP relative to hamsters that did not receive dexamethasone treatment. Secondly, decreased lymphocyte activity and delayed hypersensitivity reactions to Johnin and purified protein derivative of both *M. avium* and *M. bovis* were identified in 10 heifers suspected of MAP infection but not in three control heifers following administration of dexamethasone.

Recently, a study evaluating the role for a genetic predisposition for susceptibility to MAP infection identified intermediate heritability. This was similar to heritability estimates previously identified for other disease traits, both infectious and metabolic, in dairy cattle.

### 2.5.3.2 Herd-level

Multiple studies have addressed herd-level risk factors that are associated with MAP infection in dairy herds. Although the specific risk factors associated with infected herds varied, several herd characteristics have been repeatedly associated with infection.

As herd size increases, the risk of MAP infection in the herd also increases. Commercial dairy herds have also been identified to be at a higher risk for Johne’s disease infection than registered herds. Wells and Wagner also identified high percentages of cows born on other farms as a risk factor for infection. These observations indicate the importance of open herd status in the transmission of disease from farm to farm. Introduction of Johne’s disease into a herd is believed to be the result of the purchase of an infected animal.
Housing and management of young calves, especially exposure of young calves to manure from adult cattle, has also been identified as a risk factor for infection at the herd-level.\textsuperscript{73,74,90-93} Group housing for periparturient cows has been associated with increased risk of Johne’s disease within herds.\textsuperscript{90} Cleaning calving areas between each use has been associated with decreased risk of herd infection.\textsuperscript{92} These observations emphasize the importance of the calving environment in the transmission of MAP infection within a herd.

Manure management and hygienic practices are also associated with herd infection status for Johne’s disease. Goodger et al identified relationships between apparent prevalence of MAP infection in dairy herds and both environmental conditions and manure handling.\textsuperscript{93}

2.5.4 **Host Range**

Recent findings suggest that the host range of MAP is not necessarily limited to domestic ruminant species. Sporadic reports of natural MAP infections in wild ruminants such as deer, elk, bison, bighorn sheep, mountain goats, wild red deer, wild roe deer and fallow deer have been reported in the United States, Western Alps and the Czech Republic.\textsuperscript{94-99} More interestingly, natural MAP infections in non-ruminant wildlife species have recently been reported in Scotland. Natural MAP infections confirmed by histopathological examination and tissue culture have been identified in wild rabbits, foxes and stoats.\textsuperscript{100,101} Other wild species found to harbor MAP include the crow, rook, jackdaw, rat, wood mouse, hare and badger.\textsuperscript{102} Finally, MAP has been isolated from several *Diptera* species taken from contaminated environments.
including a cattle slaughter facility. These findings suggest that environmental contamination with MAP may be more widespread than originally thought, and may permit a wider range of exposures to the organism.

2.5.5 Economics

Based on economic information obtained as part of Dairy ’96, it is estimated that annual production losses associated with Johne’s disease for the United States dairy industry are between $200 and $250 million. In herds with greater than 10% of cull cows demonstrating clinical signs of Johne’s disease, losses were estimated to be over $200 per cow in the herd.6,57

Although several factors contribute to these economic losses, the primary determinants appear to be a result of reduced milk production and replacement of infected individuals.6,57,60 Milk production has been demonstrated to be lower in clinically and subclinically infected cows as well as in seropositive cows when compared to that of non-infected and seronegative cows, respectively.6,58-60,104 Nordlund et al identified a 3.95% decrease in milk production for ELISA-positive cows.58 In addition to decreased culling value of infected cows, cow mortality appears to be increased in Johne’s positive herds.6,57,60

2.6 Diagnostic Testing

2.6.1 Introduction

In addition to the implementation of management practices designed to limit transmission to susceptible animals, an important aspect of control of MAP infection is the identification and removal of infected animals from a herd. However,
identification of animals infected with MAP, especially those in subclinical stages of the infection, continues to present a diagnostic challenge despite nearly a century of development of diagnostic testing methods.

The diagnostic tests available for Johne’s disease either detect MAP itself or the host’s immune response to it. Tests based on organism detection are generally considered confirmatory tests. This is in contrast to tests that rely on the detection of host immune responses that indicate previous exposure of the host to MAP (or perhaps other bacteria with similar antigenic properties) rather than current infection.

Diagnosis in animals displaying clinical signs of Johne’s disease is easily accomplished with several of the available diagnostic tests. However, it is the apparently healthy, subclinically infected animals that represent the majority of infected animals in a herd. Screening these animals for MAP infection is fraught with difficulties using the tests that are currently available.

2.6.2 Diagnostic Tests based on MAP Detection

2.6.2.1 Direct Microscopic Exam

Microscopic examination of either feces or rectal scrapings is an inexpensive, rapid method for detection of MAP. A positive result requires that acid-fast bacilli be detected in clumps or within macrophages using Ziehl/Neelsen staining. This test method may be useful when fecal shedding of MAP is occurring at high levels or when intestinal lesions have advanced into the rectum. However, identification of acid-fast bacilli alone cannot confirm the diagnosis, and a negative result does not exclude the possibility of infection.
2.6.2.2 Histopathology and Immunohistochemistry

The combination of histopathology and culture of intestinal tissue and/or mesenteric lymph nodes is considered to be the most accurate diagnostic protocol for MAP infections, especially for early infections.\(^3\) Regardless of culture status, histopathologic examination serves as a confirmatory diagnostic test for Johne’s disease.

Multiple intestinal wall and mesenteric lymph node samples, including those from the terminal ileum and ileo-cecal lymph nodes, are examined for the presence of acid-fast bacilli and the pathognomonic lesions associated with the infection.\(^{105}\) Granulomatous inflammation of the lamina propria, Peyer’s patches and cortex of the mesenteric lymph nodes with large, pale epithelioid cells and multi-nucleated Langerhans’ giant cells is considered to be pathognomonic for MAP infection.\(^{105}\) Both haematoxylin-and-eosin staining and Ziehl/Neelsen staining are recommended to identify MAP bacilli.\(^{105}\) Although it is considered a definitive method of diagnosis, histopathology is not routinely used as a herd screening test because the nature of sampling (surgical biopsy) is impractical in a field setting where large numbers of animals require testing.

Although the development of immunohistochemical techniques to identify MAP in tissues has been reported, immunohistochemistry has not been widely incorporated into diagnostic testing for Johne’s disease.\(^{106,107}\) The lack of sensitivity and specificity, the nature of sample required and the lack of commercially available antibodies has limited its usefulness.
2.6.2.3 Culture

Bacteriological culture of MAP is considered the gold standard of diagnosis for infection. Although tissue culture, particularly ileo-cecal lymph node culture, is generally considered to be more sensitive, fecal culture is a more practical means of confirming infection in herd screening programs. However, the use of fecal cultures in screening programs has been criticized for poor sensitivity, high cost and long incubation time until culture results are available.¹⁰⁸

When compared to ileal or ileo-cecal lymph node tissue culture, the sensitivity of fecal culture is estimated to be 50% and the specificity greater than 99%.¹⁰⁸ Others consider the specificity of the fecal culture to be 100% since isolation of MAP from an animal is believed to confirm infection.¹⁰⁹,¹¹⁰

Thus, a single positive fecal culture can provide definitive proof of MAP infection. However, a single negative fecal culture does not definitively classify an animal as non-infected. Several characteristics of MAP and its resultant infection make false negative fecal culture results possible. The chronic nature of MAP infection results in a delay between the time of the initial infection and fecal shedding of the agent which may be intermittent or involve small numbers of MAP, particularly early in the course of the infection. The fastidious nature and potential for variations in growth requirements between MAP strains may preclude culture. The tendency for MAP to form clumps can make detection difficult when few organisms are present in a biological sample or only a portion of the sample is cultured.
The limit of detection by culture appears to be between 50-1000 MAP bacilli per gram of feces.\textsuperscript{110-112} However, differences between sample preparation techniques and media can influence the analytical sensitivity of the culture procedure.

In 1991, the USDA National Veterinary Service Laboratory (USDA-NVSL) proposed a standardized protocol for fecal culture to detect MAP.\textsuperscript{112} Adoption of common procedures for fecal culture of MAP was promoted in an effort to create uniformity across laboratories, facilitate comparisons between laboratories and define a reference for comparisons as new procedures are developed.\textsuperscript{112} The OIE has subsequently incorporated this procedure into its Manual of Standards for Diagnostic Tests and Vaccines.\textsuperscript{105}

Overgrowth of non-mycobacterial agents, especially fungal contaminants, can obscure cultural growth of MAP, making results inconclusive. Thus, decontamination of samples prior to culture is necessary. However, decontamination inevitably results in the loss of viability of a certain percentage of MAP present in the sample.\textsuperscript{2,111-113} Chemical decontaminants, including oxalic acid, sodium hydroxide, benzalkonium chloride, phenols and sodium hypochlorite, have been incorporated into culture procedures for MAP. The USDA-NVSL protocol suggests the use of 0.75% hexadecylpyridinium chloride (HPC) for 16-24 hours, indicating that HPC has the least detrimental effect on MAP growth relative to other decontaminants.\textsuperscript{112}

In addition to chemical decontamination, antimicrobial agents are often included in the culture media to further inhibit the growth of undesired bacteria and
fungi. The OIE manual promotes the addition of chloramphenicol, penicillin and amphotericin B to culture media, while only amphotericin B is included in the USDA-NVSL protocol.105,112

Because MAP may be present in low numbers, sample processing prior to culture typically includes a concentration procedure. Centrifugation of samples at various speeds has been documented to increase the analytical sensitivity of subsequent culture.114-116 However, a significant degree of contamination, often resulting in unreadable cultures, has been observed following centrifugation.114,115

Sedimentation, allowing diluted fecal samples to stand overnight so that MAP clumps settle out of solution, is the concentration technique utilized in the USDA-NVSL procedure. In addition to lower contamination rates relative to centrifugation, sedimentation is less labor intensive – an important consideration when a large number of samples require processing.

Recently, the use of polycarbonate filters to concentrate MAP prior to radiometric culture has been described.117 Immunomagnetic separation (IMS) has been used to concentrate MAP in milk samples prior to culture.29,118 Neither of these novel concentration methods has been incorporated in routine diagnostic culture procedures for fecal samples.

Several processing methods that incorporate specific combinations of decontamination and concentration procedures have been described. Stabel et al compared centrifugation, sedimentation, the Cornell method (a two-step decontamination process following centrifugation) and the NADC method (a double
centrifugation, double decontamination process). Results of this comparison suggest that the NADC method is 10 times more analytically sensitive and significantly reduced contamination relative to the other three methods. The Cornell method was the least sensitive of the methods evaluated.  

Mycobactin supplementation of culture media is a requirement for the growth of MAP. Mycobactin J, extracted from *M. paratuberculosis* Strain 18, is associated with a more rapid appearance of a greater number of MAP colonies than when media is supplemented with mycobactin P, extracted from *M. phlei*. Thus, mycobactin J is primarily used for fecal culture supplementation.  

Since the 1930’s, various culture media have been evaluated to facilitate the growth of MAP. Neilsen et al indicate that Herrold’s egg yolk media (HEYM) and modified Lowenstein-Jensen media, both supplemented with mycobactin J, are the preferred media of veterinary diagnostic laboratories. The OIE lists HEYM, modified Dubos’s media and Middlebrook 7H9, 7H10, and 7H11 media, all supplemented with mycobactin J, as suitable for the isolation of MAP. The USDA-NVSL standardized procedure advocates the use of HEYM.  

Although MAP growth can occur in as few as 6 to 8 weeks, samples are typically incubated at 37°C for 12 to 16 weeks. In order to demonstrate mycobactin dependency, three tubes containing HEYM with mycobactin J and one tube without mycobactin J are inoculated and cultured.  

The application of radiometric and non-radiometric automated culture systems, modified by the addition of egg yolk, mycobactin J and antimicrobials, appears to
improve the sensitivity of MAP detection and reduce the incubation time required to
diagnose MAP infection.\textsuperscript{23,111} These systems are widely used for the diagnosis of
tuberculosis and other mycobacterial infections in human medicine.

Radiometric culture systems use a liquid culture media containing a radio-
labeled carbon source.\textsuperscript{119} Bacteria metabolize this carbon source and the production of
\(^{14}\text{C}\)-labeled carbon dioxide is measured. Detection of MAP can occur between 5 to 8
weeks of culture, earlier than in traditional culture systems because carbon dioxide
production is evident prior to colony growth.\textsuperscript{23} However, carbon dioxide production is
not specific to MAP. Thus, positive results require confirmation, often incorporating
polymerase chain reaction (PCR) for IS900.

Several studies have evaluated the use of BACTEC, a commercially available
radiometric culture system, for MAP diagnostics.\textsuperscript{117,119-122} In addition to enhanced
sensitivity and more rapid growth, it appears that radiometric culture may facilitate the
culture of sheep strains of MAP.\textsuperscript{120} Despite obvious advantages of radiometric culture
systems in the detection of MAP, the costs of instrumentation and issues concerning
safety and disposal of radioactive substances have limited implementation of this
method.

Automated culture systems that use liquid media, but without radioactive
substances, have been evaluated more recently. These systems measure changes in
quantities of substrates (oxygen) or products (carbon dioxide) of bacterial metabolism
using colorimetric sensors.\textsuperscript{123} A recent review did not identify published evaluations
of these systems.\textsuperscript{111} However, the New York State Diagnostic Laboratory at Cornell
University replaced traditional HEYM culture with one of these automated, non-radiometric culture systems, the ESP Culture System II, in May 2001.\textsuperscript{124}

2.6.2.4 Application of IS900

The discovery of IS900, a DNA sequence believed to be specific for MAP, has the potential to revolutionize diagnostic testing for Johne’s disease. However, the incorporation of identification of IS900 into routine diagnostic testing is limited at the present time.

A commercially available DNA probe identifies specific DNA segments contained within the IS900 sequence in fecal samples. These tests utilize PCR to amplify the target DNA sequence, which is then detected by a gene probe. Although PCR and DNA probe technologies are generally considered to be exquisitely sensitive methods of organism detection, use of DNA probes to detect MAP in bovine feces has been disappointing.

Diagnostic sensitivity estimates for various DNA probes have ranged from 3\% to 23\%.\textsuperscript{125} The most significant factor contributing to the low sensitivity is the presence of PCR inhibitors in feces such as urea, hemoglobin or heparin.\textsuperscript{111} Secondly, processing methods may not facilitate adequate lysis of MAP cells, thus limiting the quantity of DNA available for detection.\textsuperscript{111} Finally, the lower limit for detection of MAP using DNA probes had been estimated to be between $10^2$ to $10^5$ organisms, higher than the detection limit for traditional fecal culture methods.\textsuperscript{111}

Specificity of the DNA probe is often considered to be 100\%; however, reported specificity estimates have been as low as 89\%.\textsuperscript{125} Several factors may explain
this apparently imperfect specificity of the DNA probe. False positive results may occur as a result of laboratory contamination of sample, identification of DNA from non-viable MAP organisms or the possibility that the target DNA sequence is not specific for MAP.\textsuperscript{111,125}

Despite these problems, the DNA probe for MAP has the advantage of being the most rapid method of organism detection available.\textsuperscript{108} Additionally, it appears that this technique may prove useful in sheep, where MAP is difficult to culture.\textsuperscript{5} In addition to concerns regarding sensitivity and specificity, the test has limited availability and is relatively expensive. The diagnostic utility of IS900 PCR for MAP detection would be greatly improved if techniques to overcome PCR inhibitors in fecal samples were to be developed. Perhaps this testing methodology will become a primary confirmatory test for MAP infection in the future.

Although direct application of DNA probes on clinical samples is not yet a widely adopted means of diagnostic testing for Johne’s disease, detection of IS900 DNA has become an important confirmatory step following MAP isolation using various culture methods. This is particularly true for broth culture systems that are not specific for MAP growth and do not permit assessment of colony morphology.\textsuperscript{111,119,120,122,126}
2.6.3 Diagnostic Tests based on Detection of Cell-Mediated Immune Response to MAP

2.6.3.1 Intradermal skin testing

Although intradermal skin testing has been successfully used as a screening test in bovine tuberculosis control and eradication programs in the United States, the detection of a delayed-type hypersensitivity response (a cell-mediated immune response) following the intradermal injection of MAP extract, johnin, has not proven to be a useful screening test for paratuberculosis. Briefly, skin measurements are taken 24-48 hours following the intradermal injection of johnin. Identification of skin thickness greater than 5mm represents a positive response. This test method has been criticized for both poor sensitivity and specificity. Results correlate poorly with the true infection status of an animal. Cross-reactivity with environmental mycobacteria and other related species further limits the usefulness of this method. Additionally, as cell-mediated immunity wanes late in the course of infection, intradermal skin tests typically become negative. Although international animal health requirements may necessitate the use of this test, it is not routinely used for the diagnosis or control of Johne’s disease.

2.6.3.2 Intravenous Johnin Test

This test identifies a systemic response to the intravenous injection of johnin. A positive test is defined as an increase in body temperature of 1.5°C or a shift in the neutrophil:lymphocyte ratio to >2:1 within 6 hours following the intravenous
administration of 2-4 mL of johnin. However, administration of the johnin may result in anaphylactic reactions or exacerbate clinical signs in infected cattle. This test is not routinely used for the Johne’s disease diagnosis.

2.6.3.3 Interferon-Gamma Testing (IFN-γ)

Interferon-Gamma testing, an \textit{in vitro} correlate to intradermal skin testing, identifies cell-mediated immune responses to MAP. Peripheral lymphocytes are stimulated with MAP antigens and subsequent IFN-γ production is measured using ELISA methodology. Increased levels of IFN-γ suggest previous exposure to MAP. In addition to stimulation with MAP antigens, lymphocytes are also stimulated with \textit{M. avium} purified protein derivative to determine if cross-reactivity with related mycobacterial species is occurring and a mitogen to verify that the lymphocytes are capable of responding to antigen.

Early work by Billman-Jacobe et al estimated the sensitivity of the IFN-γ assay to range from 71.8% in subclinical, nonshedders to 100% in clinical cases. Specificity was estimated to be 97.6%. More recent work by Stabel et al monitored fecal culture and ELISA results relative to IFN-γ results in several dairy herds with low prevalence of MAP infection. Accuracy of the IFN-γ ranged from 50% to 75% at the cow-level. High specificity was assumed when blood lymphocytes from animals in a herd considered free of the infection failed to respond to MAP antigen stimulation. Application of this test in animals less than 25 months of age appears to be limited.
Although this test is commercially available, it is not widely adopted for Johne’s disease testing. Lack of specificity of the IFN-γ assay remains a primary concern. Additionally, cost is a prohibitive factor.

2.6.4 Diagnostic Tests based on Detection of Humoral Immune Response to MAP

2.6.4.1 Fluorescent Antibody Test (FA)

Both direct fluorescent antibody testing to identify MAP antigen in feces and indirect fluorescent antibody testing to identify anti-MAP antibodies have been described. In a study comparing the indirect fluorescent antibody test to the complement fixation test, a significant difference between the performance of the two tests was not identified. Given its poor sensitivity and specificity, this test is not widely used.

2.6.4.2 Complement Fixation Test (CFT)

The complement fixation test identifies the presence of complement-fixing antibodies directed against MAP in serum. Reported sensitivity and specificity estimates vary, and direct comparison between reports is difficult because of variations in study design and the cutoff values used to define positive results in these studies. Overall sensitivity of the CFT ranged from 10.8% to 64.8% and specificity ranged from 87.2% to 99.0%. Cross-reactivity with other mycobacteria, as well as with cornyebacteria, is a frequent problem. When compared to other serologic tests, CFT has been consistently demonstrated to be less sensitive than the ELISA, but more sensitive than the AGID. The CFT appears to detect antibodies that are
produced later in the course of infection than those detected by the ELISA. The specificity of the CFT was generally similar to the other two tests.

Although the CFT has been criticized for its poor sensitivity and specificity relative to other available serologic tests, it still serves as the official test required for export to certain countries. The most appropriate field application of this test may be for confirmation of infection in a clinically affected animal.

2.6.4.2 Agar Gel Immunodiffusion Test (AGID)

The AGID was one of the first serological tests developed for Johne’s disease diagnosis. Although this test has become an important diagnostic tool in clinically ill ruminants, it is generally considered to lack the sensitivity required for a screening test.

Estimates of sensitivity vary, but reports have ranged from 5% to 27.8% in cattle. Specificity of the AGID is considered to be 100%, the most specific of all serological tests for Johne’s disease, although reported estimates have been as low as 96.8%. Sherman et al documented a positive correlation between level of fecal shedding of MAP and AGID results.

The AGID appears to be an important diagnostic tool in sheep where strain variations in MAP prohibit bacteriologic culture. Several studies have demonstrated high sensitivity of the AGID in sheep, although sensitivity appears to be improved for the multibacillary form of the disease relative to the paucibacillary form. Specificity of the AGID also appears to be high when used in sheep, however, cross-reactions with Corynebacterium pseudotuberculosis are of concern.
2.6.4.3 Enzyme-Linked Immunoabsorbent Assay (ELISA)

The ELISA is considered to be the most suitable serologic test for use as a screening test in subclinically infected animals.\textsuperscript{131,137} The ELISA detects anti-MAP antibodies, specifically IgG isotypes, in serum.

Several studies have reported sensitivity and specificity values for an absorbed ELISA similar to the test commercially manufactured in the United States.\textsuperscript{131,136,137} Sweeney et al report the sensitivity and specificity values for the absorbed ELISA to be $45\% \pm 4.8\%$ and $99\% \pm 0.9\%$, respectively.\textsuperscript{142} Several technical changes have occurred with the kit since the time of that evaluation. Dargatz et al estimated the overall sensitivity and specificity of the revised ELISA kit to be $50.0\%$ (95\% CI = 45.9-54.1\%) and $96.8\%$ (95\% CI = 95.3-98\%), respectively.\textsuperscript{143} However, ELISA sensitivity was as low as 15\% in subclinical animals that were shedding low numbers of MAP (1-10 colonies per fecal culture tube) and as high as 88\% in fecal culture positive cows with clinical signs of Johne’s disease.\textsuperscript{143} Specificity estimates ranged from 82.6\% in cows with clinical signs of Johne’s disease to 100\% in cows from a herd without history of Johne’s disease, bulls from a commercial bull stud and cows from a closed, uninfected herd.\textsuperscript{143} Because over 1000 of the samples evaluated had also been tested by Sweeney et al, a direct comparison between test results yielded a kappa value of 0.815, indicating a high level of agreement between the two versions of the ELISA.\textsuperscript{143}

Recently, additional applications of the ELISA methodology for detection of antibodies to MAP have occurred. A cow-side test incorporating ELISA
methodology, the Tip-Test®️, has become commercially available for Johne’s disease testing. Additionally, ELISA testing of milk samples has received considerable attention. However, milk ELISA testing appears to be less sensitive and less specific than serum ELISA. Correlation with serum ELISA results was poor in one study, but comparable in another. Further evaluation of these applications of the ELISA is necessary.

