

**Detection of Johne's Disease in an Iowa (United States) dairy herd: Comparisons of the milk ELISA, serum ELISA, Gamma-Interferon and fecal culture tests and the effect of a skin-test using a cell-free sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (19698) on the production of Gamma-Interferon**

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Signatures have been redacted for privacy

## DEDICATION

There are special people without whom projects of this nature would be impossible. I would like to thank my wife Yvonne who is my best friend, constant companion and my closest advisor. Without her encouragement and all of her gracious help this would never have happened. Yvonne has been a light in my life since the day that I met her. My sons, Brad and Chris gave their time with little complaint, and they are inspirations of what life is really about. I would also like to thank my parents, Keith and Arlene Kirkpatrick for their support and encouragement to pursue a veterinary career. Their memory is my constant companion and they are still my guardians. Dr. Karl Kersting has been extremely generous in giving his time and effort to serve as my major professor, mentor and colleague. Achieving a fraction of Dr. Kersting's knowledge would truly be an accomplishment. Dr. Leo Timms has been a tremendous colleague, friend and a mentor in pushing my thought patterns concerning dairy problem solving and for providing so many learning opportunities. I also owe a debt of thanks to Dr. Mike Apley for giving me an initial shove into graduate course work. He too, has been a terrific colleague and friend. To all these special people I dedicate this thesis and research.

## TABLE OF CONTENTS

LIST OF FIGURES.....	vi
LIST OF TABLES .....	vii
ACKNOWLEDGEMENTS .....	viii
ABSTRACT.....	ix
CHAPTER 1. INTRODUCTION.....	1
Gamma-Interferon Testing of Johne’s Disease.....	2
Focus of this Study.....	3
Thesis Organization.....	4
CHAPTER 2. LITERATURE REVIEW.....	5
Economics and Milk Production.....	6
Transmission of <i>M. paratuberculosis</i> .....	8
Clinical and Gross Pathology.....	13
Stage I – Silent Infection .....	13
Stage II – Subclinical Disease .....	14
Stage III – Clinical Disease .....	14
Stage IV – Advanced Clinical Disease .....	14
Immunology of Johne’s Disease.....	14
Immunology and Transmission.....	17
Vaccination against Johne’s Disease .....	18
The Department of Agriculture Agrees To:.....	18
The Veterinarian Agrees To: .....	19
The Owner Agrees To: .....	19
Testing Procedures.....	20
Organism Detection .....	21
Antibody Assays.....	24
Determinations of Cell-mediated Immunity (CMI).....	28
Testing Summary.....	30
Predictive Values of a Positive or Negative Test.....	30
Estimated Prevalence (EP).....	30
Apparent Prevalence (AP).....	31
True Prevalence (TP).....	31
Likelihood Ratios .....	32
Treatment of Johne’s disease .....	33
Regulatory Concerns.....	34
Crohn’s disease – Is <i>M. paratuberculosis</i> a Zoonotic Agent?.....	36
Literature Review Conclusions.....	39
CHAPTER 3. COMPARISONS OF THE MILK ELISA, SERUM ELISA, GAMMA-INTERFERON AND FECAL CULTURE TESTS FOR THE DETECTION OF JOHNE’S DISEASE IN AN IOWA (UNITED STATES) DAIRY HERD .....	40
Introduction.....	40
Materials and Methods.....	41
Classification trial.....	41
Milk ELISA .....	41
$\gamma$ -IFN Assay .....	43
19698 MpS Production.....	43

Absorbed ELISA .....	44
Fecal and Tissue Cultures .....	44
Statistics.....	44
Results.....	44
Discussion.....	45
CHAPTER 4. EFFECT OF A SKIN-TEST USING A CELL-FREE SONICATE OF <i>MYCOBACTERIUM AVIUM</i> SUBSP. <i>PARATUBERCULOSIS</i> (19698) ON THE PRODUCTION OF GAMMA-INTERFERON.....	
Introduction.....	52
Materials and Methods.....	53
Classification trial.....	53
Milk ELISA, Serum ELISA Fecal and Tissue Culture and $\gamma$ -IFN assay.....	54
19698 MpS Production.....	54
Treatment Assignment.....	54
Exploratory laparotomies.....	55
Histopathology.....	56
Statistics.....	56
Results.....	56
Discussion.....	62
CHAPTER 5. GENERAL CONCLUSIONS .....	
Comparisons of Testing Procedures for the Detection of Johne's Disease.....	66
Effect of a Skin-test on the Production of Gamma-Interferon .....	