2.6.5 Selection of Diagnostic Tests

Given the variety of diagnostic tests available and the limitations that exist for each of these testing methods, choice of a diagnostic test to detect MAP infection is influenced by numerous factors. One of the more important considerations is the purpose for testing. Situations that may necessitate Johne’s disease testing include: confirmation of diagnosis in a single animal with clinical signs, confirmation of a positive serological test result, estimation of the prevalence in a herd, participation in a control program or formal herd certification program, pre-purchase testing, screening young stock, or export testing. Some of these situations, such as export testing or participation in a formal herd certification program, have pre-defined testing requirements. Others permit greater flexibility in test selection. Additionally, each situation demands varying levels of confidence in either positive and/or negative test results that require high levels of diagnostic specificity, sensitivity, or both.
In addition to the purpose for testing and the level of sensitivity and/or specificity it requires, other factors are considered during test selection. A significant factor influencing test selection is the cost of a herd testing program since multiple animals will be tested repeatedly over time, possibly requiring a sizeable financial investment on the part of the herd owner. In addition to cost, test availability, sample requirements and time until test results are available are also considered.

2.6.6 Applications of ELISA and Fecal Culture for Herd Screening

At the herd-level, Johne’s disease testing programs can be used to estimate the prevalence of MAP infection within the herd or to provide evidence that a herd is unlikely to be infected with MAP. Simultaneously, these test results are also interpreted at the individual-cow level in order to identify infected cows for removal from the herd. Herd screening requires a test with high sensitivity to identify infected individuals within the herd. False negative results impede control efforts and may facilitate the spread of infection to other herds. However, a certain level of specificity is also needed given that there are also costs associated with false positive results -- an individual cow may be unnecessarily culled or a herd may be misclassified as infected.

To minimize the consequences of incorrect test results, herd testing programs generally incorporate a combination of both fecal culture and ELISA testing. A common testing strategy is serial testing. Because it provides an inexpensive, rapidly available test result, the ELISA is used to identify test-positive animals that are then re-tested with a fecal culture to confirm infection. Animals that were positive for both tests are considered infected. This strategy improves the overall specificity of testing.
Because of the imperfect sensitivity of both tests, there will be a proportion of infected animals that are not identified. For these reasons, repeated herd testing at a minimum of yearly intervals is recommended.\textsuperscript{1,108,109}

Recently, the combined use of ELISA and fecal culture for herd testing has been evaluated. Together, these studies suggest that, although the ELISA is a sensitive screening test at the herd-level, the possibility of incorrectly classifying non-infected herds as infected remains a problem, especially when results from the ELISA are not confirmed by fecal culture. Additionally, when test results are interpreted at the individual cow-level, fecal culture and ELISA results do not consistently identify the same individuals.\textsuperscript{148-150}

Whitlock et al analyzed ELISA and fecal culture results from 10 MAP-infected dairy herds that were tested at 6-month intervals over a four-year period. Fecal culture identified nearly twice as many test-positive cows as the ELISA during the initial herd test. The estimated sensitivity of the ELISA decreased over the observation period. The authors attribute this change to the culling of animals in late stages of infection early in the observation period, leaving a significant proportion of animals in early stages of infection with each subsequent herd test making infected individuals more difficult to detect.\textsuperscript{150}

Stabel et al observed that only 37 of 61 herds with one or more ELISA-positive cows also identified fecal culture-positive individuals. Even in herds with three or more ELISA-positive cows, only 79% were identified as infected by fecal culture. At the individual cow-level, only 25% of fecal culture-positive cows were also identified
as ELISA-positive. Similarly, 22% of positive ELISA results were confirmed by fecal culture in this study. In fecal culture negative cows, 6% had positive ELISA results.148

Wells et al estimated the herd-level sensitivity of the herd testing strategy specified by the US Voluntary Johne’s Disease Herd Status Program for Cattle. This study concludes that, at the herd-level, relying on ELISA results alone will improve sensitivity, but misclassify a high percentage of uninfected herds.149

Given the expense and the long incubation time until fecal culture results are available, many producers prefer to rely on the ELISA for herd testing. Although the ELISA can provide useful information concerning infection status at the herd-level, interpretation of results at the individual cow-level is complicated.

Several researchers advocate the quantitative evaluation of ELISA optical density (OD) values or sample-to-positive (S/P) ratios, rather than relying solely on positive or negative classifications defined by a single cut-off value.79,108,137,151 Early reports demonstrated relatively consistent increases in likelihood ratios as optical density and S/P ratios increased relative to both concurrent fecal culture result and known infection status.137,152

Recently, likelihood ratios were published in conjunction with management recommendations providing an algorithm to assist in the interpretation of ELISA S/P ratios.153 However, these likelihood ratios were calculated using sensitivity and specificity estimates rather than calculating likelihood ratios individually from multiple ranges of S/P values, excluded S/P ratios with negative values from
calculations and used cattle from non-infected herds in The Netherlands as the non-infected population.\textsuperscript{153} Thus, these estimates overestimate the risk of MAP infection relative to specific ELISA S/P ratios, and the study population may not adequately represent MAP-infected herds in the United States.

Additionally, the interpretation of ELISA results is further complicated when animals are repeatedly tested over time, as occurs in a Johne’s disease testing program. Hirst et al observed that nearly 40\% of dairy cattle identified as ELISA positive were subsequently negative with repeated ELISA testing, irrespective of the length of time between the tests. In this study, cows with ELISA S/P ratios > 0.700 were more likely to maintain their positive status on subsequent testing than cows with S/P ratios >0.25 or >0.40.\textsuperscript{151} Further, coefficients of variation greater than 20\% have been reported for ELISA S/P ratios when individual sera samples were tested multiple times, often resulting in different classification (positive/negative) of the test result.\textsuperscript{154} These studies demonstrate additional factors to consider when multiple ELISA results are interpreted as part of an individual animal’s test history. In both studies, the authors support the development of a method that allows for the quantitative evaluation of S/P ratio as opposed to relying on a single cut-off value to classify test results, especially when considering the potential for variation in ELISA S/P ratios.\textsuperscript{151,154}

\textbf{2.7 On-Farm Control Measures}

Ideally, dairy herds should strive to prevent the introduction of Johne’s disease into their herds by maintaining a closed herd status or restricting purchases of animals to herds that have documented their non-infected status. Control of MAP at the herd
level is often a long-term proposition requiring five or more years to achieve success.\textsuperscript{155} Eradication of Johne’s disease at the herd-level may be impractical at this time given the shortcomings of the diagnostic tests that are currently available.

2.7.1 Development of Control Programs

Johne’s disease control programs incorporate knowledge about the contributions of the agent, host and environment to the epidemiology of Johne’s disease in order to develop risk-based strategies that prevent infection at both the individual animal and herd-levels.\textsuperscript{25} Kennedy et al have identified five key objectives for on-farm Johne’s disease control programs: 1) to reduce the economic impact of the infection in the herd, 2) to protect non-infected individuals or herds, 3) to reduce excretion of the bacteria into the environment, 4) to disrupt the chain of transmission to young cattle and 5) to reduce the contamination of raw milk with MAP.\textsuperscript{25} Many of the published approaches to Johne’s disease control address these objectives.\textsuperscript{79,155,156}

Several factors impede the implementation and subsequent progress of Johne’s disease control.\textsuperscript{25,79} The chronic, insidious nature of the infection paired with limitations of available diagnostic tests make detection of subclinically infected animals difficult, thus maintaining a source of infection and environmental contamination in the herd. Additionally, significant gaps in the current scientific knowledge about the epidemiology and economic consequences of this infection require implementation of control measures that may be based on assumptions rather than evidence-based. In addition to possible regulatory consequences, there is a
negative stigma associated with Johne’s disease within the dairy industry that may interfere with participation in organized control programs.79

The first critical step in developing an individualized control program for Johne’s disease is an on-farm risk assessment. The purposes of the risk assessment are to systematically categorize the likelihood of MAP infection, to identify management practices that potentially contribute to transmission of MAP on a farm and to prioritize necessary management changes. In addition, the owner formulates his/her goals with respect to Johne’s disease control. Risk assessments can also incorporate a discussion of the financial burden associated with proposed management changes as well as establishing the likely outcomes and the time-frame required to achieve the producer’s goals.79,155,156

2.7.2 Recommended Management Practices

Although control programs are designed to focus on the needs of individual farms, three key management areas are commonly addressed. These include calving and calf rearing, manure management and environmental sanitation and herd testing strategies.25,79,155

Because calves less than six months of age are believed to be at the greatest risk of becoming infected with MAP, considerable effort to limit their exposure to MAP occurs as part of Johne’s disease control programs. Specific management practices recommended to reduce the risk of transmission to young stock include: use of individual calving pens cleaned between each calf, prompt removal of calf from dam (within 6 to 12 hours of birth), avoid allowing calves to nurse from dam, clean
udder and teats prior to colostrum collection, use colostrum from Johne’s-negative cows only, avoid feeding pooled colostrum or milk to calves, use milk replacer rather than raw milk, limit physical contact of growing calves to manure from adult cattle, have dedicated young stock housing and pastures that prevent contact with adult cattle or their manure, avoid contamination of feed and water supplies for young stock with manure from adult cattle and consider culling the most recent offspring from known-infected cattle.25,79,155,156

Environmental sanitation is also a major consideration for control programs. Environmental contamination with MAP can be significant and long-lasting on infected farms.79 Many of the recommendations regarding manure management focus on limiting exposure of young stock to manure from adult cattle, as previously discussed. Additional recommendations include: regular removal of manure from animal housing areas, use of separate equipment for manure removal and feeding purposes, limit runoff from contaminated lots, use of footbaths, cleaning of surfaces with phenolic-based disinfectants following removal of manure and avoidance of spreading manure on hayfields or pastures.25,79,155,156

Finally, removal of infected animals immediately reduces within herd prevalence of the infection, and is considered to produce the quickest, most effective impact on Johne’s disease control.79 Whole-herd testing of all animals two-years-of-age or older is recommended on a yearly basis, although very aggressive producers or producers faced with a high prevalence of infection may elect to repeat testing every six-months early in the control program.79,156 By removing infected animals, the
source of environmental contamination is eliminated, thus limiting exposures of susceptible animals to the organism.\textsuperscript{79} An economic decision analysis model by Collins and Morgan indicated that test-and-cull programs were economically justified when herd prevalence exceeded 5\%.\textsuperscript{157} In herds where high prevalence prohibits immediate culling of all infected cattle, segregation of infected cattle from the remaining herd has been suggested to limit environmental contamination and prevent potential exposures of susceptible animals to MAP.\textsuperscript{79}

Few studies have addressed the adoption of recommended management practices on dairies following education about Johne’s disease. However, it appears that there is a considerable gap between the dissemination of recommendations to control Johne’s disease and the implementation of these recommendations on farms. A mail survey completed in Victoria, Australia, examined compliance with six recommendations aimed at Johne’s disease control on 800 randomly selected dairy farms.\textsuperscript{158} Results indicate that the majority of respondent herds complied with zero or only one of these six recommendations despite extensive educational efforts by the government. Herd compliance with three or more of these recommendations was significantly higher in herds with a previous diagnosis of Johne’s disease than those without a previous diagnosis. Additionally, one component of the USDA-APHIS NAHMS Dairy ’96 study assessed whether familiarity of a dairy farm manager with Johne’s disease or prior diagnosis of Johne’s disease in a herd influenced management practices in that herd.\textsuperscript{90} This study suggested that few preventive management practices were associated with familiarity or prior diagnosis of Johne’s disease in a
given herd. Both of these studies emphasize the need for evaluation of existing educational efforts aimed at Johne’s disease control in order to identify factors that impede adoption of recommended management practices.

2.7.3 Role of Vaccination in Control

The incorporation of vaccination as part of Johne’s control programs is not widely recommended in the United States, although it can be a viable option in herds with significant clinical disease and economic loss. In the United States, a single killed vaccine is approved for use in dairy cattle.\textsuperscript{79} Vaccine use often requires permission from state veterinary regulatory agencies and/or administration by a licensed, accredited veterinarian. The vaccine is typically administered to calves prior to six weeks of age. Use of this vaccine appears to limit the development of clinical signs and reduce fecal excretion of the organism.\textsuperscript{1,79} However, vaccination does not provide protective immunity, and vaccinated animals can become infected.

Vaccination complicates the interpretation of the diagnostic tests currently used in control programs for Johne’s disease. Vaccination may result in false positive results on serologic tests for Johne’s disease, and the reduction in fecal shedding may result in false negative fecal culture results. Other disadvantages include potentially severe granulomatous reactions at injection sites and severe tissue site reactions in veterinarians accidentally injected with the vaccine. Current research efforts in the United States are directed toward the development of an improved vaccine for MAP.\textsuperscript{1}

Another significant concern associated with the use of a vaccine to control Johne’s disease is the misconception that the vaccine is a “silver bullet”, eliminating
the need for appropriate management practices to control the infection. Kalis et al compared four preventive management practices in The Netherlands between 25 dairy herds that vaccinated for MAP and 29 herds that did not vaccinate. Herds that did not vaccinate against MAP were more likely to have individual calving areas and remove calves from their dam immediately following birth, feed colostrum only from the calf’s dam, avoid feeding raw milk after the colostrum period and use separate housing and pastures for young stock and adult cattle. Although only 5 (17%) of nonvaccinated herds followed all four recommendations, none of the vaccinated herds practiced all four recommendations. This provides evidence that producers who vaccinate fail to adopt the management practices recommended for control of Johne’s disease.

2.8 Regulatory Issues

2.8.1 United States

Federal regulations specifically regarding Johne’s disease restrict the interstate movement of known-infected animals. Effective as of May 2000, revisions to parts 71 and 80 of the Code of Federal Regulations (CFR) restricted the interstate movement of MAP-infected animals to recognized slaughter establishments. This rule amended a previous version that failed to adequately prevent transport of infected animals.

At the state level, regulations concerning Johne’s disease vary. In some states, Johne’s disease is reportable to state animal health officials while in others it is not. Some states restrict intrastate movement of infected animals, while these restrictions do not exist in others. Civil liability may exist when producers knowingly sell an
infected animal for purposes other than slaughter. To clarify the responsibilities of sellers, Wisconsin has enacted an implied warranty law that stipulates cattle to be sold are guaranteed to be free of MAP-infection unless sellers provide a written retraction of this guarantee at the time of sale.\textsuperscript{161}

One regulatory issue that is not clearly defined is the liability of veterinarians signing health certificates for individual animals or groups of animals originating from a herd of unknown Johne’s disease status or from a herd known to be infected. Health certificates often include a statement regarding freedom of designated animals from infectious disease. Because animals originating from infected herds may be subclinically infected, veterinarians may not be able to verify freedom from infectious disease. Disclaimers added to the health certificate regarding a herd’s Johne’s status have been suggested as one possible alternative to address this problem.\textsuperscript{161}

In the United States, Johne’s disease testing and control programs operate primarily at the state-level. Two basic types of programs exist. Control programs focus on reducing prevalence and limiting transmission in infected herds. Certification or status programs rely on repeated herd testing to validate that a herd is unlikely to be infected with Johne’s disease. According to the USDA-APHIS, 20 states had Johne’s disease control programs and 25 had herd certification/status programs as of January 1, 2002. Additionally, four states reported that control programs were in development, and three were developing certification/status programs.\textsuperscript{162}
In an effort to unify and standardize Johne’s disease control programs at the state-level, the National Johne’s Working Group of the United States Animal Health Association (USAHA) appointed a subcommittee to design an affordable, scientifically-based herd certification program to serve as a model for individual states developing and implementing certification programs. The resulting program was adopted by the USAHA in October 1998, and was subsequently named the US Voluntary Johne’s Disease Herd Status Program for Cattle by USDA-APHIS.163

The testing scheme for the program combines ELISA testing of 30 second or higher lactation cows, ELISA testing of a defined statistical subset of second or higher lactation cows and fecal culture of a defined statistical subset of second or higher lactation cows over time to allow herds to progress through four levels of certification, each with increasing confidence that the herd is truly non-infected. At Level 1, there is a 70% probability that a herd is truly non-infected, while at Level 4 a 99% probability of being truly non-infected is reported. Herds that do not wish to progress to the next certification level may elect annual ELISA testing of 30 second or higher lactation cows to maintain their current status.163

Wells et al evaluated the herd-level sensitivity of the testing strategy required to achieve Level 1 status -- ELISA testing 30 randomly selected second or higher lactation cows followed with confirmatory fecal culture on ELISA positive individuals. With this approach, herd-level sensitivity ranged from 33% to 84%. Sensitivity improved in herds with greater than 5% of fecal culture-positive cows. If fecal cultures were not used to confirm positive ELISA results, herd-level sensitivity
ranged from 70% to 93%, but the probability of misclassifying uninfected herds as infected was 89%. The authors concluded that the testing strategy used in the first level of the program will fail to identify most infected herds with low prevalence of infection, and, although relying on ELISA results alone without fecal culture will improve the herd sensitivity, a high percentage of uninfected herds will be misclassified.\textsuperscript{149}

Wells et al only evaluated the sensitivity of the first tier of testing within the program. In order to obtain higher levels of herd certification, herds are required to test a “statistical subset” of cows. Herds with fewer than 300 cows are required to test all second or higher lactation cows, and larger herds are required to test a minimum of 300 cows in subsequent testing, with the actual number tested determined by herd size.\textsuperscript{163} Although subsequent levels of testing are believed to improve the ability of the program to detect infected herds, the herd-level sensitivity of each progressive level has not been evaluated. Additionally, herd owners may elect to remain in Level 1 indefinitely, rather than progressing to the next level. It is conceivable that infected herds may remain at Level 1 for several years, possibly marketing based on their non-infected status.

In April 2002, USDA-APHIS published the Uniform Program Standards for the Voluntary Bovine Johne’s Disease Control Program.\textsuperscript{164} The purpose of the document is to “provide minimum national standards for the control of Johne’s disease.” The program incorporates the testing strategy set forth within the US Voluntary Johne’s Disease Herd Status Program as well as addressing education and
management requirements for herds that choose to participate. Administrative requirements for personnel, Johne’s-certified veterinarians and an advisory committee are established at the state-level. Additionally, performance standards for approval of diagnostic laboratories providing testing for the programs are identified. Fecal and/or tissue culture, DNA probe for IS900 and tissue histology are specified as official Johne’s disease diagnostic tests, and the USDA-approved ELISA is identified as a screening test.¹⁶⁴

Participation in the Voluntary Bovine Johne’s Disease Control Program is at the discretion of individual producers. However, several factors may necessitate a mandatory program in the future. There is speculation that trade barriers may soon prohibit international movement of products from MAP-infected animals.¹⁶⁵ Concerns regarding the zoonotic potential of MAP and apparent inability of pasteurization procedures to completely eliminate the bacterium from milk also place pressure on the dairy industry to control this disease.

2.8.2 International Perspective

In addition to the United States, several other countries have initiated voluntary Johne’s disease control programs for cattle at both regional and national levels. Programs in Norway and Sweden focus primarily on eradication and disease prevention since the prevalence in these countries is believed to be low. A government supported voluntary certification program in The Netherlands has achieved considerable industry support. Denmark and France have implemented industry funded regional control programs. In Japan, an aggressive regional test-and-
cull program providing financial compensation for test positive animals is being expanded. In New Zealand, the government determined that a national control program for paratuberculosis was not economically feasible, however, vaccination is available by permit.\textsuperscript{1,25,165}

Australia has implemented the National Johne’s Disease Market Assurance Program for Cattle, a voluntary program to assess and certify herds as test-negative.\textsuperscript{63} By early 2000, over 1000 cattle herds have been certified as “monitored negative” status.\textsuperscript{25} This program has been expanded to include the sheep, alpaca and goat industries. Successful implementation of this program in addition to zoning and transport restrictions have enabled Queensland, the Northern Territory and Western Australia to be recognized as disease-free. In regions considered infected with Johne’s disease, there is considerable industry participation in government-sponsored testing and control programs.

It appears that a voluntary control program similar to those in the United States and Australia is on the horizon in the United Kingdom. The Department for Environmental, Food, and Rural Affairs (formerly Ministry of Food and Fisheries) in the United Kingdom issued a draft “Guidance Document for Johne’s Disease Control in Dairy Cattle” for public comment.\textsuperscript{166} The SAC Veterinary Division has also completed a report entitled, “Assessment of Surveillance and Control of Johne’s Disease in Farm Animals in Great Britain” that recommends strategies for a national control program.\textsuperscript{167}
2.9 The Crohn’s Disease Controversy

The possibility that MAP may be a zoonotic pathogen has generated considerable speculation by the scientific community for nearly a century. The 1913 report by Dalziel, “Chronic Interstitial Enteritis”, is considered to have initiated the debate concerning the zoonotic potential of MAP.\textsuperscript{168-170} Dalziel observed that the intestinal lesions in these patients were grossly and histologically similar to those of pseudoparatuberculosis (which later became known as paratuberculosis or Johne’s disease) and suggested a common etiology.\textsuperscript{171}

In 1932, a landmark paper by Crohn, Ginzberg and Oppenheimer described the clinical and pathologic findings associated with regional ileitis in fourteen patients and convincingly identified regional ileitis as a unique disease entity.\textsuperscript{172} The condition they described has become known as Crohn’s disease, although earlier cases, including those described by Dalziel, were reported in the medical literature.\textsuperscript{168} Importantly, Crohn et al indicated that the etiology of this disease was unknown and failed to speculate as to potential causes.

Despite the considerable body of scientific knowledge regarding Crohn’s disease that has accumulated since its first description, its etiology remains a mystery. Today, Crohn’s disease is believed to be multifactorial in etiology with environmental exposures, genetic predisposition and immune dysfunction all contributing to the development of disease.

Crohn’s disease is a form of chronic inflammatory bowel disease. The pathologic changes associated with Crohn’s disease are characterized by a chronic,
segmental inflammation that most commonly occurs in the distal ileum or proximal colon, although lesions can occur at any location throughout the gastrointestinal tract. Lesions typically begin as focal, superficial ulcers of the lymphoid follicles at Peyer's Patches and progress to transmural lymphocytic lymphangitis. Non-caseating granulomas are the pathological hallmark of this disease, although these pathognomonic lesions are only present in approximately 50% of Crohn’s disease patients.\textsuperscript{168,173} Periods of active inflammation are intermittent, but recurrent.

The clinical signs associated with Crohn’s disease are a consequence of the location within the gastrointestinal tract at which inflammation occurs. Abdominal pain, diarrhea, vomiting and weight loss coincide with periods of active inflammation, and, thus, may be intermittent. Extra-intestinal manifestations of Crohn’s disease that may result in clinical signs include fever, arthralgia and/or arthritis, mucocutaneous lesions, ophthalmologic lesions, hepatobiliary disease, renal disease and bone abnormalities. The associated clinical signs may either precede gastrointestinal symptoms or occur simultaneously with them. Additionally, delayed growth and sexual maturation may be important clinical consequences of Crohn’s disease in children.\textsuperscript{174}

The diagnosis of Crohn’s disease is primarily one of exclusion and is typically based on a combination of clinical signs and observations made during physical examinations, laboratory tests, endoscopic examinations and radiographic evaluations.\textsuperscript{174} Standard diagnostic criteria have not been universally adopted.\textsuperscript{175} A
diagnosis of Crohn’s disease has been suggested to represent several different disease entities rather than a single unique disease.\(^{168}\)

The highest incidence rates of Crohn’s disease have been observed in the United States, the United Kingdom and Scandinavia while it is seldom diagnosed in Africa, Asia or South America.\(^{168,174,175}\) Worldwide, the incidence of Crohn’s disease has increased from 1960 to 1987, although incidence rates appear to have stabilized at the present.\(^{175}\)

The most recent population-based study to estimate the burden of disease in the United States was conducted in Olmsted County, Minnesota.\(^{176}\) As of January, 1, 1991, the age and sex-adjusted prevalence of Crohn’s Disease in Olmsted County was 144.1 per 100,000 and the adjusted incidence rate for the period from 1984 to 1993 was 6.9 per 100,000 person-years. Another survey estimates that 0.15% of the American population, approximately 500,000 individuals, have been diagnosed with Crohn’s disease.\(^{177}\) However, this estimate originated from a telephone survey that relied on self-reporting to identify cases. In 1999, the Crohn’s and Colitis Foundation of America estimated that over 300,000 Americans are living with Crohn’s disease.\(^{178}\)

Classically, Crohn’s disease is considered to have a bimodal age distribution with peaks in incidence at ages 15-25 and 55-60, although a trend toward initial diagnosis in patients older than 60 has recently been recognized.\(^{175,179}\) The incidence of Crohn’s disease has been observed to be higher in women than men.\(^{175,180}\) Certain racial and ethnic groups are considered to have higher than normal incidence of Crohn’s disease. Jews, particularly Ashkenazi Jews, have incidence rates that are
several times higher than the general population. Caucasians are believed to be at higher risk for the development of Crohn’s disease than African-Americans, Hispanics and Asians.\textsuperscript{179,180} Several studies have observed higher incidence rates for inflammatory bowel disease in urban communities and in individuals of higher socioeconomic status.\textsuperscript{175}

In 1984, Chiodini et al published a series of reports that is credited with initiating the present-day controversy regarding the role of mycobacteria, specifically MAP, in the etiology of Crohn’s disease.\textsuperscript{181-184} The initial report described the isolation of an unclassified \textit{Mycobacterium} sp. from three patients with Crohn’s disease. Based on mycobactin dependency and biochemical and cultural characteristics, the authors suggested that the organisms may represent either a biovariant or subspecies of MAP or a new species altogether.\textsuperscript{183} A pygmy goat kid given milk inoculated with the organism developed granulomatous disease of the small intestine by the end of a five-month observation period, and the same \textit{Mycobacterium} sp was re-isolated from a mesenteric lymph node.\textsuperscript{184} The ability to induce similar pathology in a ruminant model provided critical evidence supporting causation. Subsequent analysis of restriction fragment length polymorphisms (RFLP) could not distinguish between the three isolates and definitively identified all three as MAP.\textsuperscript{181} The Chiodini group also isolated cell-wall deficient forms of mycobacteria, spheroplasts, from twelve Crohn’s disease patients that were successfully transformed into the classical bacillary form and identified as MAP using RFLP analysis.\textsuperscript{182}
Simultaneously with the isolation of MAP from Crohn’s disease patients, a report describing an outbreak of chronic diarrhea and progressive weight loss in a research colony of stump-tailed macaques was published.\(^{185}\) This report was the first to document natural paratuberculosis in sub-human primates and provided further evidence that MAP may be a zoonotic pathogen.

Initiated by these early reports, the quest to establish a causative role for MAP in the development of Crohn’s disease has incorporated microbiological, immunohistochemical, serological and molecular methodologies. Regardless of the methodology used, results have been inconsistent and, to date, are not considered to definitively establish that MAP plays a causative role in Crohn’s disease. In fact, recent reports from governmental agencies in the United States, the United Kingdom, Ireland and the European Commission have all concluded that existing evidence is insufficient to either prove or disprove that MAP is the cause of Crohn’s disease and all recommend continued scientific research.\(^{186-189}\)

Several reports describing both successful and unsuccessful attempts to culture MAP from Crohn’s disease patients have been published in the scientific literature.\(^{190-193}\) A 1992 review indicates that, as of that time, only 10 isolates of MAP had been obtained from cultures of 180 Crohn’s patients.\(^{194}\) Although several reports have described the successful culture of MAP from Crohn’s disease patients, the isolation of this organism from control patients suggests that factors other than or in addition to MAP exposure contribute to the development of Crohn’s disease.
Studies utilizing immunohistochemistry techniques have generally failed to identify MAP in tissues from Crohn’s disease patients.\textsuperscript{195,196} As with attempts to culture the organism, the lack of sensitivity of these techniques paired with the small numbers of organisms and the existence of cell-wall deficient forms greatly limit the opportunity for detection.

Studies of the humoral immune responses of Crohn’s disease patients to MAP have also failed to consistently support a causal relationship. Early studies evaluating these immune responses suffered from cross-reactivity with other \textit{Mycobacterium} sp. that are ubiquitous in the environment.\textsuperscript{168} Recent attempts have focused on the identification of antibody responses directed against antigens believed to be specific to MAP.\textsuperscript{17,197,198} While the use of specific antigens may limit cross-reactivity to environmental mycobacteria, the value of humoral immune responses as evidence to either support or refute a relationship between MAP and Crohn’s disease is questionable. Diagnostic tests that utilize antibody responses to predict an animal’s infection status have limited usefulness in the diagnosis of Johne’s disease in ruminants given the intracellular nature of the infection, the predominant cell-mediated immune response and the highly variable antibody response between individuals.\textsuperscript{168} Thus, serologic evidence of exposure to MAP in Crohn’s disease patients is not particularly useful to determine causation.

Given the difficulties associated with previous attempts to identify MAP in tissues of Crohn’s disease patients, the advent of molecular techniques was initially regarded as a means to finally resolve this controversy. Following the identification of
a DNA segment believed to be specific for MAP, IS900, a barrage of investigations used PCR to amplify this sequence in attempts to detect MAP in full thickness intestinal samples, biopsy samples and tissue cultures from Crohn’s disease patients.

Using PCR for IS900 in full thickness intestinal samples, Sanderson et al identified MAP in samples from 26 of 40 (65%) Crohn’s disease patients, 1 of 23 (4.3%) ulcerative colitis patients and 5 of 40 (12.5%) of control patients. Several subsequent studies were also interpreted as supportive evidence for a causative role of MAP in the development of Crohn’s disease. Fidler et al provided the most conclusive evidence supporting a causal relationship. In a double blind trial that matched tissues from Crohn’s disease patients, ulcerative colitis patients and control patients relative to age, sex and tissue sample location, 4 of 31 samples from Crohn’s disease patients were IS900 positive while no samples from either ulcerative colitis patients (n=10) or control patients (n=20) amplified the sequence.

Interestingly, following Fidler’s report the majority of subsequent studies evaluating the presence of IS900 in tissues of Crohn’s disease patients failed to support a role for MAP in the causation of Crohn’s disease. Most of these studies failed to identify IS900 in any of the samples tested, although one reported uniformly high detection rates across all study groups. Finally, two studies utilizing IS900 for in situ DNA hybridization have both supported a causative association between MAP and Crohn’s disease.

Thus, despite high hopes for a resolution to the controversy, the utilization of molecular techniques to address the relationship between MAP and Crohn’s disease
merely provided additional conflicting evidence. The potential for imperfect specificity of IS900 in the detection of MAP may have significant consequences for the interpretation of many studies in both Crohn’s disease patients and animals that considered this sequence to be specific.\textsuperscript{16,17}

Response to antimicrobial therapy, specifically antimycobacterial therapy, has also been considered as evidence regarding the possible role of mycobacteria in the causation of Crohn’s disease. A recent review by Hulten et al summarized findings of over 30 published studies that evaluated treatment of Crohn’s disease patients with antibacterial drugs.\textsuperscript{215} It appears that a subset of patients may experience clinical improvement following antimicrobial therapy. In general, the effectiveness of antimycobacterial treatment is not considered as substantial evidence to support or refute a mycobacterial cause for Crohn’s disease.

Recent discoveries have only added to the enigma of the relationship between MAP and Crohn’s disease. In 1998, Hermon-Taylor et al published a case report about a eight-year-old boy with cervical lymphadenitis. Although mycobacteria were not cultured from lymph nodes, these tissues were strongly positive for IS900 suggesting a “relatively high abundance” of MAP organisms. The boy later was diagnosed with Crohn’s disease. Following long-term treatment with rifabutin and clarithromycin, his symptoms and chronic inflammation appeared to have completely resolved.\textsuperscript{216} As indicated in one review, “This paper has been viewed by some as the first documented case of MAP causing disease in a human being.”\textsuperscript{217}
In 1999, Naser and his collaborators described the identification of MAP by IS900 PCR from the breast milk of two lactating women diagnosed with Crohn’s disease, but not from the breast milk of five control patients. This report identified a striking similarity with MAP infections in ruminants where shedding of the organism in milk has been consistently documented.

The recent discovery of NOD2, a gene that appears to confer susceptibility to Crohn’s disease has received considerable attention from the scientific community. In the May 2001 issue of Nature, two independent research teams, one using genome-wide screening the other using candidate gene analysis, reported that mutations in the NOD2 gene located within the IBD1 locus on chromosome 16 were associated with susceptibility to Crohn’s disease. At least two subsequent studies have confirmed these findings. Further investigation suggests that these mutations may specifically confer susceptibility to ileal manifestations of Crohn’s disease.

The NOD2 gene is only expressed in monocytes and the NOD protein for which it encodes is believed to serve as a cytosolic receptor for bacterial components, particularly lipopolysaccarides, and as an activator for NF-κB that initiates inflammatory responses. Given the role of the NOD2 gene in the inflammatory process, these reports implicate both bacterial exposure and genetic susceptibility in the development of Crohn’s disease.

Despite multiple, independent reviews of the entire body of scientific literature that have determined that the evidence regarding the link is inconclusive and that more research is necessary, controversy regarding this issue clearly remains. Titles of
recent review articles, editorials, and letters to the editors in the peer-reviewed scientific literature clearly reflect the authors’ biases regarding this controversial issue. This controversy was also the subject of a debate in a recent issue of Gut. The debate concerning the zoonotic potential of MAP will likely continue in the near future.

Related to the controversy concerning the zoonotic potential of MAP and its hypothesized relationship with Crohn’s disease, is the issue of how people are exposed to this organism. Direct exposure to infected ruminants is likely for individuals with occupational exposures to these animals. Additionally, environmental contamination in facilities that house infected animals may be overwhelming and long-lasting. However, an excess risk of Crohn’s disease has not been documented for occupations in direct contact with infected food-animals. Thus, a majority of human exposure to MAP is believed to occur through indirect routes: food-borne and water-borne.

The water-borne route is hypothesized to be an important exposure pathway for the general public to MAP. Members of Mycobacterium avium-intracellulare Complex (MAC) are documented contaminants of public water supplies and recognized as a cause of water-borne nosocomial infections. Public water supplies could become contaminated by runoff from agricultural land on which manure has been applied as a fertilizer or MAP-infected cattle have grazed. It has also been hypothesized that MAP may actually replicate in biofilms present in water distribution systems. Although multiple studies provide convincing evidence that
MAC are common contaminants of potable water supplies, documentation that MAP, specifically, is present in public water supplies is lacking.

A recent report demonstrated that water contaminated with more than $10^6$ cfu/ml of MAP was not adequately disinfected after 30 minutes of contact time with 2 $\mu$g/ml of chlorine.38 Because the concentration of MAP at various points in the water supply and distribution system has not been determined, the practical significance of this study is unknown. The water-borne exposure pathway has not been documented to occur for MAP.

Ingestion of MAP via contaminated meat or milk is considered the most likely route for human exposure. Contamination of meat with MAP could occur as a result of fecal contamination during animal slaughter. Additionally, the infection has a systemic component that results in dissemination of the organism throughout the body.80,81,84,218 Although considered a possible route of exposure, few published reports address the prevalence of MAP in meat and meat products or the degree of inactivation of MAP achieved by cooking these food items.24

More scientific effort has been dedicated to understanding the epidemiology of MAP in the milk supply. The excretion of MAP into milk has been documented for both clinically and subclinically infected cattle.80-82 Another, perhaps more significant, source of MAP in milk is fecal contamination during milking.2,23,64,234

Although the prevalence and concentration at which raw cow’s milk is contaminated with MAP is a critical determinant of the effectiveness of pasteurization in eliminating viable MAP from raw milk as well as the role of milk consumption as a
possible source of exposure to MAP, there is little scientific documentation of the severity of this problem. In 1960, Smith was unsuccessful in attempts to culture MAP from 52 bulk milk samples obtained from 10 dairy herds with documented MAP infection. However, the failure to document contamination of the milk with MAP is not surprising given the unavailability of adequate culture systems and concentrating techniques for MAP at that time. Rahn et al report testing 612 pooled samples representing at total of 1224 dairies in Ontario, Canada; none of which resulted in isolation of MAP. The decontamination procedure used in this study was subsequently criticized because it may have precluded detection. Corti and Stephan identified IS900 in 273 of 1384 (19.7%) bulk tank raw milk samples in Switzerland, with regional prevalence varying from 1.7% to 49.2% IS900 positive bulk tanks. Nauta et al estimated that bulk tank milk from a “severely contaminated” farm infected with MAP is likely to contain 540 cfu/L of MAP. However, the majority of information used to construct the model was based on expert opinion rather than published scientific data. The lack of data documenting contamination of raw milk has been cited in several publications as an area requiring additional investigation.

Considerable evidence suggests that a proportion of MAP may survive when milk is heat-treated to current pasteurization requirements. MAP has been successfully cultured from milk subjected to standard pasteurization temperature and time combinations – 63°C for 30 minutes as well as 72°C for 15 seconds. These studies suggest that heat inactivation may be incomplete when large numbers of
MAP, $10^4$ to $10^7$ cfu/ml, are initially present in milk, but survival also occurred when fewer organisms, $10^2$ to $10^3$, were present.\textsuperscript{28,30} Additionally, several of these studies suggest that increasing the time of heating from 15 seconds to 25 seconds appears to result in greater inactivation of MAP than increasing temperature.\textsuperscript{32,243} However, other investigators have failed to culture MAP following similar time-temperature combinations, suggesting that pasteurization is indeed effective in eliminating viable MAP from milk.\textsuperscript{244-247}

In addition to laboratory-based studies that have relied on the inoculation of milk with MAP in order to evaluate heat inactivation, several have focused on assessing heat inactivation of MAP in naturally infected milk. Millar et al identified MAP using PCR for IS900 in 7\% of 312 retail milk samples collected from England and Wales. Although PCR failed to demonstrate viability of the MAP, they did successfully culture MAP in 15 of 54 long term cultures.\textsuperscript{241} Grant et al tested milk from two MAP-infected farms. Viable MAP was cultured from 4/60 (6.9\%) of raw milk samples and 10/144 (6.9\%) of pasteurized milk samples.\textsuperscript{33} MAP was detected via IMS-PCR for IS900 in 4/60 (6.9\%) samples of raw milk and 30/144 (20.8\%) of pasteurized samples.\textsuperscript{33} A second study assessing the prevalence of MAP in both raw and commercially pasteurized milk in the United Kingdom identified MAP using IMS-PCR in 19/244 (7.8\%) of raw milk samples and 67/567 (11.8\%) of pasteurized samples.\textsuperscript{248} Viable MAP was successfully cultured using HEYM from 4 bulk tank samples and 10 pasteurized samples, although colony counts were low and corresponded to 4-20 cfu/50 ml milk.\textsuperscript{248} Gao et al identified IS900 in 15\% (110/710)
of retail milk samples collected from stores and dairy plants in southwestern Ontario. Subsequent culture of a subset of 244 samples failed to identify viable MAP, however, the authors noted that the BACTEC radiometric culture system used was likely not sensitive enough to detect very low numbers of MAP.249

Together these studies provide further evidence that, while pasteurization may be effective in significantly reducing the number of viable MAP in naturally infected milk, it may not completely eliminate the organism. As Grant states, “The potential public health impact of this situation is uncertain given that an association with Crohn’s disease in humans remains unproven. However, the presence of *M. paratuberculosis*, a known animal pathogen with possible zoonotic potential, in pasteurized milk is probably undesirable.” In light of existing evidence, the UK dairy industry voluntarily increased the holding time during pasteurization of milk from 15 to 25 seconds in hopes of increasing the inactivation of MAP in milk.248

Several studies have addressed the fate of MAP in cheese. Both suggest that pH and temperature during the cheese making process are key determinants of the inactivation of MAP.36,250 While pasteurization of milk appears to be necessary to ensure that no viable MAP is present in soft cheeses, 60-day curing in the manufacturing of hard cheeses appears to be equivalent to pasteurization with respect to inactivation of MAP.36,250
2.10 Fecal Cortisol as a Measure of Stress in Mammals

2.10.1 Historical Perspective

The evaluation of fecal steroid hormones was originally developed as a tool for the reproductive management of captive exotic species.\textsuperscript{251} By measuring fecal levels of gonadal hormones, caretakers could determine phase of the reproductive cycle and pregnancy status of group members while avoiding labor intensive, high risk procedures requiring anesthesia or invasive sampling techniques.\textsuperscript{252} Recently, focus has shifted to the identification of the response of various animal species to environmental and social stressors by determining fecal cortisol or cortisol metabolite levels.

Traditionally, plasma cortisol levels have been used to quantify the body’s response to stressors.\textsuperscript{253} However, these measurements have been criticized because of their invasiveness. The collection process to obtain a blood sample can itself stimulate a stress response and make interpretation of cortisol measurements difficult. Thus, development of a noninvasive method to evaluate the stress response, without influencing it, is desirable.

2.10.2 Physiology of Cortisol Release and Fecal Excretion

An increase in serum cortisol is a transient occurrence in response to a stressor. While this is a complex pathway involving many hormones, ultimately adrenocorticotropic hormone (ACTH) from the anterior pituitary gland acts on the adrenal gland to initiate the release of cortisol (hydrocortisone) into the bloodstream. The liver quickly metabolizes cortisol, and these metabolites are eventually excreted...
into the bile and urine at concentrations several times greater than that of the parent cortisol. Species variation exists in the degree of metabolism, the end metabolites excreted from the body, the major route of excretion and the time between cortisol secretion from the adrenal gland and excretion into urine or feces.

The advent of immunology-based assays has been essential in quantifying fecal cortisol. These techniques have allowed researchers to overcome the technical problems associated with measuring very low levels of hormones in the complex matrix of a fecal sample. Because cortisol metabolites, rather than cortisol itself, are excreted into the feces, the term “fecal cortisol” includes all metabolites of cortisol that are excreted in the feces. Because of the variety of excreted metabolites, it is essential that the antibody utilized in the assay identify a significant proportion of the most common cortisol metabolites for that species. This is referred to as a group-specific antibody.

2.10.3 Applied Studies

Initial research that identified the fecal excretion of cortisol and its metabolites involved the infusion of radio-labeled cortisol and the subsequent identification of radioactive metabolites in the urine or feces. Infusion studies were followed by challenge studies that induced an increase in serum cortisol concentrations in the experimental subject by the administration of exogenous ACTH.

Presently, the experimental focus for fecal cortisol measurement is to identify stress responses to environment, behavior, reproductive cycle or disease by measuring fecal cortisol levels in animals and relating these measurements to potential and
observed stressors to which the animals were exposed. Fecal cortisol levels have been evaluated relative to events known to produce acute stress such as anesthesia in chimpanzees, transport in cattle and post-operative pain in ferrets.\textsuperscript{265-267} Fecal cortisol levels are considered to provide a more accurate measurement of chronic environmental or social stress than a single serum cortisol sample because a fecal sample incorporates excreted metabolites arising from multiple spikes in serum cortisol.\textsuperscript{262,267} Examples of studies using fecal cortisol measurements to assess chronic environmental and social stresses include: fecal cortisol levels in the face of a naturally occurring Pasteurellosis outbreak in bighorn sheep; the relationship between fecal cortisol levels and reproductive cycling in the cheetah and Tufted capuchin monkey; fecal cortisol and its relationship to received aggression in barbary macaques; environmental enrichment and fecal cortisol levels in brown capuchin; and the comparison of fecal cortisol and testosterone levels between rainy and dry seasons and breeding and non-breeding seasons in male muriquis.\textsuperscript{268-273}

2.10.4 Validation of Fecal Cortisol as a Measure of Stress in Ruminant Species

Miller et al were the first to evaluate the use of fecal cortisol measurements to assess the stress response in a ruminant species.\textsuperscript{262,268} The first report described an ACTH challenge study that validated urinary and fecal cortisol levels as measures of acute stress in Rocky Mountain bighorn sheep. A statistically significant increase in both urinary and fecal cortisol was observed following ACTH stimulation. Urinary cortisol was highly correlated with serum cortisol levels. Although fecal cortisol levels generally followed the same trend as plasma and urine cortisol, a well-defined
correlation between fecal and plasma cortisol was not observed. This was attributed to
the considerable variability in fecal cortisol levels observed between individual
animals and the integration of multiple serum cortisol spikes to a single fecal sample
cortisol measurement.\textsuperscript{262} The second report identified a statistically significant
difference in fecal and urine cortisol levels from a 16 day observation period between
Rocky Mountain bighorn sheep that subsequently developed clinical pneumonia
relative to those that did not.\textsuperscript{268}

Palme et al compared urinary and fecal excretion of $^{14}$C-labeled cortisol
intravenously administered to sheep, pigs and ponies to compare time, route, cortisol
metabolites and distribution within the feces between these species.\textsuperscript{257} In sheep,
cortisol was primarily excreted in the urine, although approximately 23\% of recovered
radiolabeled cortisol was present in the feces representing the highest degree of fecal
excretion of the three species evaluated. Peak fecal excretion of radioactive cortisol
occurred approximately 12 hours post-infusion. A follow-up study by this research
group compared assays detecting cortisol, corticosterone and 11, 17-dioxoandrostanes
in the feces of sheep.\textsuperscript{256} The highest detection rates were observed using the 11, 17-
dioxoandrostane assay, providing evidence that the use of group-specific assays allows
increased detection of fecal cortisol relative to antibodies that specifically detect
cortisol.

An ACTH stimulation trial performed in cattle validated the measurement of
fecal cortisol metabolites, specifically 11, 17-dioxoandrostanes, as an indicator of
stressful stimuli.\textsuperscript{260} Additionally, measurement of fecal 11, 17-dioxoandrostanes
following transport of cattle, a documented stressor, resulted in fecal cortisol metabolite levels that were significantly higher at 8 to 16 hours post-transport than those detected during other intervals that were monitored.²⁶⁵

### 2.10.5 Cautions Regarding Fecal Cortisol as a Measure of Stress

Although fecal cortisol measurements have been validated to reflect the stress response in a number of species, several cautions exist for the use of this technique in epidemiologic studies. First, given the variability between species with regard to the type of cortisol metabolites, primary route of excretion of these metabolites and time-lag following exposure, the validation of fecal cortisol excretion and the determination of the antibody that identifies the greatest proportion of excreted metabolites is required prior to incorporation in an epidemiologic study.

Perhaps a more important issue when attempting to quantify a stress response is inter-animal variability.²⁵³ Considerable individual variability between animals has been reported for both serum and fecal cortisol concentrations following ACTH stimulation.²⁶⁰,²⁶²,²⁷⁴ This variability may interfere with the ability to demonstrate significant differences between experimental or observational groups. Palme et al have suggested that this can be circumvented if either large numbers of animals are sampled or the response is expressed as a percent increase above baseline values allowing each animal to act as its own control.²⁶⁰

Another problem is the lack of normal fecal cortisol values. No published reports regarding normal fecal cortisol values exist. Few, if any, published reports are available regarding fecal cortisol measurements within any given species. This lack of
published data can complicate the interpretation of fecal cortisol measurements. While this is less of an issue in experimental settings where baseline levels can be obtained prior to exposure to a stressor, this issue must be addressed in studies that attempt to quantify responses to chronic, ill-defined stressors.

Other physiologic phenomenon may influence circulating plasma cortisol levels, which in turn impact fecal cortisol levels. Because cortisol release occurs in a diurnal pattern, time of day at which fecal sample collection occurs may influence results. Reproductive cycle and pregnancy status may influence cortisol levels. While it is impossible to control for every factor that may ultimately influence cortisol secretion, an effort should be made to control for as many of these factors as possible when utilizing fecal cortisol measurements in experimental or observational studies.

Although the use of fecal cortisol as a measure of chronic stress in populations of animals is still in its infancy, it has become an increasingly accepted method of measuring both acute and chronic stress. Despite the need for continued validation of this measurement, there is great potential for a noninvasive, low-risk diagnostic test that provides information relative to the physiological stress to which animals are exposed.

2.10.6 Possible Relationship between Stress and Immunity to Johne’s Disease

Cortisol acts an important immunoregulator. In addition to modulating the response of reactor cells, cytokine production activates the hypothalamic-pituitary-adrenal axis during an acute inflammatory reaction. The subsequent increase in circulating cortisol serves as a primary down-regulator of the inflammatory
Specifically, the immunosuppressive effects of cortisol include: lysis and margination of blood leukocytes; decreased production of prostaglandins, thromboxanes, and leukotrienes; suppressed cell adhesion; and suppressed macrophage activation and antigen presentation. Overall, cortisol tends to shift cell-mediated responses to humoral responses. Without the negative feedback provided by cortisol, exaggerated cytokine production would progress to the point of neurotoxicity, circulatory collapse, shock and death.

Although the immunosuppressive actions of cortisol are necessary to prevent excessive immune and inflammatory responses, excessive and/or chronic elevation of circulating cortisol levels results in generalized immunosuppression and enhances susceptibility to infection. This phenomenon has been documented to occur in both humans and animals. In addition to circulating cortisol levels, other factors such as type of stressor, timing of stress response relative to exposure, type of host immune response and type of pathogen also influence the ultimate effect of chronic stress on health.

The relationship between immunosuppression as a result of physiologic or environmental stressors and the development or progression of MAP infections and clinical Johne’s disease is ill defined. Anecdotally, exposure to acute and chronic stressors is associated with the onset or exacerbation of clinical signs of Johne’s disease. A limited number of experimental studies suggest that dexamethasone treatment, an established model for acute stress, decreases the immune response to MAP infection possibly permitting multiplication of bacilli in infected animals.
Chronic stress is considered a risk factor for the development and progression of tuberculoid infections in humans. These examples demonstrate the potential role of chronic stress in the development and progression of Johne’s disease.

Immunologically, the tendency for cortisol to shift immune responses toward a humoral (T$_{H2}$) response may explain these observations. Cell-mediated immune (CMI) responses are considered essential to limit the extent of MAP proliferation in infected animals. Humoral responses are generally ineffective. It is possible that infected animals that are exposed to an acute stress or exceed some threshold for chronic stress attain a level of circulating cortisol that limits CMI to the point that proliferation of MAP bacilli is unrestrained by the host.

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CHAPTER 3

LIKELIHOOD RATIOS FOR MULTIPLE RANGES OF ELISA S/P RATIOS IN OHIO DAIRY HERDS INFECTED WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

3.1 Abstract

Whole herd *Mycobacterium avium* subsp. *paratuberculosis* (MAP) test records for nine Ohio dairy farms from 1994-1999 were obtained from the Ohio Department of Agriculture, Animal Disease Diagnostic Laboratory. 5354 observations, including 4856 ELISA with concurrent fecal culture, representing 1780 cows were obtained. In addition to comparing fecal culture and ELISA results for individual cows on these farms, likelihood ratios that indicate the odds that a cow with a given ELISA S/P ratio will be identified as infected relative to non-infected were calculated for a subset of the data. Nearly 50% of cows with a positive fecal culture lacked a positive ELISA result during the observation period. Similarly, 55% of cows with a positive ELISA result did not have infection confirmed during the observation period. Of cows that had both positive ELISA and fecal culture at some point in their test histories, nearly 80% were identified as fecal culture positive before or at the same time as their
positive ELISA result. As ELISA S/P ratios increased, the likelihood that a cow would be identified as infected during the observation period increased. Cows with ELISA S/P ratios ≥ 0.800 were 55 times more likely to be identified as infected relative to non-infected. However, ELISA S/P ratios < 0.800 were of limited value in predicting the true infection status of cows. Although cows with ELISA S/P ratios between 0.250-0.799 were at a moderately increased risk of MAP infection, the majority of results in this range classified cows believed to be non-infected as ELISA positive. This lack of consistency between ELISA and fecal culture results supports the recommendation that the ELISA be used as a screening test rather than the sole tool to determine MAP infection status or to make management recommendations concerning individual cows.

3.2 Introduction

Johne’s disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic infectious disease of ruminants. A recent national survey of U.S. dairy cattle estimated that 22% of dairy herds and 3% of dairy cows are infected.¹ The United States Department of Agriculture estimates that the US dairy industry loses $200 to $250 million annually as a result of MAP infections.²

In addition to the implementation of management practices designed to limit transmission to susceptible animals, an important aspect of control of MAP infection is the identification and removal of infected animals from a herd because these animals are the source of significant contamination for the herd environment. However, the identification of animals with MAP infections presents a diagnostic
challenge. Cows with MAP infections typically exhibit prolonged incubation, intermittent fecal shedding and delayed antibody production. These factors create a situation where an animal may be infected with the organism but will falsely test negative. Additionally, misclassification of an animal’s MAP infection status is also influenced by the limitations of the screening tests that are currently available.

Consequences of the misclassification of an animal’s true infection status include economic losses associated with the unnecessary culling and replacement of false positive cows and the continued environmental contamination and transmission of infection as a result of failure to remove false negative cows from the herd. Thus, the appropriate interpretation of test results for an individual animal in light of herd infection history and animal health status is necessary to avoid these undesirable situations.

Fecal culture is routinely used to confirm infection in herd screening programs for MAP. However, the use of fecal cultures in screening programs has been criticized for poor sensitivity, high cost and long incubation time until culture results are available. Several serological testing methods that detect antibodies to MAP have been developed. Some investigators consider the ELISA (enzyme-linked immunosorbent assay) to be most suitable for use as a screening test in subclinically infected animals. The ELISA provides an inexpensive, rapid test result; however, a recent evaluation of one commercially available ELISA reports an overall diagnostic sensitivity of 50% relative to fecal culture, noting variability of the sensitivity of the test between different clinical stages of disease or levels of bacterial shedding, and a
reported specificity of 96.8%. Additionally, coefficients of variation greater than 20% have been reported for ELISA S/P ratios when individual sera samples were tested multiple times, often resulting in a different classification (positive/negative) of the test result.

Several researchers have advocated the quantitative evaluation of ELISA S/P ratios obtained when screening for MAP infection, rather than relying on a positive or negative classification for these results that is defined by a single cut-off value. Likelihood ratios represent the odds that a given level of a diagnostic test would be expected in an individual with the target disorder relative to an individual without the target disorder. By calculating likelihood ratios for multiple levels of a test result reported on a continuous scale, the risk of possessing the target disorder can be compared between multiple levels of test results. The advantage of this approach is that it provides additional diagnostic information that cannot be appreciated when test results are reported in a positive/negative dichotomy.

In the present study, we describe fecal culture and ELISA results collected from Ohio dairy herds involved in Johne’s disease testing programs. We calculate likelihood ratios for multiple levels of ELISA S/P ratios, including negative values, based on results obtained from repeated herd testing in MAP-infected dairy herds.

3.3 Methods

Test records for nine southwestern Ohio dairy farms were obtained from the Ohio Department of Agriculture, Animal Disease Diagnostic Laboratory (ADDL) for MAP tests performed from 1994 through 1999. Herds were tested approximately
every six months using both fecal culture and ELISA concurrently. All adult cattle two years of age or older that were herd members on the day of sample collection were tested. As a result, cows were likely to be tested multiple times during the observation period.

Fecal culture and ELISA were performed at the ADDL. Briefly, 2g of feces were added to 40 mL of distilled water, shaken for 30 minutes, and left at room temperature for 24 hours. Following sedimentation, 5 mL from the top of the supernatant were added to 25 ml of 0.9% hexadecylpyridinium chloride (HPC), and the solution was decontaminated for 24 hours at room temperature. Approximately 0.2 mL of sediment were transferred into each of four slants of modified Herrold’s egg yolk medium (HEYM) containing amphotericin B, vancomycin and nalidixic acid. Three HEYM slants contained supplemental mycobactin J, and one did not. Slants were incubated at 37°C, and growth was observed at 1, 4, 8, 12 and 16 weeks. Confirmation of isolates as MAP was based on colony morphology, mycobactin dependency and acid-fast staining.

A commercial ELISA test kit was used as directed by the manufacturer (IDEXX Laboratories, Inc., Westbrook, ME). A change in the format of the ELISA kit occurred early in 1998 after which the classification of a positive test was based on a sample-to-positive (S/P) ratio versus the optical density values used previously. As indicated by the manufacturer, ELISA S/P ratios ≥ 0.250 were considered positive.

The following information was obtained for each animal tested: identification number/name, sample collection date, fecal culture result and ELISA result. The
retrospective nature of this study limited the availability of individual cow or farm management information. S/P ratios were obtained for ELISA performed in 1998 and 1999.

Descriptive statistics were calculated at the test-level, herd-level and cow-level. The percentage of tests that were positive was calculated for both the ELISA and the fecal culture over the entire observation period and for each herd test. The infection status of each herd was determined based on fecal culture results and herd histories.

Individual cow test histories were examined. The number of concurrent ELISA-fecal culture pairs and the total number of fecal cultures performed was determined for each cow. Additionally, the test or tests that became positive, the test that was the first to become positive and the confirmation of infection status by positive fecal culture were characterized for each cow. Cows with a positive fecal culture were classified as MAP-infected. Cows without a positive fecal culture during the observation period were classified as unknown infection status.

ELISA S/P ratios were initially assigned to one of 17 strata, each representing a range of S/P ratios. For each stratum, the number of both infected and non-infected cows represented by the S/P ratios within the defined range was determined. Cows with a positive fecal culture formed the MAP-infected group. Only those cows with 3 or more negative fecal cultures in their test history were included in the non-infected group. A likelihood ratio representing the odds that cows with S/P ratios within a stratum would be classified as infected relative to non-infected was calculated as:


3.4 Results

Records obtained included results from 4856 ELISA and concurrently conducted fecal cultures representing a total of 1642 cows. Results were also available for 498 ELISA or fecal cultures performed without additional concurrent testing representing another 138 cows. In total, 5354 observations represented 1780 cows from nine dairy herds. S/P ratios were available for 1812 ELISA representing 923 cows.

Of the 4856 observations with results for concurrent ELISA-fecal culture pairs, 4.2% of ELISA (n=205) and 3.8% (n=185) of fecal cultures were positive. Of the 185 positive fecal cultures, 86 were concurrently ELISA positive. Of the 4671 negative fecal cultures, 4552 were concurrently ELISA negative.

The average size of the dairy herds included in the analysis ranged from 32 to 216 total cows with a mean of 88 cows. Additional information regarding herd production and management practices was unavailable in this retrospective study.

Table 3.1 summarizes ELISA and fecal culture results for each of the nine herds. Eight of the nine herds were infected with MAP. These eight infected herds had at least one fecal culture-positive animal identified during the observation period. These herds usually culled fecal culture-positive cows by the end of the lactation during which infection was identified. Although cows in these herds were not culled
solely because of a positive ELISA result, culling of ELISA-positive cows did occur on an individual basis in light of production and/or overall health status. One herd included in this study, Farm L, was considered non-infected based on historical absence of clinical signs of Johne’s disease, closed herd status and failure to identify fecal culture-positive animals during the observation period. In addition to the negative fecal cultures reported here, the herd has remained closed and subsequent herd testing over an additional four years has failed to identify MAP infection.

The test histories of 1780 cows were evaluated. A total of 153 cows were classified as MAP-infected. These cows had at least one positive fecal culture in their test history. The true MAP infection status of the remaining 1627 cows is unknown. These cows were negative on all fecal cultures performed during the observation period. Of these 1627 cows, 97 had one or more positive ELISA results, but remained fecal culture-negative despite repeated testing. More than half of these cows had three or more negative fecal cultures (55/97) and 13% (13/97) had seven or more negative fecal cultures in their test histories.

Of the 153 infected (fecal culture-positive) cows, 78 were also identified as ELISA-positive at some time during the observation period while 75 remained ELISA-negative throughout the observation period. Thus, nearly 50% of known-infected cows (75/153) lacked a positive ELISA result during the observation period (mean number of tests per cow = 3.4).

A total of 175 cows had at least one positive ELISA in their test histories. Of these cows, only 45% (78/175) had MAP infection confirmed during the observation
period. Additionally, of the 78 cows with positive results on both fecal culture and ELISA at some time during the observation period, 15.4% (12/78) of positive fecal culture results were obtained from fecal samples collected prior to ELISA-positive serum, and 64.1% (50/78) had positive fecal and serum samples that were collected on the same date.

Table 3.2 presents the likelihood ratios representing the odds that cows with ELISA S/P ratios in a defined stratum were identified as infected relative to non-infected. These calculations were based on a subset of 1323 ELISA tests representing 64 infected cows and 503 non-infected cows. As S/P ratios increased, cows were more likely to have MAP infection confirmed during the observation period. Cows with S/P ratios ≥ 0.800 were 55 times more likely to be identified as infected rather than non-infected during the observation period.

3.5 Discussion

ELISA screening in a Johne’s disease control program is most useful when the test detects infected animals, especially those that are shedding the agent in their feces, in order to remove these individuals from the herd. Nearly 50% of fecal culture positive cows in this study lacked a positive ELISA result in their test history. Additionally, in those infected cows with a positive ELISA result in their test history, nearly 80% were identified by a positive fecal culture before or at the same time as a positive ELISA result.

Our observations suggest that false negative ELISA results may incorrectly classify a proportion, perhaps a considerable proportion, of infected cows. If only the
ELISA had been used in the herds described here, nearly 50% of infected cows would not have been identified. Additionally, our data support the observation that fecal culture appears to identify infected animals earlier in the course of infection than does the ELISA. In that report, only 15.4% of animals classified as low fecal shedders of MAP were ELISA-positive. Unfortunately, colony counts were not available to us to assess level of fecal shedding relative to ELISA status. Use of the fecal culture in MAP control programs will facilitate earlier removal of infected animals from the herd, thus reducing overall farm contamination with MAP.

Although 55% of cows with positive ELISA results were never confirmed as infected during the observation period, we were unable to definitively classify the true infection status of those cows. Although a single positive fecal culture confirms that a cow is infected with MAP, multiple negative fecal cultures cannot definitively rule out infection. Several factors contribute to this dilemma. Infected animals do not shed MAP early in the course of infection. Thus, it is impossible to detect these individuals even using the most sensitive methods for organism detection. The sedimentation procedure utilized for the fecal cultures reported here is less analytically sensitive than methods that incorporate centrifugation. It is possible that this methodology resulted in false negative fecal culture results, especially if cows were shedding low numbers of organisms in their feces at the time of sampling. Additionally, some ELISA-positive cows in this study were lost to follow-up without additional fecal cultures. It is possible that a proportion of these cows may have had their infection status confirmed with additional fecal cultures.
Because assessing the true infection status of test negative cows in infected herds is problematic, we chose to include only those cows with three or more negative fecal cultures as members of the non-infected group in our calculations for likelihood ratios. Similar definitions for non-infected populations have been utilized in other reports that have evaluated ELISA performance relative to fecal culture, although these authors were careful to note the limitations of this approach. The misclassification of infected cattle as non-infected would underestimate the odds of MAP infection in our calculations. Although the possibility of this type of misclassification cannot be eliminated, given the cows in this study originated from infected herds, we reduce the probability that truly infected cattle are misclassified as non-infected by requiring that cows included in the non-infected group have at least three negative fecal cultures over time. Over one-third of the cattle in this group had more than six negative fecal cultures in their test history.

The likelihood ratios we report here differ considerably from those recently published. The method of calculation for the reported likelihood ratios incorporated sensitivity and specificity estimates for the ELISA at several arbitrary cut-off values. Because this calculation relied on the dichotomous classification of test results, it did not permit a comparison of the risk of MAP infection between multiple ranges of ELISA S/P ratios. Further, ELISA S/P ratios with negative values, less than 0.000, were excluded from those likelihood ratio calculations, even though these results represented over one-third of the available data. We utilize an alternative approach to calculating likelihood ratios that permits a direct comparison of the risk of MAP
infection between multiple ranges of ELISA S/P ratios, including those that are less than 0.000. 13

Additionally, these reported likelihood ratio calculations utilized cattle originating from dairy herds believed to be free of MAP infection to form the non-infected group. 12 Although this approach increases the level of confidence that these cattle were truly non-infected, it is possible that the absence of environmental exposure to MAP in these non-infected herds may result in decreased sero-reactivity to MAP. Thus, the distribution pattern of ELISA S/P ratios in non-infected herds may differ from that observed in MAP-infected herds. 18,19 Further, the majority of non-infected cattle originated from herds in The Netherlands. Again, the possibility of different distribution patterns of serological responses to MAP may exist between cattle from a geographic area believed to have a low level of MAP infection relative to that observed in the United States. By including the entire range of ELISA S/P ratios obtained when testing MAP-infected dairy herds, we believe the estimates presented here are directly applicable when interpreting ELISA results in MAP-infected herds in the Midwest, and likely throughout the United States.

Cows with ELISA S/P ratios ≥ 0.800 were 55 times more likely to be identified as infected relative to non-infected. Although the potential for misclassification still exists with S/P ratios ≥ 0.800, the likelihood ratio presented here represents strong statistical evidence that these cows are likely to be truly infected. 20 Although our likelihood ratio estimates are considerably lower than those reported by Collins, a similar trend is observed as likelihood ratios increase, and values greater
than 0.75 in that study also appeared to be more strongly associated with infection than lower values. Additionally, these observations agree with the report by Hirst et al where cows with ELISA S/P ratios >0.700 were more likely to maintain an ELISA-positive status with subsequent testing, although fecal culture status was not determined for cows in that study. Together, these observations suggest that quantitative use of ELISA S/P ratios to predict an animal’s infection status provides the most confidence when S/P ratios are considerably higher than the manufacturer’s recommended cut-off value for positive results.

ELISA S/P ratios < 0.800 were of limited value in predicting the true infection status of individual cows in this study. Although cows with ELISA S/P ratios between 0.250 and 0.799 were more likely to be infected than non-infected, the calculated likelihood ratio of 3.59 reflects only a moderate increase in risk of MAP infection. Of the 52 ELISA S/P ratios in this range, 39 (75%) classified cows with three or more negative fecal cultures as ELISA-positive.

3.6 Conclusions

This study has identified several considerations regarding the interpretation of test results obtained as part of Johne’s disease herd screening programs. Likelihood ratios presented here estimate the odds of MAP infection for a cow originating from infected dairy herd with a given ELISA S/P ratio. Although individual cows with ELISA S/P ratios ≥ 0.800 were 55 times more likely to be infected with MAP, the potential for misclassification, although small, still exists. ELISA with S/P ratios ≤ 0.800 provided little additional diagnostic evidence regarding a cow’s true MAP
infection status. These estimates, in addition to the lack of consistency between
ELISA and fecal culture results observed, support the recommendation that the ELISA
not be used as the sole tool to determine MAP infection status or to make subsequent
management recommendations concerning individual cows.

3.7 Literature Cited


Table 3.1: Number of cows tested, percent of positive fecal culture results and percent of positive ELISA results for whole-herd tests completed for each farm by six-month intervals from 1994-1999.
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Table 3.2: Likelihood ratios indicating the odds of MAP infection relative to non-infection for multiple ranges of ELISA S/P ratios.
CHAPTER 4

FECAL CORTISOL MEASUREMENTS IN DAIRY CATTLE
FOLLOWING ACTH ADMINISTRATION

4.1 Abstract

The objectives of this study were to verify that fecal cortisol levels can be measured in dairy cattle using a previously described technique and to demonstrate that fecal cortisol levels increase following a physiologic stress, simulated in this study by the administration of synthetic ACTH. Fecal and serum samples from eight randomly selected cull dairy cows were collected to establish baseline cortisol levels. Each cow received 0.25 mg of synthetic ACTH intravenously. A second serum sample was obtained one-hour post-ACTH administration. Fecal samples were collected every 12 hours following ACTH administration for a 72-hour period. An increase in serum cortisol was observed (p=0.005). Increases from baseline fecal cortisol levels ranging from 18% to 230% were observed for seven cows. However, median fecal cortisol levels were not different from the median baseline value at any collection time (p=0.196). Possible explanations for the inability to demonstrate a significant change in the fecal cortisol levels at collection periods following ACTH administration include small sample size, individual variability between cows,
selection of currently stressed cows, inappropriate dose of ACTH and inability of the RIA used to detect excreted cortisol metabolites.

4.2 Introduction

Plasma cortisol levels have been used extensively to quantify the body’s response to stressors. Recent research has developed the use of fecal cortisol measurements as a noninvasive method of evaluating the stress response. An increase in serum cortisol is a transient occurrence in response to a stressful situation or event. This complex pathway ultimately results in the release of cortisol from the adrenal gland in response to ACTH. The liver quickly metabolizes cortisol, and these metabolites are excreted into the bile and urine.¹

The evaluation of fecal steroid hormones was originally developed as a tool for the reproductive management of captive exotic species maintained in zoological and game parks. By measuring fecal levels of gonadal hormones, animal caretakers can determine the phase of reproductive cycle and pregnancy status of group members while avoiding labor intensive, high risk procedures requiring anesthesia or invasive sampling techniques.² Recently, the measurement of fecal cortisol levels has been used to identify the response of exotic and laboratory animal species to environmental factors, behavioral stressors, reproductive cycle and infectious disease.³-⁸

When assessing chronic stress responses, fecal cortisol measurements provide several advantages over the use of a single serum sample. This noninvasive sampling method reduces the possibility of iatrogenic elevations in cortisol levels associated with the sampling procedure. The risks associated with chemical and/or physical
restraint for both the animal and its handlers are limited. Additionally, fecal cortisol levels are considered to provide a more accurate measurement of chronic environmental or social stress than a single serum cortisol sample because cortisol excreted into the feces represents an accumulation of several transient increases in serum cortisol.9–11

Species variation exists in the degree of metabolism, the metabolites excreted from the body, the major route of excretion and the time between secretion of cortisol into the bloodstream and excretion into urine or feces.12,13 Because of these species variations, validation of fecal cortisol measurement is necessary before applied research utilizing this measure of stress can occur in a given species.

With respect to cattle, Palme et al have validated that fecal cortisol levels reflect increases in serum cortisol concentrations following ACTH administration.14 Additionally, fecal cortisol levels were significantly elevated from baseline levels in cattle following transport, a known acute stressor.6

The objective of this trial was to describe the fecal cortisol response following the administration of ACTH in order to evaluate the potential usefulness of this measurement in an epidemiologic investigation. Specifically, our aim was to verify that fecal cortisol levels in dairy cattle can be measured using a previously described technique.

4.3 Materials and Methods

Eight cows were randomly selected from a group of 15 cull dairy cows being maintained for teaching purposes at the College of Veterinary Medicine, The Ohio
State University. Cows had been obtained approximately two weeks prior to this study. All cows used in this study were non-lactating, non-pregnant by rectal palpation, negative on *M. avium* subsp. *paratuberculosis* ELISA and had normal temperature (*T* = 100°F – 102.5°F) at the start of the ACTH stimulation trial.

Fecal and blood samples were collected to establish baseline cortisol levels. Free-catch fecal samples were preferred; however, rectal collection with a clean obstetrical sleeve was performed if necessary. Blood samples were obtained from the coccygeal vein.

Each cow received 0.25 mg of Cortrosyn®, synthetic ACTH, in a 1 mL dilution of saline intravenously at approximately 9 A.M. EST. A second blood sample was obtained one hour post-ACTH administration. Fecal samples were collected every 12 hours for a 72-hour period.

Blood samples were centrifuged within two hours of collection and serum was separated. Serum and fecal samples were frozen at -20°C and shipped overnight on ice to the Endocrinology Laboratory, Department of Physiology, Colorado State University. A fecal cortisol RIA, as previously described, was used. The Cochran-Mantel-Haenszel statistic was used to compare rank scores for cortisol levels between collection periods and the baseline cortisol level for both serum and fecal samples while controlling for variation between individual cows. Additionally, the percentage change from baseline to post-ACTH serum cortisol level and the maximum percentage change from baseline fecal cortisol level were calculated to allow each cow to serve as its own experimental control. The sign test was used to
determine if these changes represented significant increases in serum and fecal cortisol levels following ACTH administration. Spearman’s rank-correlation coefficient was used to evaluate the relationship between the maximum change in fecal cortisol levels and the change in serum cortisol levels exhibited by individual cows.

### 4.4 Results

Median baseline serum cortisol was 6.6 ng/ml. Following ACTH stimulation, serum cortisol increased to a median value of 31.4 ng/ml (p=0.005). Figure 4.1 presents the serum cortisol response of each cow. Median baseline fecal cortisol was 8.9 ng/g. Median fecal cortisol levels were 10.7, 8.0, 9.0, 7.7, 7.7 and 6.6 ng/g at 12, 24, 36, 48, 60 and 72 hours post-ACTH stimulation, respectively. These values were not different from the baseline value (p=0.196). Figure 4.2 graphs the fecal cortisol levels of each cow over the observation period.

Table 4.1 reports the percent of change in serum and fecal cortisol levels for each cow. The change in serum cortisol levels for individual cows ranged from a 120% increase to more than a 1700% increase, representing a significant increase in serum cortisol levels across all cows (p=0.008). The maximum change from baseline fecal cortisol levels ranged from –48% to 230%. This was not a significant change (p=0.07). An inverse relationship was observed between the change in serum cortisol levels and the change in fecal cortisol levels of individual cows (r_s= -0.43, p=0.289).

The time until maximum change in fecal cortisol level following ACTH administration varied among individual cows (Figure 4.3). Of seven cows with increases in fecal cortisol levels following ACTH administration, peak fecal cortisol
levels were observed at 12 hours for 3 cows, 36 hours for 2 cows, and at 48 and 72 hours for one cow each. A single cow had fecal cortisol levels that decreased throughout the observation period.

4.5 Discussion

Changes from the baseline serum cortisol level verify that administration of synthetic ACTH initiated an adrenal release of cortisol. Although cortisol was detectable in the feces of dairy cattle, median fecal cortisol levels did not differ significantly from the baseline value at any time following ACTH stimulation.

Variability in cortisol responses between cows has been previously reported. Given this variability, the analysis of the percentage change in fecal cortisol level for individual subjects may be more appropriate than comparisons of the central tendencies of values, especially for small sample sizes of animals. By allowing each cow to serve as its own control in this study, we were able to observe increases in fecal cortisol levels for all but one cow.

In addition to varying degrees of cortisol responses between cows, there also appeared to be variability in the pattern of cortisol excretion relative to the timing of ACTH administration. Interestingly, the maximum increase in fecal cortisol levels was observed at 24 hours or greater following ACTH stimulation for 4 of 7 cows. Other reports suggested that maximum fecal cortisol excretion would be observed 8 to 16 hours following a stressful event in cattle.

Additionally, fecal cortisol levels returned to baseline as of the collection period immediately following the maximum increase in fecal cortisol levels for 5 of 7 cows.
cows. Fecal cortisol levels have been considered an appropriate measure of chronic, environmental stresses. In this study, we simulated a single, acute stress response. Possibly, repeated dosing or administration of a repository, slow-release form of ACTH would have better mimicked the chronic stress response. Alternatively, perhaps the dose of synthetic ACTH administered to cows in this study was insufficient.

The inverse relationship noted between the change in serum cortisol levels and the maximum change in fecal cortisol levels was also unexpected. Our use of an RIA that detected cortisol, rather than cortisol metabolites, may have impaired our ability to detect the majority of excreted cortisol metabolites. Metabolism of cortisol results in the excretion of cortisol metabolites rather than cortisol itself. The use of an antibody specific for cortisol would not identify cortisol metabolites, even though they represent the primary form of excreted hormone. Substituting an antibody with group-specific binding properties would have identified a greater proportion of excreted cortisol metabolites. Perhaps, the measurement of fecal cortisol metabolites would have better reflected the increases that were observed for serum cortisol following ACTH administration.

Other factors may have also influenced the cortisol responses observed here. Based on published reports of “normal” serum cortisol levels in the bovine, four cows in this experimental group had apparently low baseline cortisol levels. Possibly, chronic physiological and environmental stresses associated with culling and relocation of the cows used in this study resulted in diminished response following ACTH stimulation in certain individuals or influenced the magnitude of cortisol
release and/or metabolism. Also, circulating levels of reproductive hormones may have influenced cortisol release, metabolism or excretion. The cows in this study were non-pregnant; however, their reproductive stages had not been recorded nor had their reproductive cycles been synchronized. These potential differences may have further contributed to the variability in fecal cortisol levels observed between cows in this trial.

Although fecal cortisol levels were measurable in dairy cattle using previously described techniques, our study is consistent with others in that individual variability in cortisol response was considerable. The use of fecal cortisol measurements as an indicator of chronic stress in dairy cattle requires further exploration.

4.6 Literature Cited


7. Strier KB, Ziegler TE, Wittwer DJ. Seasonal and social correlates of fecal testosterone and cortisol levels in wild male muriquis (Brachyteles arachnoides). *Horm Behav* 1999;35:125-34.


Figure 4.1: Serum cortisol levels at baseline and at 1 hour following ACTH administration.
Figure 4.2: Fecal cortisol values for individual cows over the observation period.
Table 4.1: Percent change from baseline in serum and fecal cortisol levels and time of maximum change in fecal cortisol level for individual cows.
Figure 4.3: Time of maximum increase in fecal cortisol levels following ACTH administration.
5.1 Abstract

A potential role of acute and chronic stress in the development of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infections and the progression of Johne’s disease has been proposed. This study is the first to investigate this relationship in dairy herds by utilizing fecal cortisol measurements. The objectives of this study were two-fold: to identify herd-level management practices and milk production parameters that influence mean herd fecal cortisol levels and to assess differences in fecal cortisol levels between herds that are infected with MAP relative to those that are not. Composite fecal samples were obtained from multiple housing areas representing five management groups on four separate visits to each of 55 Ohio dairy farms. In addition to fecal cortisol analysis, herd management practices were described, and both DHI production records and MAP-testing records were obtained. Farm, sampling location on a farm and sampling date significantly influenced fecal
cortisol levels. Few individual management practices or production parameters that affected mean herd fecal cortisol levels were identified. Mean herd fecal cortisol levels did not differ between MAP-infected and non-infected herds. Several opportunities for the refinement of fecal cortisol measurements in dairy herds as part of epidemiologic studies are also discussed.

5.2 Introduction

The use of fecal cortisol measurements is becoming an increasingly accepted method of quantifying both acute and chronic physiological stresses in animal populations. Because the collection of a blood sample to measure plasma cortisol levels can itself induce a detectable stress response, there is great value in a noninvasive, low-risk diagnostic test that quantifies the physiological stress of animals. 1-3

Fecal cortisol levels are considered to provide a more accurate measurement of chronic environmental or social stress than a single serum cortisol sample because a fecal sample incorporates multiple spikes in serum cortisol. 4,5 Fecal cortisol measurements have been validated as an indicator of the stress response in a number of species, both in experimental and observational studies. 4,6-13

Miller et al were the first to evaluate the use of fecal cortisol measurements to assess the stress response in a ruminant species, the Rocky Mountain Bighorn sheep. This work both validated fecal cortisol as a measure of acute stress and identified a difference in fecal cortisol levels of sheep that developed clinical pneumonia relative to those that did not. 5,8 Palme et al reported that approximately 23% of 14C-labeled
cortisol administered intravenously to domestic sheep was recovered in the feces, with peak fecal excretion occurring approximately 12 hours post-infusion. An ACTH stimulation trial validated the measurement of fecal cortisol metabolites as an indicator of stressful stimuli in cattle. Additionally, fecal cortisol metabolite levels were significantly higher at 8 to 16 hours post-transport, a documented stressor for cattle, than those detected during other intervals that were monitored.

Excessive and/or chronic elevation of circulating cortisol levels results in generalized immunosuppression and enhances susceptibility to infection in both humans and animals. The relationship between immunosuppression as a result of physiologic or environmental stressors and the development or progression of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infections and clinical Johne’s disease is ill defined. Anecdotally, exposure to acute and chronic stressors is associated with the onset or exacerbation of clinical signs of Johne’s disease. A limited number of experimental studies suggest that dexamethasone treatment, an established model for acute stress, decreases the immune response to MAP infection possibly permitting multiplication of bacilli in infected animals. These examples demonstrate the potential role of chronic physiologic stress in the development and progression of Johne’s disease.

Despite the potential value of fecal cortisol measurements as an indicator of chronic stress, and possibly an increased susceptibility to infectious disease processes, several cautions exist concerning the use of these measurements in epidemiologic studies. There are many factors that may influence the cortisol responses of individual
animals. For example, because cortisol release occurs in a diurnal pattern, time of day at which fecal sample collection occurs may influence results.\textsuperscript{26} Also, reproductive cycle and pregnancy status of individual animals may influence cortisol levels.\textsuperscript{5,10} In experimental settings, baseline levels obtained prior to exposure to a stressor and random assignment to treatment groups allow for statistical control for many of these factors.\textsuperscript{7,15} However, potential confounding factors that influence cortisol levels in observational studies must be identified in order to control for them when quantifying responses to chronic, ill-defined stressors.

One objective of this study was to evaluate the influence of management practices and milk production on fecal cortisol levels measured in Ohio dairy herds. The identification of herd-level factors that influence fecal cortisol levels is a critical preliminary step that would make fecal cortisol measurements more useful in large-scale epidemiological studies in dairy cattle. A second objective of this study was to assess differences in fecal cortisol levels between herds that are infected with MAP relative to those that are believed to be non-infected. By describing the relationship between herd fecal cortisol levels and herd MAP-infection status, a more complete understanding of the role of stress in the development of this infection or subsequent clinical disease may be elucidated.

5.3 Materials and Methods

5.3.1 Herd Selection and Visitation

Private veterinary practitioners and state and federal veterinary medical officers involved with herd Johne’s disease testing programs were asked to identify
dairy herds that would likely participate in the study. Eligibility for participation was restricted to Ohio dairy herds that completed annual whole-herd testing for Johne’s disease. Both MAP-infected and Ohio Test Negative Status herds were recruited for participation.

Herds that agreed to participate were visited at six-month intervals. Over the two-year duration of the study, herds were visited a total of four times. Herd visits typically occurred from December through March and from June through August. A comprehensive questionnaire, with special focus on management practices recommended for Johne’s disease control, was completed during the initial farm visit. Subsequent visits utilized a questionnaire designed to identify management changes that occurred since the previous visit. Additionally, the incidence of selected infectious and metabolic diseases common on dairy farms was estimated for the six-month period prior to the date of visitation.

5.3.2 Fecal Sample Collection and Measurement of Fecal Cortisol

During each farm visit, fecal samples were collected for cortisol analysis from five animal housing areas -- lactating cow areas, dry cow areas, areas housing heifers from weaning to breeding age, areas housing sick cattle, and areas used for calving. If lactating cows were managed as multiple production groups, separate samples were collected for each group. Housing areas that were present on-site and had contained animals within the 24 hours prior to the herd visit were sampled. On some farms, certain housing areas did not exist or were not available for sampling at the time of the farm visit.
For each housing area sampled, five fresh fecal samples were collected from the four outermost corners and the center of each area. If five samples were not available, the maximum possible number of fresh samples available was collected. In large areas housing more than 100 individual animals, an additional one to five samples were collected per area. Fecal samples were mixed thoroughly to create a composite sample for each housing area.

Samples were transported on ice from the farm to the laboratory at which time samples were frozen at -20°C until processing. Sample processing included thawing, transferring to glass vials suitable for lyophilizing, and refreezing at -20°C.

Frozen samples were lyophilized at -40°C in sets ranging from 13 to 23 individual samples. Lyophilizing continued until the average loss of weight over a six-hour period was less than 2% for all samples within the set. Lyophilized samples were ground until fine, and approximately 0.5g was transferred to a glass centrifuge tube. Five milliliters of double distilled, deionized water was added to the sample. Under refrigeration, each sample was shaken for 1 hour then centrifuged for 15 minutes at 3000 x g. The supernatant was transferred to a plastic vial, sealed and frozen at -20°C until shipment. Frozen samples were shipped overnight on ice to the Colorado State University Endocrinology Laboratory for fecal cortisol analysis as previously described.5,8

5.3.3 Collection of Milk Production and MAP Testing Records

In addition to the management information and fecal samples for cortisol analysis collected during farm visits, additional herd records were obtained. Test
results for whole-herd and individual animal MAP testing, using either the ELISA and/or fecal culture, for samples submitted to the Animal Disease Diagnostic Laboratory, the Ohio Department of Agriculture (ODA-ADDL) from January 1999 through June 2002 were obtained directly from the laboratory. Also, milk production records were obtained directly from DHI Cooperative, Inc for a subset of herds.

5.3.4 Statistical Analysis

For statistical models assessing the influence of management practices on herd fecal cortisol levels, the practices in effect at the time of each fecal sample collection were evaluated. Additionally, the influence of the incidence of selected infectious and metabolic diseases for the six-month period prior to the date of fecal sample collection was evaluated relative to fecal cortisol levels.

Herd MAP-infection status was classified according to results obtained for whole-herd MAP testing conducted at the ODA-ADDL. Herds that identified one or more fecal culture positive cows or clinical cases of Johne’s disease during the observation period were classified as infected herds. Herds that were identified as Ohio Test Negative Status herds throughout the observation period were considered to be non-infected in this study.

Milk production records (rolling herd average milk production, protein production, and fat production as well as bulk tank somatic cell counts) were available for a subset of herds. For models assessing the influence of these parameters on herd fecal cortisol levels, herd-level DHI records for July 2000, January 2001, July 2001
and January 2002 were selected to represent herd production for herd visits 1 through 4, respectively.

Descriptive statistics were calculated for all fecal cortisol measurements obtained during the study as well as by herd, visit and management group on a farm. A log transformation was applied to the fecal cortisol measurements to achieve a normal distribution for further analysis. Multiple comparisons of fecal cortisol values between herds, visits and management groups were performed using Tukey’s HSD test with an experiment-wise error of 0.05.

A mixed linear model that nested management groups within a farm was used to evaluate the effects of herd, visit and group within herd on the log-transformed fecal cortisol measurements. Several covariance structures including compound symmetry, first-order autoregression, variance components and unstructured variance were evaluated for model fit, and the covariance structure resulting in the lowest univariate model AIC value was selected.

The effects of herd management practices, production parameters and herd MAP-infection status on the mean herd fecal cortisol level were also evaluated using mixed linear modeling that specified repeated visits within a herd. The mean herd fecal cortisol level for each visit was calculated, and a log transformation was applied to achieve a normal distribution of these values. Univariate models were used to screen variables for significance. Variables with p values < 0.250 were selected for inclusion in subsequent models. Because multi-collinearity between several of these variables was identified, variables were grouped into 14 subsets. Multivariate mixed
models for each management subset included variables that had p values < 0.250 in univariate models. Variables with p-values < 0.100 in the subset models were included in a full model. In a step-wise fashion, the variable with the highest p value was removed from the model until a reduction in the model AIC was no longer achieved.

Similarly, mixed models assessed the effect of management practices and production on fecal cortisol levels for individual management groups within farms including sick cattle, calving pen, combined lactating cows (not subdivided by production), high-producing lactating cows, low-producing lactating cows, dry cows and heifers. Only those management practices or production variables directly related to animals housed within each location were evaluated in these models. When necessary, a log transformation was used to achieve a normal distribution of the fecal cortisol levels for a specific location.

5.4 Results

A total of 219 herd visits were made to 55 Ohio dairy herds during the observation period. DHI records were available for a subset of 37 herds. 43 herds were classified as MAP-infected, while the remaining 12 were Ohio Test Negative Status herds.

Fecal cortisol analysis was performed on 846 fecal samples. The herd contribution to these samples ranged from 8 (n = three herds) to 28 samples (n = one herd). Composite fecal samples were obtained from 11 separate management groups; however, areas that housed lactating cows (including subgroups for high and low
producing cows) (n=303), dry cows (n=190) and heifers (n=190) represented 80% of the samples analyzed. A similar number of samples were collected at each herd visit, ranging from 194 samples at visit 4 to 225 samples at visit 1.

Fecal cortisol levels ranged from 2480.91 pg/g of feces to 20,634.9 pg/g of feces with a right-skewed distribution. For all fecal cortisol levels, the mean value was 7304.78 pg/g of feces with a standard deviation of 2361.00 pg/g of feces.

Table 5.1 presents the number of samples, the mean fecal cortisol values, and the standard deviation for the means for each herd. Mean fecal cortisol levels differed between Herd I and Herds HH, PP and U; Herd U and Herds BB, CC, H; and Herd PP and Herd CC.

Table 5.2 presents the number of samples, the mean fecal cortisol values and the standard deviation for the means for each management group within a herd. Although samples from 11 groups were collected, values from four -- combined calving pen/sick pen, medium-producing lactating cows, post-freshening cows and combined lactating/dry cows -- were excluded from subsequent analysis because of small sample size.

Mean fecal cortisol levels were observed to increase for areas housing heifers, lactating cows, sick cows, calving pen and dry cows, respectively. The mean fecal cortisol level for locations housing heifers was lower than that for areas housing lactating cows, dry cows and the calving pen. The mean fecal cortisol level of areas housing dry cows was higher than all milking cow groups and the area housing sick
individuals, although it did not differ from that of the calving areas. Results of additional multiple comparison tests are also identified in Table 5.2.

Table 5.3 presents the number of samples, the mean fecal cortisol value, and the standard deviation for the means of each visit. Mean fecal cortisol values differed between Visits 1 and 2, Visits 2 and 3, and Visits 3 and 4.

The mean fecal cortisol level for 173 samples from non-infected herds was 7216.17 pg/g of feces with a standard deviation of 2323.15 pg/g of feces. This did not differ from that for the 673 samples obtained from infected herds (7327.57 pg/g of feces ± 2371.76).

When modeling log-transformed fecal cortisol values as the outcome of interest, “farm” was specified as a random effect, “visit” was specified as a fixed effect and the covariance structure between repeated measurements taken from management groups within farms was best described by specifying compound symmetry. Visit (p<0.001), herd (p=0.04) and management group within herd (p=0.002) all significantly influenced log-transformed fecal cortisol values. Of the total variability present in the log-transformed fecal cortisol values, herd accounted for 2.9% and group within herd represented 11.8% of this variability.

For the evaluation of the effect of herd management practices, DHI production parameters and herd MAP-infection status on herd fecal cortisol levels, the log-transformed mean fecal cortisol level for each visit within each herd was specified as the outcome of interest. Visit within herd was identified as a repeated measure, and the variance components covariance structure was selected to model this relationship.
122 variables were screened for significance in univariate models. The 51 variables with p values < 0.250 that were selected for inclusion in 14 subsequent variable-subset screening models are identified in Table 5.4. Herd MAP-infection status was not significantly associated with mean herd fecal cortisol at the 0.250 level in a univariate model. The full mixed model included 12 variables that had p values < 0.100 in a subset model. Using a backward building technique, 7 variables remained in the final model. (Table 5.5)

When the effects of management practices and production on fecal cortisol levels for individual locations within farms were evaluated, variables that had p values <0.250 in univariate models were no longer significant when combined in full models.

5.5 Discussion

To the author’s knowledge, this is the first report utilizing fecal cortisol measurements in an observational study in dairy herds. Several factors that influence fecal cortisol levels were identified. Farm, management group and time of sample collection were significantly associated with fecal cortisol levels.

Given the variability that exists between herds regarding management practices and milk production, differences in fecal cortisol levels were expected. Analysis of results obtained from epidemiologic studies involving multiple dairy farms typically includes procedures to control for the effect of “farm” on the outcome of interest. However, the management group sampled within a farm also had a significant effect on fecal cortisol levels observed in this study. In fact, group within herd explained nearly three times more variability in fecal cortisol levels than did the effect of farm. It
appears that controlling for management groups within herds, in addition to controlling for the herd effect, is necessary. This recommendation would likely apply to management groups within other animal populations for which fecal cortisol levels are measured as well.

Although differences in fecal cortisol levels between various management groups within dairy farms were expected, some observations were counter-intuitive. It was not surprising that the mean cortisol level for fecal samples obtained from areas housing heifers were the lowest identified in the study, given that animals in this age group were not faced with the physiological stresses of lactation or parturition as were other management groups examined in this study. It is interesting, however, that the mean fecal cortisol value for dry cows was significantly higher than that of lactating cows. The physiologic stresses of lactation are often cited as potential welfare concerns for dairy cattle. Yet, this observation suggests that chronic stress may be of more concern in cows that are not currently lactating. Perhaps the increased fecal cortisol level relative to that of lactating cows reflects physiologic stress resulting from an abrupt cessation of lactation coupled with the environmental/management changes that accompany this event. Additionally, because dry cows are in the last several months of gestation, it is possible that high circulating levels of progesterone and estrogen may have influenced cortisol levels. 5,10

Interestingly, few specific management practices or production parameters were significantly associated with the mean fecal cortisol levels of dairy herds represented in this study. Differences observed in log mean herd fecal cortisol levels
associated with the geographic location of herds within Ohio may reflect regional variations in temperature and/or weather conditions. Although increased fecal cortisol levels were expected in herds with high incidence of common infectious and/or metabolic diseases, increasing incidence of left displaced abomasums (LDA) and problem breeders was associated with decreasing fecal cortisol levels in this study. Increased herd fecal cortisol levels were associated with heifer purchases from heifer raisers or multiple sources. This observation may reflect increased herd exposure to infectious agents or changes in social structures subsequent to the introduction of new herd members. Interestingly, the purchase of heifers from traditional auctions was associated with lowered fecal cortisol levels although this situation is commonly discouraged because of biosecurity concerns for farms. The inability of producers to provide a response to a given question was repeatedly associated with elevated herd fecal cortisol levels relative to herds in which a response was provided. Perhaps failing to provide a response indicates a less rigorous approach to herd management that results in increased physiologic stress for the herd.

By using the arithmetic mean of multiple fecal cortisol measurements obtained on a given farm to represent a single, overall herd level, the identification of management factors that influenced fecal cortisol levels related to a specific management group may have been precluded. However, even when modeling fecal cortisol measurements for management groups within herds, significant explanatory variables were not identified.
We were unable to detect differences in fecal cortisol levels between MAP-infected and non-infected dairy herds. It is possible that fecal cortisol levels may differ between non-infected herds and herds with high burdens of infection; however, we would have been unable to detect these differences because the majority of infected herds described here (n=28) had within herd MAP-infection prevalence of <5%.

Although it may be more efficient to compare fecal cortisol levels between individual infected and non-infected cattle to further explore a possible relationship between chronic stress and MAP-infection or the development of clinical signs of Johne’s disease, several important limitations that would complicate the interpretation of results exist for this approach as well. First, considerable variability between individual animals has been reported for cortisol levels in serum and feces.\textsuperscript{7,15} Fecal cortisol measurements in individual animals are likely more influenced by reproductive cyclicity, pregnancy status and diurnal variations than are herd-level measurements.\textsuperscript{5,10} Finally, the temporal relationship between increased fecal cortisol levels and development of infection and/or disease could only be assessed by long-term prospective studies.

Several aspects of the use of fecal cortisol measurements in epidemiological studies require additional evaluation. For example, the ideal number of management groups to sample on a farm, samples per group relative to the number of animals within the group and the frequency of visitations require further investigation to identify the sampling strategy that best reflects the fecal cortisol levels of all
individuals within the herd. Further, a comparison of results obtained measuring both fecal cortisol and fecal cortisol metabolites as described by other researchers will identify the preferred approach to quantifying this hormone.

This study identified several factors that should be considered when utilizing fecal cortisol measurements in epidemiologic studies in dairy cattle. Because few specific management practices or production parameters that significantly effected these measurements were identified, it appears that controlling for management groups within herds of animals, in addition to controlling for the herd effect, may be sufficient to limit confounding of mean fecal cortisol levels in epidemiologic studies. Further, differences in fecal cortisol level were not observed between MAP-infected herds relative to herds considered to be non-infected. Several opportunities for refinement of these measurements as part of epidemiologic investigations were also identified and discussed.

5.6 Literature Cited


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Table 5.1 (continued)

Table 5.1: Mean fecal cortisol levels, standard deviation and number of samples analyzed by farm.
Table 5.1 (continued)

<table>
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<tr>
<th>Farm</th>
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<td>Number of Samples</td>
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<tr>
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<td>Growing Heifers&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Managed as a single group&lt;sup&gt;b,d&lt;/sup&gt;</td>
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<td>Low Producing&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>71</td>
<td>7224.9</td>
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<td>Sick Pen&lt;sup&gt;b,d&lt;/sup&gt;</td>
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<tr>
<td>Calving Pen&lt;sup&gt;b,c,d&lt;/sup&gt;</td>
<td>90</td>
<td>7924.4</td>
<td>2353</td>
<td></td>
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<tr>
<td>Dry Cows&lt;sup&gt;b,x&lt;/sup&gt;</td>
<td>190</td>
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<td>Post Freshening Cows</td>
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<td>Combined Calving Pen/ Sick Pen</td>
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Table 5.2:  Mean fecal cortisol levels, standard deviation and number of samples analyzed by location within farm where different superscripts indicate significantly different means.
<table>
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<tr>
<th>Visit</th>
<th>Number of Samples</th>
<th>Fecal Cortisol (pg/g feces)</th>
<th>Mean</th>
<th>Standard Deviation</th>
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<tbody>
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<td>2</td>
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<td>3</td>
<td>212</td>
<td>6730.1</td>
<td>1977</td>
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</tr>
<tr>
<td>4</td>
<td>194</td>
<td>7413.5</td>
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<td>All samples</td>
<td>846</td>
<td>7304.8</td>
<td>2361</td>
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</tr>
</tbody>
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Table 5.3:  Mean fecal cortisol levels, standard deviation and number of samples analyzed by visit.
### Table 5.4: Variables identified as significant at the 0.250 level in univariate mixed models and significant at the 0.100 level in management practice subset models.

<table>
<thead>
<tr>
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<th>DF</th>
<th>Univariate Model</th>
<th>Subset Models</th>
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<tr>
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<td>P value</td>
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<td>Herd Characteristics (n=6)</td>
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<td>0.260</td>
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<td>0.020</td>
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<td>Ohio region of herd location</td>
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<td>2.41</td>
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<tr>
<td>Use of BST</td>
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<td>1.64</td>
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<tr>
<td>Percentage of cattle that receive BST (Continuous)</td>
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<tr>
<td>MAP-Infection Status (n=4)</td>
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<td>Individual who initiated herd testing</td>
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<td>Primary reason tested was initiated</td>
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<td>3.66</td>
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<td>Previous MAP testing</td>
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<td>MAP Testing and Culling Strategies (n=7)</td>
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<td>Culling practices for clinical cases</td>
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<tr>
<td>Mastitis</td>
<td>8</td>
<td>1.38</td>
<td>0.208</td>
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<tr>
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<tr>
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<tr>
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<td>Clinical cases of Johne’s Disease</td>
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<td>Mastitis is a primary culling factor</td>
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<td>0.234</td>
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<tr>
<td>Johne’s disease is a primary culling factor</td>
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<td>0.017</td>
</tr>
<tr>
<td>Reproduction is a primary culling factor</td>
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<td>0.017</td>
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<td>0.008</td>
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<td>Herd purchases are isolated ≥ 2 weeks</td>
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<td>Vet inspection prior to purchase</td>
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<td>Potential purchases tested for MAP</td>
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Table 5.4 (continued)
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<th>Subset Models</th>
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<td>4.25</td>
<td>0.040</td>
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<td>Loose housing for lactating cows</td>
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<td>Adopted bottle feeding colostrum</td>
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<td>Began pasteurizing milk/colostrum</td>
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<td>Focused on keeping calving areas cleaner</td>
<td>2</td>
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<td>0.147</td>
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<td>Moved housing of heifers</td>
<td>2</td>
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<td>0.163</td>
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<td>Reduced cow-to-calf exposures</td>
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<td>Changes in manure management</td>
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<td>0.161</td>
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<td>-----------------------------------------------</td>
<td>--------------------------------------------------</td>
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<tr>
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<tr>
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</tr>
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<td>0.454</td>
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<td>West</td>
<td>Reference</td>
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<tr>
<td>Yes</td>
<td>-0.174</td>
<td>0.063</td>
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</tr>
<tr>
<td>Unable to provide response</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incidence of LDA in previous six month period</td>
<td></td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>No cases</td>
<td>0.012</td>
<td>0.749</td>
<td></td>
</tr>
<tr>
<td>≤ 5%</td>
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</tr>
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<td>5%&lt;10</td>
<td>0.056</td>
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<td></td>
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<tr>
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<td>-0.090</td>
<td>0.0214</td>
<td></td>
</tr>
<tr>
<td>20%&lt;30</td>
<td>-0.1089</td>
<td>0.0933</td>
<td></td>
</tr>
<tr>
<td>30%&lt;40</td>
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<tr>
<td>40%&lt;50</td>
<td>N/A</td>
<td>N/A</td>
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<td>&gt;50%</td>
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<td>0.6692</td>
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</tr>
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<tr>
<td>Incidence of problem breeders</td>
<td></td>
<td>0.022</td>
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<tr>
<td>No cases</td>
<td>0.306</td>
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<td>≤ 5%</td>
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<td>30%&lt;40</td>
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<td>40%&lt;50</td>
<td>0.104</td>
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<td>&gt;50%</td>
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<td>Source of purchased heifers</td>
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<td>0.028</td>
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<td>No purchases</td>
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<td>0.201</td>
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<td>Auction</td>
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<td>Dispersement/consignment sale</td>
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<td>Private treaty</td>
<td>0.115</td>
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<tr>
<td>Heifer raiser</td>
<td>0.326</td>
<td>0.088</td>
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<td>Multiple sources</td>
<td>0.245</td>
<td>0.072</td>
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<td>Reference</td>
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Table 5.5 (continued)

Table 5.5: Variables included in final mixed model for mean herd fecal cortisol levels.
### Variable Effects on Log-Mean Herd Cortisol Level

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<th>Variable</th>
<th>Estimated Effect on log_mean herd cortisol level</th>
<th>P value</th>
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<tbody>
<tr>
<td><strong>Previous MAP testing</strong></td>
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<td></td>
</tr>
<tr>
<td>First test</td>
<td>-0.069</td>
<td>0.014</td>
</tr>
<tr>
<td>One year</td>
<td>-0.116</td>
<td>0.014</td>
</tr>
<tr>
<td>Two years</td>
<td>-0.090</td>
<td>0.016</td>
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<tr>
<td>Three years</td>
<td>-0.035</td>
<td>0.261</td>
</tr>
<tr>
<td>More than three years</td>
<td>Reference</td>
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</tr>
<tr>
<td><strong>Reproduction as a primary culling factor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>-0.1744</td>
<td>0.018</td>
</tr>
<tr>
<td>Unable to provide a response</td>
<td>Reference</td>
<td></td>
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</tbody>
</table>
CHAPTER 6

ASSOCIATIONS BETWEEN TEST RESULTS FOR MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS AND MILK PRODUCTION INDICES IN OHIO DAIRIES

6.1 Abstract

The purpose of this study was to compare testday milk, milk protein and milk fat production between cows that were test-positive relative to those that were test-negative for both the ELISA and fecal culture for Mycobacterium avium subsp. paratuberculosis (MAP). Milk production records were obtained directly from DHI Cooperative, Inc. Production data included testday milk, milk protein, and milk fat production as well as somatic cell counts and linear scores for individual cows from June 2000 through June 2002. Additionally, the most recent calving date and the lactation number were available for each cow. Results for individual animal MAP testing completed during whole-herd MAP screening tests that were submitted to the Animal Disease Diagnostic Laboratory, the Ohio Department of Agriculture from January 1999 through June 2002 were obtained directly from the laboratory. When DHI data was matched with Johne’s disease test results collected within a 30-day period, a subset of 4199 observations representing 3810 individual cows was
available. Mixed linear univariate models that nested individual cow observations within herd were used to assess associations between ELISA and fecal culture results for each production parameter while controlling for the effects of herd, lactation number and days-in-milk.

On a given testday, a positive fecal culture result was associated with an average reduction of 8.0 pounds of milk, 0.25 pounds of milk protein and 0.31 pounds of milk fat per individual cow. Positive ELISA results were associated with a significant reduction in testday milk production, 3.9 pounds, relative to cows with ELISA negative results. Further, ELISA-positive cows with S/P ratios ≥0.800 produced significantly less milk, 7.8 pounds less, than those cows classified as ELISA-negative. These observations provide additional information for dairy producers to consider as they make culling decisions regarding MAP test-positive cows.

6.2 Introduction

Annual production losses associated with Johne’s disease for the United States dairy industry are estimated to be between $200 and $250 million. Reduced milk production has been identified as a primary determinant of these losses.1-3

Numerous studies have evaluated the effect of MAP-infection or seropositivity to MAP on milk production indices in subclinically infected cows.3-8 Most frequently, these studies have reported reductions in the mature equivalent (ME) milk production of cows with positive MAP test results.6-8 However, increased milk production in ELISA-positive cows relative to test-negative cows has been reported.5 Associations
identified between MAP test status and somatic cell counts have been less consistent.\textsuperscript{5-8} Recently, Johnson et al were unable to identify significant differences in ME milk, fat or protein production relative to results from ELISA and radiometric fecal cultures for MAP.\textsuperscript{4}

Mature equivalents (ME) are the preferred index for comparing milk production between individual cows. ME are standardized measures that estimate an individual cow’s milk production over a 305-day lactation while controlling for factors that influence production potential such as age of the cow, season of calving, and geographic location. Although using ME allows for the direct comparison of production between cows, this measure represents the theoretical milk production during a lactation, rather than the actual production of a cow. Comparisons of milk production indices that reflect actual differences in production between individual cows relative to MAP test results rather than estimated differences would be of practical significance for dairy producers.

The purpose of this study was to compare testday milk, milk protein and milk fat production between cows that were test-positive relative to those that were test-negative for both the ELISA and fecal culture for MAP. Further, comparisons of these production parameters relative to the magnitude of ELISA S/P ratios were evaluated.

\textbf{6.3 Methods}

Private veterinary practitioners as well as state and federal veterinary medical officers involved with herd Johne’s disease testing and control programs were contacted and asked to identify dairy herds that would likely agree to participate in the
study. Eligibility for herd participation was restricted to Ohio dairy herds that completed annual whole-herd testing for Johne’s disease. Both MAP-infected and Ohio Test Negative Status herds were recruited for participation.

Results for individual animal MAP tests completed during whole-herd MAP screening that were submitted to the Animal Disease Diagnostic Laboratory, the Ohio Department of Agriculture from January 1999 through June 2002 were obtained directly from the laboratory. Positive fecal cultures were those where isolates were confirmed as MAP based on colony morphology, mycobactin dependency and acid-fast staining. ELISA with S/P ratios ≥ 0.250 were classified as positive. ELISA S/P ratios were further categorized into three strata: ≤ 0.249, 0.250-0.799 and ≥ 0.800.

Milk production records were obtained directly from DHI Cooperative, Inc. for a subset of 32 herds. Production data included testday milk, milk protein, and milk fat production as well as somatic cell counts and linear scores for individual cows from June 2000 through June 2002. Additionally, the most recent calving date and the lactation number were provided for each cow. Days-in-milk was calculated as the difference in days between the most recent calving date and the DHI testdate. For each cow, this value was categorized into one of seven strata: < 30 days, 30 < 90 days, 90 < 195 days, 195 < 305 days, 305 < 400 days, 400 < 600 days, ≥ 600 days.

Production records were matched with MAP test results where both sample collections occurred within a 30-day period. Unmatched records and matched tests that were completed more than 30 days apart were excluded from further analysis.
Mixed linear univariate models that nested individual cow observations within herds were used to assess associations between ELISA and fecal culture results and each production parameter outcome while controlling for herd, lactation number and days-in-milk. Several covariance structures including compound symmetry, first-order autoregression, variance components and unstructured variance were evaluated for model fit, and the covariance structure consistently resulting in models with the lowest AIC value was selected.

6.4 Results

Johne’s disease test records were obtained for 54 Ohio dairy herds, 12 of which were classified as Ohio Johne’s Disease Test Negative herds. The remaining 42 herds were MAP-infected with 28 herds classified as low-prevalence herds (<5%) and 14 were considered to be high prevalence herds (>5%). Samples for ELISA and fecal cultures were collected at 508 separate testing events, 162 representing whole-herd screening for MAP infection. The remaining tests were performed as diagnostic tests in suspected clinical cases or to confirm infection in animals identified as ELISA-positive during herd screening.

DHI production data including test-day milk, milk protein, milk fat, and somatic cell count, as well as the lactation number and the most recent calving date, were obtained for individual cows from a subset of 32 dairy herds. Following matching with results for Johne’s disease tests that were collected within a 30-day period of DHI sampling, data for a subset of 4199 observations representing 3810 individual cows was analyzed.
Of the 3085 ELISA included in this subset, 165 (5.4%) were positive. S/P ratios were available for 3027 of these ELISA. Positive results were obtained for 106 (6.5%) of the 1638 fecal cultures evaluated. These results identified 259 cows as ELISA-positive and 101 as MAP-infected. The majority of cows had matched DHI and Johne’s disease records for a single test (n= 3436).

Of the cows contributing to the production data analyzed, the majority were in their first- or second-lactation (58.9%), although over 200 observations were obtained from cows in their sixth-lactation or higher (4.8%). Days-in-milk for these cows ranged from 1 to over 1000 days with an average lactation length of 175 ± 138 days. Slightly more than 80% of these cows (n= 3535) had lactation lengths less than 305 days.

Testday milk production ranged from 2.6 pounds to 178.2 pounds with an average daily production of 72.4 ± 24.2 pounds per cow. A range of 0.0 to 5.0 pounds was observed for milk protein production (mean = 2.2 ± 0.6 pounds), while milk fat production ranged from 0.0 to 7.9 pounds with a mean of 2.6 ± 1.0 pounds. Table 6.1 presents the means and standard deviations for these production parameters relative to MAP test results.

Adjusted means, their standard errors and the estimated effect of a positive test result on production indices were obtained from mixed linear models specifying a first order autoregressive covariance structure. Tables 6.2 and 6.3 present the mean testday milk production, milk protein and milk fat adjusted for herd, lactation number and
days-in-milk relative to matched ELISA and fecal culture results. Additionally, associations between ELISA S/P ratios and these production indices are described in Table 6.4.

A significant reduction in milk, milk protein and milk fat production was associated with positive fecal culture results collected within 30 days of DHI testing. On a given testday, a positive fecal culture result was associated with an average reduction of 8.0 pounds of milk, 0.25 pounds of milk protein and 0.31 pound of milk fat per individual cow. Positive ELISA results were associated with a significant reduction in testday milk production, 3.9 pounds, relative to cows with ELISA negative results. Further, ELISA-positive cows with S/P ratios ≥0.800 produced significantly less milk, 7.8 pounds less, than those cows classified as ELISA-negative. Significant relationships for other production parameters relative to ELISA results were not identified. No significant relationships between MAP test results and somatic cell counts categorized in quartiles nor linear scores were identified.

6.5 Discussion

This study is unique in that actual milk production measurements for nearly 4000 dairy cows are evaluated relative to MAP test results obtained within a 30-day period. Other studies evaluating the effect of MAP-infection or seropositivity on milk production in dairy cattle have typically included much smaller sample sizes and have utilized estimated (ME) production values. By restricting our analysis to those production measures and MAP test results occurring within a 30-day period, we identify the actual production losses occurring at the time of identification as MAP
test-positive. The associations we identify here provide additional information for dairy producers to consider as they make culling decisions regarding MAP test-positive cows.

Because herd, DIM and lactation number are important determinants of milk production, these variables were forced into our linear model to produce adjusted estimates of the means for the production indices relative to MAP test result. Further, the clustering of cows within herd was specified in order to produce valid estimates of standard error.9

Positive fecal culture results were associated with significant reductions in milk, milk protein and milk fat production in this study. This observation provides additional incentive for producers to quickly remove these individuals from the herd. In addition to serving as a source of environmental contamination with MAP, these infected cows can represent a considerable economic loss for producers. For example, our observations suggest that in a 100-cow dairy with a 5% prevalence of fecal culture positive cows, an estimated 40 pounds of milk production will be lost daily, 280 pounds lost weekly, and 1120 pounds lost monthly.

Positive ELISA results were less consistently associated with reductions in the production parameters evaluated. Although a significant reduction in milk production was observed for ELISA-positive cows, this effect was less pronounced than that observed for fecal culture positive cows. Misclassification of infection status associated with the ELISA, both false positive and false negative results, would explain the change in the estimate toward the null.
To the authors’ knowledge, this is the first report to quantitatively evaluate ELISA S/P ratios relative to milk production. We identified a significant reduction in milk production between ELISA negative cows and those with ELISA S/P ratios ≥ 0.800. Interestingly, the magnitude of this reduction, 7.8 pounds per cow/day, was very similar to that observed for fecal culture positive cows, 8.0 pounds per cow/day. In previous reports, ELISA S/P ratios significantly higher than the manufacturer’s recommended cut-off have been associated with both a high likelihood of MAP infection as well as persistently high S/P ratios on repeated testing.\textsuperscript{10,11} Results of this study suggest that cattle with high ELISA S/P ratios also demonstrate significant reductions in milk production similar to that observed for MAP-infected cows. These observations corroborate other findings reported here (Chapter 3), and may justify the prompt removal of these individuals from the herd.

Given differences in the outcomes of interest between this study and previous studies, it is difficult to compare our estimates to those that have been reported. Assuming a 305-day lactation, results of this study would suggest that an average of 2440 pounds of milk production would be lost during a lactation in a fecal culture-positive cow. This is similar to the estimated reduction in ME values identified by others.\textsuperscript{7,8} Further, based on the differences identified here, we would estimate production losses of 1139 pounds per lactation for an ELISA-positive cow which is slightly more than the reduction in ME estimated by Nordlund et al for ELISA-positive cows.\textsuperscript{6}
However, it may be inappropriate to extrapolate the production losses observed here over an entire lactation. Other than indicating that the cows in this study were not displaying signs associated with clinical Johne’s disease, we were unable to determine the stage of MAP infection for the cows represented in this study, nor were we able to confirm MAP infection status for those cows without positive fecal culture results. It has been suggested that the stage of MAP-infection is an important determinant of both the magnitude and the direction of the effect observed for milk production relative to MAP test results. If a large proportion of the test-positive cows represented in this study were in late stages of infection, the effects we report may overestimate the relationships between MAP test result and milk production. However, given the large number of herds with varying levels of within herd MAP prevalence represented in this study and the exclusion of individuals with overt clinical signs of Johne’s disease from this analysis, we do not believe this to be a likely scenario.

Results of this study confirm previous reports that have identified reductions in milk production with positive MAP test results. Significant production losses, 8.0 pounds per fecal culture-positive cow per day, were observed for a given testday relative to results of a fecal culture. This provides further incentive for producers to remove these individuals from the herd since milk production appears to be compromised at the time of identification as a MAP-infected individual. Further, this reduction associated with positive fecal culture results was similar to that observed for ELISA negative cows relative to those with ELISA S/P ratios ≥0.800, corroborating
previous reports that suggest cows with ELISA S/P ratio significantly greater than the manufacturer’s recommended cut-off are likely to be infected.

6.6 Literature Cited


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<tr>
<th>Production Parameter</th>
<th>Fecal Culture Result</th>
<th>ELISA Result</th>
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</thead>
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<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>Milk Production (lbs/cow)</td>
<td>73.0</td>
<td>23.0</td>
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<td>2.2</td>
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<tr>
<td>Milk Fat (lbs/cow)</td>
<td>2.7</td>
<td>1.0</td>
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Table 6.1: Mean testday milk, milk fat and milk protein production relative to results of concurrent fecal culture and ELISA testing.
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<th>Number of tests</th>
<th>Negative Adjusted Mean</th>
<th>Negative Standard Error</th>
<th>Positive Adjusted Mean</th>
<th>Positive Standard Error</th>
<th>Comparison of Means (p value)</th>
<th>Estimated Effect of a Positive Result on Parameter</th>
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<td>Milk Production (lbs/cow)</td>
<td>1532</td>
<td>64.2</td>
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<td>1.7</td>
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<td>Milk Fat (lbs/cow)</td>
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<td>106</td>
<td>2.2</td>
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Table 6.2: Adjusted means for milk production parameters when controlling for herd, lactation number and days-in-milk relative to results of a concurrent fecal culture.
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<th>Production Parameters</th>
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<tbody>
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<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of tests</td>
<td>Adjusted Mean</td>
<td>Standard Error</td>
<td>Number of tests</td>
<td>Adjusted Mean</td>
<td>Standard Error</td>
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<tr>
<td>Milk Production (lbs/cow)</td>
<td>2920 57.0 1.1</td>
<td>165 53.2 1.8</td>
<td>0.026</td>
<td>-3.9</td>
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<td>Milk Protein (lbs/cow)</td>
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<td>Milk Fat (lbs/cow)</td>
<td>2873 2.2 0.05</td>
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<td>0.301</td>
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Table 6.3: Adjusted means for milk production parameters when controlling for herd, lactation number and days-in-milk relative to results of a concurrent ELISA.
Table 6.4: Adjusted means for milk production parameters when controlling for herd, lactation number and days-in-milk relative to S/P ratios of concurrent ELISA where different superscripts indicate significantly different means using Tukey’s test for multiple comparisons.
CHAPTER 7

COMPARISON OF MANAGEMENT PRACTICES BETWEEN OHIO DAIRY FARMS INVOLVED IN JOHNE’S DISEASE TESTING PROGRAMS VERSUS THOSE FARMS NOT INVOLVED IN TESTING

7.1 Abstract

The purpose of this mail survey was to compare the adoption of management practices recommended for Johne’s disease control between herds involved in whole-herd testing programs versus those that do not routinely test for Johne’s disease. Eight hundred-ten (810) Ohio dairy herds were selected to participate in a mail survey during the fall of 2002. A total of 266 questionnaires were returned (32.8% response rate). Of the twenty management practices recommended for Johne’s disease control that were evaluated, nine differed between TESTING and NON-TESTING herds. TESTING herds more frequently reported adopting changes within the past five years with respect to all nine management practices evaluated relative to NON-TESTING herds. Producers with TESTING herds reported greater familiarity with Johne’s disease than those with NON-TESTING herds. Because it is conceivable that only
producers who believe their herds to be infected would be motivated to adopt the management practices recommended for control of Johne’s disease, the above comparisons were repeated when controlling for producer-perceived infection status. Interestingly, the relationships between testing status and management practices were relatively consistent regardless of whether producers believed their herd to be infected or not. We suspect that the on-farm educational efforts that occur concurrently with testing for Johne’s disease contribute to the adoption of these management practices and the level of producer familiarity with the infection. Results of this study suggest that herds that participate in this program are not only encouraged to adopt management practices to control Johne’s disease, they actually comply with many of these recommendations. Although this study provides preliminary evidence confirming the value of Johne’s disease testing programs, several opportunities for improvement, including the prioritization of control recommendations, were identified.

7.2 Introduction

Considerable pressure has been placed on the dairy industry to control *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative agent of Johne’s disease in ruminants. The United States Department of Agriculture estimates that the US dairy industry suffers losses of $200 to $250 million annually as a result of MAP infections in dairy cattle.\(^1\) There is speculation that trade restrictions may soon prohibit the international movement of MAP-infected animals or products that originate from
these animals. Additionally, the role of MAP as a potential causative agent of Crohn’s
disease in humans is under increasing scientific scrutiny.

In response to these factors, many states, including Ohio, have instituted
programs that promote on-farm control of Johne’s disease. Control programs focus on
reducing prevalence and limiting transmission in infected herds. Certification or
status programs rely on repeated herd testing to validate that a herd is unlikely to be
infected with Johne’s disease. According to the United States Department of
Agriculture, 20 states had Johne’s disease control programs and 25 had herd
certification/status programs in place as of January 1, 2002. Several other states had
control and/or herd certification/status programs in development at that time.

In April 2002, USDA-APHIS published the Uniform Program Standards for
the Voluntary Bovine Johne’s Disease Control Program. The purpose of this
document was to “provide minimum national standards for the control of Johne’s
disease.” The program outlines a testing strategy as well as addressing education and
management requirements for herds that choose to participate. At the state level,
administrative requirements for personnel, Johne’s-certified veterinarians and an
advisory committee are established. Finally, performance standards for diagnostic
laboratories providing testing as part of this program are identified. As a result of
this document, states will continue to devote sizeable financial investment to the
development of educational programs, on-farm risk assessments and herd testing
programs in accordance with the minimum standards it sets forth.
Despite these investments, few reports have addressed the adoption of recommended management practices on dairy farms following participation in these testing programs or education about the transmission of Johne’s disease. However, it appears that a considerable gap may exist between the dissemination of control recommendations and their implementation on farms. In Victoria, Australia, the majority of respondent herds in a mail survey of 800 randomly selected dairy farms complied with zero or only one of six recommendations aimed at Johne’s disease control -- despite extensive efforts by the government to educate producers about the importance of these practices in disease control. Results of one component of the USDA-APHIS NAHMS Dairy ’96 survey indicated that few preventive management practices were associated with either familiarity of a dairy farm manager with Johne’s disease or prior diagnosis of Johne’s disease in a given herd. Findings from these studies emphasize the need for the evaluation of existing efforts aimed at Johne’s disease control to determine their effectiveness in facilitating the adoption of management practices on dairy farms.

The Ohio Department of Agriculture (ODA) has invested considerable resources to provide Ohio dairy producers with Johne’s disease education programs, herd screening tests and on-farm risk assessments. The Ohio Johne’s Disease Test Negative Status Program establishes annual whole-herd testing requirements for herds that are believed to be non-infected. Participation in this program allows producers to advertise their Test Negative Status so that prospective cattle buyers have an increased confidence that the herd is not infected. This program has more herds enrolled than
any similar program in the United States. Additionally, these services are provided in a less formal program for infected dairy herds. Currently, fecal culture and serum ELISA testing are provided at no cost to producers that submit a herd plan outlining control measures in place or those that producers intend to adopt to reduce Johne’s disease transmission within their herds. If requested by the herd veterinarian, a state or federal veterinary medical officer will collect diagnostic samples and perform an on-farm risk assessment at no charge to the producer.

The purpose of this mail survey was to compare management practices recommended for Johne’s disease control between herds involved with state-supported testing programs in Ohio, both the Test Negative Status program and the control program, relative to those that were not testing. Specifically, two research questions were of interest: (1) Are farms that actively test for Johne’s disease more likely to adopt management practices believed to reduce the risk of Johne’s disease transmission? (2) Does participation in a Johne’s disease testing program result in changes in management practices?

7.3 Methods

In total, 810 questionnaires were mailed to dairy producers in September 2002 along with a letter from the investigators explaining the purpose of the survey and a postage-paid business reply envelope. The Animal Disease Diagnostic Laboratory of the Ohio Department of Agriculture (ADDL-ODA) identified 270 dairy farms that had submitted fecal or serum samples as part of herd screening tests for Johne’s disease from January 2001 through July 2002. Questionnaires were mailed to all 270 of these
herds. An additional 540 herds not identified as participants in testing programs were randomly selected from the roster of Grade A dairy herds in Ohio provided by the Dairy Division, ODA to receive questionnaires.

Because a producer’s fear of regulatory consequences or public identification as an infected herd may have negatively impacted the response rate, questionnaires were not individually coded. However, a follow-up postcard encouraging non-respondents to participate was mailed to all dairy herds two weeks after the initial mailing in an attempt to improve the response rate in the absence of tracking non-respondents.

The questionnaire consisted of 38 closed-ended questions that inquired about general herd characteristics, management practices relating to Johne’s disease control, changes that have occurred within the last five years with respect to management practices recommended for the control of Johne’s disease, producer knowledge of Johne’s disease, the perceived infection status of the herd by the producer and herd Johne’s disease testing history. Herds that reported previous herd testing were asked to complete six additional questions concerning their current testing strategy for Johne’s disease and the disposition of animals identified as test positive.

Univariate logistic regression models assessed the relationship between herd testing status (TESTING versus NON-TESTING) and each management practice, each change in management practices and producer knowledge about Johne’s disease. Wald’s chi-square p-values <0.05 identified outcomes that were different between the TESTING and NON-TESTING groups. For those management practices that were
identified as different between the two groups, odds ratios representing the odds that TESTING herds reported a specific management practice relative to NON-TESTING herds were calculated with 95% confidence intervals. For questions with ordinal responses, median responses were compared between the TESTING and NON-TESTING groups using the Wilcoxon rank sum.

Producer-perceived herd infection status was assessed as a potential confounder of the relationship between Johne’s disease testing status and the various management practices, changes in management practices and knowledge about Johne’s disease evaluated as part of the questionnaire. Herds were classified as INFECTED or NON-INFECTED based on the producer’s perception of herd infection status. Herd testing did not necessarily form the basis for these beliefs. Subsequent logistic regression models controlled for producer-perceived herd infection status while assessing the relationship between Johne’s disease testing status and management practices, changes in management practices and knowledge about Johne’s disease. Adjusted odds ratios and their 95% confidence intervals were calculated.

7.4 Results

Of the 810 surveys that were mailed to dairy producers, 266 (32.8%) were completed and returned. The response rate in the subset of herds that had submitted samples for Johne’s disease herd testing within the previous 18 months, 51.1%, was higher than the 23.7% response rate for the Grade A dairy herds that were randomly selected for participation in this survey (p<0.0001). Of the 266 herds that responded to
the survey, 157 (59.0%) reported previously completing a whole herd test for Johne’s disease. These herds were classified as the TESTING group for subsequent analysis. The remaining 109 herds did not report whole herd testing for Johne’s disease and were subsequently considered to be the NON-TESTING group.

Herd size was different between TESTING and NON-TESTING herds. Herds in the TESTING group, median size of 81, were larger than those in the NON-TESTING group, median of 61 (p=0.0004).

Table 7.1 presents descriptive statistics regarding responses to questions concerning management practices recommended for Johne’s disease control. Of the twenty management practices that were compared between TESTING and NON-TESTING herds, the distribution of responses to questions concerning nine of these practices differed between the two groups. Relative to NON-TESTING herds, TESTING herds were more likely to: be an open herd with respect to animal purchases, remove a calf from its dam within one hour following calving, use an individual calving pen, hand feed colostrum, clean teats prior to collecting colostrum, feed calves commercial milk replacer, use a separate tractor/skidloader for cleaning and feeding, wash/disinfect the bucket/tires of tractor/skidloader between cleaning and feeding, and switch the bucket of tractor/skidloader between cleaning and feeding. Additionally, TESTING herds were less likely to feed discard milk to calves or calve on pasture than NON-TESTING herds. Table 7.2 reports the unadjusted odds ratios with 95% confidence intervals representing the odds that TESTING herds reported a
specific management practice relative to the odds that NON-TESTING herds reported the same management practice.

Those management practices that did not differ between TESTING and NON-TESTING herds in this survey included: frequency of the use of holding pens for sick cattle as calving areas, frequency of new bedding for calving areas, use of pooled colostrum versus colostrum from a single cow, exposure of pre-weaning, post-weaning, and bred heifers to adult cattle or manure from adult cattle, use of shared feed bunks and water troughs between heifers and adult cattle, equipment use for feeding and manure cleaning and spreading manure from adult cattle on forage land to be harvested for or grazed by heifers.

TESTING herds were more likely than NON-TESTING herds to report adopting changes within the last five years (since 1997) for each of nine specific management practices recommended for Johne’s disease control. TESTING herds reported adopting a greater number of management changes (median = 4.0) than NON-TESTING herds (median = 0.0), (p<0.0001). The odds ratios representing the odds of adopting each of the nine possible management changes in the TESTING herds relative to NON-TESTING herds are presented in Table 7.3.

In TESTING herds, the time of adoption for each of the nine management changes was compared to the time at which herd testing was initiated. Of TESTING herds that reported making management changes, the majority indicated that these changes occurred at the time of or following the initiation of herd testing for all nine management practices. (Figure 7.1)
Concerning the Johne’s disease infection status of their herds, 130 producers considered their herds to be INFECTED, while 118 believed their herds were NON-INFECTED. The remaining 18 survey respondents were unsure of their herds’ infection status or failed to provide an answer for this question; thus eliminating them from further analysis. Producer-perceived herd infection status was associated with testing status (p<0.0001). Herds that were TESTING were 8.6 times more likely to report that their herd was INFECTED than NON-TESTING herds. Additionally, eight of twenty management practices and eight of nine management changes were associated with infection status. Thus, producer-perceived infection status of a herd confounded the relationship between testing status and the outcomes of interest in this study.

Tables 7.4 and 7.5 report the adjusted odds ratios and the 95% confidence intervals comparing reported management practices and changes in management practices, respectively, between TESTING and NON-TESTING herds when controlling for producer-perceived infection status. These odds ratios indicate that, relative to NON-TESTING herds, TESTING herds were more likely to use individual calving pens, remove calves from dam within one hour following birth, hand feed colostrum, clean teats prior to nursing/collection of colostrum, use a holding pen for sick cattle as a calving area, and use a separate tractor/skidloader, a separate bucket, or wash and/or disinfect this machinery between cleaning and feeding. TESTING herds were less likely to calve on pasture or feed raw discarded milk to heifers than NON-TESTING herds. Additionally, TESTING herds were more likely than NON-
TESTING herds to report adopting changes within the last five years for seven of nine specific management practices recommended for Johne’s disease control.

Overall, respondents indicated that they considered themselves to have at least a basic level of knowledge concerning Johne’s disease. When asked to categorize their familiarity with Johne’s disease, over 60% of respondents considered themselves to be “fairly knowledgeable.” Name recognition without additional knowledge was reported by 6.8% of respondents. A single respondent indicated that he/she had “never heard of Johne’s disease.” Additionally, producer reported knowledge about Johne’s disease was different between TESTING and NON-TESTING herds (p<0.0001). When controlling for confounding by producer-perceived infection status, TESTING herds were 16.87 times (95% CI 3.46-82.40) more likely to report that they were “fairly knowledgeable” about Johne’s disease than NON-TESTING herds (p<0.0001).

In those herds that reported previous whole-herd testing for Johne’s disease, 150 provided responses to questions concerning their testing strategies. Of these herds, 58.7% complete whole-herd tests annually and 14% test their herd every six months. The remaining 27.3% of herds had only completed a single herd test prior to the survey. All but 6 of these 41 herds intend to continue herd testing in the future, but did not know the frequency at which future testing will occur. The majority of herds, 60.7%, use a combination of the fecal culture and serum ELISA for whole-herd testing. The remaining herds rely solely on fecal culture (16%) or serum ELISA (23.3%) for herd testing.
Producers were asked to categorize the typical outcome for animals with positive test results. Cow with positive fecal culture results frequently remained in the herd, despite evidence of fecal shedding of the infectious agent. Of 118 responses, 25% of producers indicated that fecal culture positive cows remained in the herd until clinical signs of Johne’s disease were observed, while 30% of producers reported allowing these cows to complete their current lactation prior to culling. The remaining 45% of respondents reported that fecal culture positive cows were culled as soon as possible after infection had been detected.

Concerning cows with positive serum ELISA results, 56% of 136 respondents reported that serial fecal culture was used to confirm the infection status of these cows. The remaining producers reported culling serum ELISA-positive cows without additional testing: 20% were culled as soon as possible after the test result was obtained, 8% were culled at the end of the current lactation, and 16% remained in the herd until the development of clinical signs of Johne’s disease.

Finally, producers were asked to describe their culling strategy for heifer calves that were born to dams infected (fecal culture positive) with MAP. Of 119 respondents, 53% did not routinely cull these heifers. Nearly 30% indicated that, although they would consider culling these heifers, this decision was primarily based on other factors. The remaining 17% of respondents culled either the most recent or all growing heifers born to known-infected cows.
7.5 Discussion

Few studies have evaluated the effectiveness of Johne’s disease testing programs with respect to the adoption of management practices on dairy farms. Ultimately, the goal of these programs is to reduce the prevalence of infection within a herd. Simultaneous with the process of collecting fecal or serum samples to be tested and the interpretation of test results, producers are educated about management practices that decrease the risk of disease transmission, hopefully facilitating the adoption of these practices.

Results of this study consistently demonstrate that participation in a testing program for Johne’s disease is associated with the adoption of management practices recommended for disease control in Ohio dairy herds. TESTING herds were more likely to report recommended management practices than NON-TESTING herds. Additionally, TESTING herds were more likely to report making changes in management practices in order to comply with recommendations believed to reduce the risk of disease transmission. Further, of TESTING herds that reported making management changes within the past five years, changes generally occurred concurrently or after the initiation of herd testing, providing temporal evidence to suggest that some component of participation in the testing program was at least partially responsible for the adoption of these practices on Ohio dairy farms.

Producers who believed their herds were not infected with Johne’s disease may have felt it was unnecessary to adopt management practices recommended for Johne’s
disease control. Interestingly, even after controlling for producer-perceived infection status, TESTING herds remained more likely to adopt certain management practices and more likely to make changes in order to comply with recommendations for Johne’s disease control. In other words, even if a producer believed his/her herd was not infected with Johne’s disease, participation in a testing program continued to be associated with the management practices recommended for disease control and the incorporation of changes to existing practices.

Utilization of the testing services provided by the ODA appears to encourage compliance with Johne’s disease control recommendations. We suspect that the extensive on-farm educational efforts that occur concurrently with herd testing contribute to this observation. Regular contact with private veterinary practitioners or veterinary medical officers allows management practices critical for disease control to be brought to the attention of the producer. Repeated interactions of these veterinarians with producers facilitate follow-up on the resolution of these issues. Additionally, producers may feel a sense of obligation to institute recommended management practices in exchange for the free testing and/or risk assessment services provided to them.

Despite the evidence to support the value of herd testing programs with respect to compliance with management practices recommended for Johne’s disease control, several opportunities for improvement remain. Although compliance with many of the recommended management practices evaluated was high, improvement in compliance rates was possible. For example, 38% of all producers reported that calves
routinely remained with their dams for more than 6 hours following birth, 37.5% indicated that discarded milk was occasionally or frequently fed to calves and 27% reported that weaned heifers younger than breeding age were exposed for some period of time to adult cattle and/or their manure. Also of concern was the observation that the majority of herds, 60.5%, reported occasionally or frequently using holding areas for sick cattle as calving areas. All of these practices facilitate the transmission of Johne’s disease in infected herds.

Interestingly, practices associated with tractor/skidloader use for cleaning and feeding were most strongly associated with testing status. Although reducing the possibility of fecal contamination of feed is an important control measure for many infectious diseases, with respect to control of Johne’s disease, it is not as critical as practices that limit exposure of calves to manure and/or milk contaminated with MAP such as prompt removal of the calf from its dam following birth, avoiding the use of discard milk to feed calves and limiting exposure of growing heifers to adult cattle or their manure. Perhaps producers found it easier, less costly and more immediately gratifying to implement practices associated with tractor/skidloader use than those concerning calf management and housing which are long-term investments. Regardless of the explanation, it is imperative that Johne’s disease control recommendations be prioritized when communicated to producers so that those practices that achieve the highest level of disease control can be identified and implemented, rather than focusing on those that may require less financial and time investment but are of secondary importance with respect to control.
Continued education is also necessary with respect to the interpretation of the diagnostic tests incorporated in herd screening programs. Despite a positive fecal culture, nearly 55% of cows actively shedding MAP in their feces remained in the herds represented in this study at least until the end of that cow’s current lactation. The prompt removal of subclinically infected individuals, considered by many to be a tenet of Johne’s disease control, must be emphasized to producers. Although permitting infected cows to complete the current lactation prior to culling is frequently considered an economically-justifiable compromise, maintaining known-infected cattle in a herd until the development of clinical signs is unacceptable and defeats the purpose of herd screening programs. Additionally, nearly 40% of respondent herds reported that a positive serum ELISA result initiated the removal of the cow from the herd. This practice may result in the unnecessary culling of truly non-infected cows with false-positive ELISA results. Because the serum ELISA that detect antibodies to MAP is intended for use as a screening test, producers should be encouraged to confirm positive ELISA results with a fecal culture or another test permitting the identification of MAP.

The potential biases of mail surveys have been reviewed and may have influenced the observed results. Given the potential regulatory consequences and the negative stigma associated with Johne’s disease, we chose to avoid any type of survey coding in an attempt to increase response rate and encourage producers to respond truthfully without a fear of repercussion. In doing so, we excluded the possibility of follow-up with non-respondents to assess the significance of non-response bias. As
with any survey, it is possible that producers responded to certain questions in a manner they believed would be viewed favorably by the investigators. We do not believe this type of information bias invalidates the results of this study because responses that would clearly be viewed as less than desirable management practices were not uncommon. Additionally, if present, this bias is likely to occur to some extent in both experimental groups (non-differential misclassification) and bias results toward the null.

Although the availability of free laboratory services and risk assessments is unique to the state of Ohio, it provides the opportunity to assess the effectiveness of programs with limited influence of the financial costs associated with herd testing. However, this situation may make extrapolation of the results from this study to other testing programs difficult. Other studies that evaluate the effectiveness of Johne’s disease testing and education programs with respect to compliance with recommended management practices are necessary to more fully explore the relationships described here. Priorities of NAHMS Dairy 2002 include the assessment of herd participation in Johne’s disease control or certification programs and identification of those management strategies adopted for prevention or control on dairy farms. Additionally, results from this cross-sectional study will be compared to those observed in Dairy ’96 to determine whether changes have occurred with respect to herd-level Johne’s disease control practices throughout the United States.14

Finally, other than inquiring about producer familiarity with Johne’s disease, we did not address specific educational efforts that may have influenced the adoption
of the management practices recommended for control. Similarly, although we identified an association between participation in a testing program and compliance with certain recommendations for control, we did not address the motivation behind the reported producer behavior, nor the specific aspects of participation that encouraged this behavior. Perhaps identification of these factors would allow their incorporation into existing programs to enhance the outcomes associated with testing and educational programs.

7.6 Literature Cited


Table 7.1 (continued)

Table 7.1: Responses to survey questions concerning management practices recommended for Johne’s disease control.

<table>
<thead>
<tr>
<th>Management Practice</th>
<th>Responses from TESTING Herds</th>
<th>Responses from NON-TESTING Herds</th>
<th>Total Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Percent</td>
<td>N</td>
</tr>
<tr>
<td>Herd Purchases</td>
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<td></td>
</tr>
<tr>
<td>Open</td>
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</tr>
<tr>
<td>Closed</td>
<td>58</td>
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<td>54</td>
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<tr>
<td>Calving Location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pasture</td>
<td>10</td>
<td>6.5</td>
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</tr>
<tr>
<td>Individual pen</td>
<td>47</td>
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<td>Group pen</td>
<td>65</td>
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</tr>
<tr>
<td>Combination</td>
<td>31</td>
<td>20.3</td>
<td>31</td>
</tr>
<tr>
<td>Sick Pen as Calving Area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>56</td>
<td>37.1</td>
<td>46</td>
</tr>
<tr>
<td>Yes</td>
<td>95</td>
<td>62.9</td>
<td>61</td>
</tr>
<tr>
<td>Manure Removal from Calving Area</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Between calvings</td>
<td>18</td>
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<td>24</td>
</tr>
<tr>
<td>Every 2 – 5 calvings</td>
<td>56</td>
<td>38.1</td>
<td>28</td>
</tr>
<tr>
<td>After 5 calvings</td>
<td>73</td>
<td>49.7</td>
<td>47</td>
</tr>
<tr>
<td>Bedding of Calving Area</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Between calvings</td>
<td>110</td>
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<td>83</td>
</tr>
<tr>
<td>Every 2 – 5 calvings</td>
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<td>22.8</td>
<td>11</td>
</tr>
<tr>
<td>After 5 calvings</td>
<td>5</td>
<td>3.4</td>
<td>4</td>
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<tr>
<td>Removal of Calf from Dam after Birth</td>
<td></td>
<td></td>
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<tr>
<td>Less than 1 hour</td>
<td>27</td>
<td>17.6</td>
<td>5</td>
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<tr>
<td>1-6 hours</td>
<td>84</td>
<td>54.9</td>
<td>45</td>
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<td>7-12 hours</td>
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<td>36</td>
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<td>13-24 hours</td>
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<tr>
<td>More than 24 hours</td>
<td>9</td>
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</tr>
<tr>
<td>Method of Colostrum Feeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nurse dam</td>
<td>23</td>
<td>15.1</td>
<td>42</td>
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<td>Hand feed/Esophageal tube</td>
<td>129</td>
<td>84.9</td>
<td>67</td>
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<td>Colostrum Type</td>
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<tr>
<td>Pooled</td>
<td>7</td>
<td>4.6</td>
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</tr>
<tr>
<td>Single cow (dam or another cow)</td>
<td>143</td>
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<td>99</td>
</tr>
<tr>
<td>Heat-treated</td>
<td>1</td>
<td>0.7</td>
<td>0</td>
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</table>
### Table 7.1 (continued)

<table>
<thead>
<tr>
<th>Management Practice</th>
<th>Responses from TESTING Herds</th>
<th>Responses from NON-TESTING Herds</th>
<th>Total Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash Teats and Udder Prior to Nursing/Colostrum Collection</td>
<td>N</td>
<td>Percent</td>
<td>N</td>
</tr>
<tr>
<td>No</td>
<td>20</td>
<td>13.2</td>
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<td>Yes</td>
<td>132</td>
<td>86.8</td>
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<tr>
<td>Milk Normally Fed to Calves</td>
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<tr>
<td>Milk replacer</td>
<td>120</td>
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<tr>
<td>Discard milk from sick/treated cows</td>
<td>18</td>
<td>11.8</td>
<td>25</td>
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<tr>
<td>Milk from healthy cows</td>
<td>12</td>
<td>7.8</td>
<td>24</td>
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<tr>
<td>Heat-treated</td>
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<td>0</td>
</tr>
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<td>Use of Discard Milk to Feed Calves</td>
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<td></td>
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<tr>
<td>No</td>
<td>106</td>
<td>69.7</td>
<td>57</td>
</tr>
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<td>Yes (occasionally or frequently)</td>
<td>46</td>
<td>30.3</td>
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<td>Exposure of Heifers to Adult Cattle or their Manure Prior to Weaning</td>
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<tr>
<td>No</td>
<td>118</td>
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<td>Exposure of Bred Heifers to Adult Cattle or their Manure</td>
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<td>Yes</td>
<td>97</td>
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<td>Use of Common Feedbunk between Heifers and Adult Cattle</td>
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<td>No</td>
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<td>106</td>
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<td>3</td>
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<td>Use of Common Water Source between Heifers and Adult Cattle</td>
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<td>7.6</td>
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Table 7.1 (continued)

<table>
<thead>
<tr>
<th>Management Practice</th>
<th>Responses from TESTING Herds</th>
<th>Responses from NON-TESTING Herds</th>
<th>Total Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refusals from Adult Cattle Fed to Heifers</td>
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<td></td>
<td></td>
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<tr>
<td>No</td>
<td>138 90.2</td>
<td>96 88.9</td>
<td>234 89.7</td>
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<td>15 9.8</td>
<td>12 11.1</td>
<td>27 10.3</td>
</tr>
<tr>
<td>Equipment (shovels, buckets, etc) Use between Cleaning and Feeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do not use</td>
<td>15 9.5</td>
<td>11 10.2</td>
<td>26 9.8</td>
</tr>
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<td>Separate equipment</td>
<td>113 72.0</td>
<td>74 68.5</td>
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<td>Wash</td>
<td>24 15.3</td>
<td>17 15.7</td>
<td>41 15.5</td>
</tr>
<tr>
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<td>6 5.6</td>
<td>11 4.1</td>
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<tr>
<td>Tractor/Skidloader Use between Cleaning and Feeding</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Do not use</td>
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<td>33 30.8</td>
<td>65 24.8</td>
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<tr>
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<td>52 33.3</td>
<td>27 25.2</td>
<td>79 30.0</td>
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<td>Wash bucket and/or tires</td>
<td>28 17.9</td>
<td>17 15.9</td>
<td>45 17.1</td>
</tr>
<tr>
<td>Switch bucket</td>
<td>41 26.3</td>
<td>20 18.7</td>
<td>61 23.2</td>
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<tr>
<td>No treatment between these uses</td>
<td>3 2.0</td>
<td>10 9.4</td>
<td>13 4.9</td>
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<tr>
<td>Manure from Adult Cattle Spread on Forage Ground to be Grazed by or Fed to Heifers</td>
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<td></td>
<td></td>
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<td>62 56.9</td>
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<td>62 39.7</td>
<td>47 43.1</td>
<td>109 41.1</td>
</tr>
<tr>
<td>Management Practice</td>
<td>Crude Odds Ratio</td>
<td>95% Wald Confidence Interval</td>
<td>Wald’s $\chi^2$ P value</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------</td>
<td>----------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Herd Purchases</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Open Herd</td>
<td>1.90</td>
<td>1.14-3.18</td>
<td>0.0142</td>
</tr>
<tr>
<td>Closed Herd</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time of removal of calf from dam following calving</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 1 hour</td>
<td>7.20</td>
<td>1.99-26.08</td>
<td>0.0027</td>
</tr>
<tr>
<td>1-6 hours</td>
<td>2.49</td>
<td>0.98-6.35</td>
<td>0.0565</td>
</tr>
<tr>
<td>7-12 hours</td>
<td>0.96</td>
<td>0.35-2.62</td>
<td>0.9411</td>
</tr>
<tr>
<td>13-24 hours</td>
<td>0.85</td>
<td>0.24-3.06</td>
<td>0.8018</td>
</tr>
<tr>
<td>More than 24 hours</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of calving area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Individual calving pen</td>
<td>1.10</td>
<td>0.57-2.14</td>
<td>0.7756</td>
</tr>
<tr>
<td>Pasture</td>
<td>0.20</td>
<td>0.08-0.46</td>
<td>0.0002</td>
</tr>
<tr>
<td>Combination of areas</td>
<td>0.49</td>
<td>0.26-0.95</td>
<td>0.0335</td>
</tr>
<tr>
<td>Group calving pen</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency of manure removal from calving areas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between each calving</td>
<td>0.48</td>
<td>0.24-0.99</td>
<td>0.0453</td>
</tr>
<tr>
<td>Between every 2 to 5 calvings</td>
<td>1.29</td>
<td>0.72-2.31</td>
<td>0.3955</td>
</tr>
<tr>
<td>After 5 or more calvings</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method of Colostrum Feeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hand-feed colostrum</td>
<td>3.52</td>
<td>1.95-6.33</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nurse colostrum from dam</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleaning teats prior to collecting colostrum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3.83</td>
<td>2.08-7.05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>No</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of milk fed to nursing calves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Commercial milk replacer</td>
<td>2.97</td>
<td>1.45-6.10</td>
<td>0.0030</td>
</tr>
<tr>
<td>Non-discard raw milk</td>
<td>0.34</td>
<td>0.11-1.09</td>
<td>0.0700</td>
</tr>
<tr>
<td>Discarded raw milk</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
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</table>

Table 7.2: Unadjusted odds ratios and 95% confidence intervals representing the odds that TESTING herds reported the specified management practice relative to the odds the same management practice was reported by NON-TESTING herds.
Table 7.2 (continued)

<table>
<thead>
<tr>
<th>Management Practice Recommended for Johne’s Disease Control</th>
<th>Crude Odds Ratio</th>
<th>95% Wald Confidence Interval</th>
<th>Wald’s χ² P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use of discarded milk to feed calves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.48</td>
<td>0.285-0.793</td>
<td>0.0044</td>
</tr>
<tr>
<td>No</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tractor or skidloader use for feeding and cleaning</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Separate</td>
<td>6.42</td>
<td>1.63-25.30</td>
<td>0.0079</td>
</tr>
<tr>
<td>Switch buckets</td>
<td>6.83</td>
<td>1.69-27.62</td>
<td>0.0070</td>
</tr>
<tr>
<td>Wash and/or disinfect bucket and/or tires</td>
<td>5.49</td>
<td>1.32-22.80</td>
<td>0.0191</td>
</tr>
<tr>
<td>No use</td>
<td>3.23</td>
<td>0.81-12.83</td>
<td>0.0954</td>
</tr>
<tr>
<td>No special action taken</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reported Changes in Management Practices Recommended for Johne’s Disease Control since 1997</td>
<td>Crude Odds Ratio</td>
<td>95% Wald Confidence Interval</td>
<td>Wald’s $\chi^2$ P value</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>-----------------</td>
<td>-----------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Created location specifically for calving</td>
<td>2.15 Reference</td>
<td>1.14-4.04</td>
<td>0.0176</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Removed calf from dam more quickly after birth</td>
<td>3.63 Reference</td>
<td>2.03-6.49</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoided feeding pooled colostrum to calves</td>
<td>5.00 Reference</td>
<td>2.61-9.60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoided feeding discard milk to calves</td>
<td>3.46 Reference</td>
<td>1.89-6.34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Started heat treating colostrum and/or milk fed to calves</td>
<td>7.29 Reference</td>
<td>1.61-33.01</td>
<td>0.0099</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moved location of calf housing to decrease exposure to adult cattle</td>
<td>3.74 Reference</td>
<td>1.87-7.49</td>
<td>0.0002</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoided or decreased spreading manure from adult cows on forage ground that will be grazed or harvested and then fed to heifers less than 1 year of age</td>
<td>6.75 Reference</td>
<td>3.00-15.21</td>
<td>0.0001</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoided or decreased equipment use that may result in fecal contamination of feed</td>
<td>3.27 Reference</td>
<td>1.78-6.00</td>
<td>0.0001</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoided or decreased tractor/skidloader use that may result in fecal contamination of feed</td>
<td>3.55 Reference</td>
<td>1.92-6.56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.3: Unadjusted odds ratios and 95% confidence intervals representing the odds that TESTING herds reported the specified change in a management practice relative to the odds that NON-TESTING herds reported the same change.
Figure 7.1: Timing of changes in management practices relative to the initiation of Johne’s disease testing.

<table>
<thead>
<tr>
<th>Practice</th>
<th>After Testing</th>
<th>Prior to Testing</th>
<th>No Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Removed calf from dam more quickly</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Changed tractor/skidloader use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoided pooled colostrum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoided discard milk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Changed equipment use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moved location of calf housing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Changed where manure was spread</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Created a location specifically for calving</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Started heat treating colostrum and/or milk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Management Practices Recommended for Johne’s Disease Control</td>
<td>Adjusted Odds Ratio</td>
<td>95% Wald Confidence Interval</td>
<td>Wald’s ( \chi^2 )</td>
</tr>
<tr>
<td>------------------------------------------------------------</td>
<td>---------------------</td>
<td>----------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Use of holding pen for sick cattle as a calving area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.86</td>
<td>1.01-3.44</td>
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</tr>
<tr>
<td>No</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time of removal of calf from dam following calving</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 1 hour</td>
<td>6.53</td>
<td>1.34-31.69</td>
<td>0.0200</td>
</tr>
<tr>
<td>1-6 hours</td>
<td>2.08</td>
<td>0.73-5.91</td>
<td>0.1704</td>
</tr>
<tr>
<td>7-12 hours</td>
<td>0.89</td>
<td>0.29-2.71</td>
<td>0.8308</td>
</tr>
<tr>
<td>13-24 hours</td>
<td>0.71</td>
<td>0.16-3.25</td>
<td>0.6617</td>
</tr>
<tr>
<td>More than 24 hours</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of calving area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Individual calving pen</td>
<td>2.49</td>
<td>1.10-5.65</td>
<td>0.0295</td>
</tr>
<tr>
<td>Pasture</td>
<td>0.35</td>
<td>0.13-0.93</td>
<td>0.0355</td>
</tr>
<tr>
<td>Combination of areas</td>
<td>0.82</td>
<td>0.37-1.82</td>
<td>0.6302</td>
</tr>
<tr>
<td>Group calving pen</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method of Colostrum Feeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hand-feedcolostrum</td>
<td>3.08</td>
<td>1.56-6.11</td>
<td>0.0012</td>
</tr>
<tr>
<td>Nurse colostrum from dam</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleaning teats prior to collecting colostrum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3.59</td>
<td>1.77-7.25</td>
<td>0.0004</td>
</tr>
<tr>
<td>No</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Use of discarded milk to feed calves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0.50</td>
<td>0.27-0.91</td>
<td>0.0235</td>
</tr>
<tr>
<td>No</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tractor or skidloader use for feeding and cleaning</td>
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<td></td>
<td></td>
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<tr>
<td>Separate</td>
<td>8.44</td>
<td>1.71-41.56</td>
<td>0.0087</td>
</tr>
<tr>
<td>Switch buckets</td>
<td>6.34</td>
<td>1.26-31.77</td>
<td>0.0247</td>
</tr>
<tr>
<td>Wash and/or disinfect bucket and/or tires</td>
<td>6.50</td>
<td>1.22-34.60</td>
<td>0.0284</td>
</tr>
<tr>
<td>No use</td>
<td>3.33</td>
<td>0.68-16.40</td>
<td>0.1392</td>
</tr>
<tr>
<td>No special action taken</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.4: Adjusted odds ratios and 95% confidence intervals representing the odds that TESTING herds reported the specified management practice relative to the odds the same management practice was reported by NON-TESTING herds when controlling for producer-perceived infection status.
Table 7.5: Adjusted odds ratios and 95% confidence intervals representing the odds that TESTING herds reported the specified change in a management practice relative to the odds that NON-TESTING herds reported the same change when controlling for producer perceived infection status.

<table>
<thead>
<tr>
<th>Reported Changes in Management Practices Recommended for Control of Johne’s Disease since 1997</th>
<th>Adjusted Odds Ratio</th>
<th>95% Wald Confidence Interval</th>
<th>Wald's $\chi^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Removed calf from dam more quickly after birth</td>
<td>Yes 2.31 Reference</td>
<td>1.18-4.50</td>
<td>0.0141</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoided feeding pooled colostrum to calves</td>
<td>Yes 3.15 Reference</td>
<td>1.50-6.61</td>
<td>0.0024</td>
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</tr>
<tr>
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<td>No</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Avoided feeding discard milk to calves</td>
<td>Yes 2.50 Reference</td>
<td>1.24-5.04</td>
<td>0.0107</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moved location of calf housing to decrease exposure to adult cattle</td>
<td>Yes 3.09 Reference</td>
<td>1.41-6.77</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoided or decreased spreading manure from adult cows on forage ground that will be grazed or harvested and then fed to heifers less than 1 year of age</td>
<td>Yes 5.55 Reference</td>
<td>2.14-14.42</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoided or decreased equipment use that may result in fecal contamination of feed</td>
<td>Yes 2.37 Reference</td>
<td>1.18-4.78</td>
<td>0.0154</td>
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<td>No</td>
<td></td>
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<tr>
<td>Avoided or decreased tractor/skidloader use that may result in fecal contamination of feed</td>
<td>Yes 2.74 Reference</td>
<td>1.36-5.52</td>
<td>0.0048</td>
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</tr>
<tr>
<td></td>
<td>No</td>
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</tr>
</tbody>
</table>
CONCLUSIONS

8.1 Likelihood ratios presented here indicate that cows originating from MAP-infected dairy herds with ELISA S/P ratios $\geq 0.800$ are 55 times more likely to be infected than non-infected with MAP, although the potential for misclassification exists. ELISA with S/P ratios $\leq 0.800$ provided little additional diagnostic evidence regarding a cow’s true MAP infection status.

8.2 Nearly 50% of fecal culture positive cows in this study lacked a positive ELISA result in their test history. Additionally, in those infected cows with a positive ELISA result in their test history, nearly 80% were identified by a positive fecal culture before or at the same time as a positive ELISA result.

8.3 Fecal cortisol levels can be measured in dairy cattle following ACTH stimulation; however, the variability of responses between individual cows may be considerable.
8.4 Farm, sampling location within a farm and sampling date all significantly influenced fecal cortisol levels measured in composite fecal samples. However, few specific management practices or production parameters that significantly influenced mean fecal cortisol levels of a herd were identified. Herd MAP-infection status was not associated with fecal cortisol levels.

8.5 On a given testday, a positive fecal culture result was associated with an average reduction of 8.0 pounds of milk and positive ELISA results were associated with a 3.9 pound reduction relative to cows with negative MAP test results when controlling for days-in-milk and lactation number. These observations provide additional information for dairy producers to consider as they make culling decisions regarding MAP test-positive cows.

8.6 Even if a producer believed his/her herd was not infected with MAP, participation in a testing program continued to be associated with herd management practices recommended for Johne’s disease control and the incorporation of changes to existing practices.
8.7 Continued education is necessary with respect to the interpretation of the diagnostic tests incorporated in herd screening programs. Producers should be encouraged to cull fecal culture positive animals and confirm ELISA-positive results with serial fecal culture.

8.8 Johne’s disease control recommendations should be prioritized for producers so that those practices that achieve the highest level of disease control can be identified and implemented.
Chapter 1


18. Rubery E. A review of the evidence for a link between exposure to *Mycobacterium paratuberculosis* (MAP) and Crohn's disease (CD) in humans. Food Standards Agency, United Kingdom.

Chapter 2


213


www.northstarcooperative.com/Antel/Johnes%20Watch/jw_prod_may01.htm.


186. Rubery E. A review of the evidence for a link between exposure to *Mycobacterium paratuberculosis* (MAP) and Crohn's disease (CD) in humans. Food Standards Agency, United Kingdom.


272. Strier KB, Ziegler TE, Wittwer DJ. Seasonal and social correlates of fecal testosterone and cortisol levels in wild male muriquis (Brachyteles arachnoides). Horm Behav 1999;35:125-34.


Chapter 3


Chapter 4


7. Strier KB, Ziegler TE, Wittwer DJ. Seasonal and social correlates of fecal testosterone and cortisol levels in wild male mirmiquis (Brachyteles arachnoides). Horm Behav 1999;35:125-34.


Chapter 5


Chapter 6


**Chapter 7**


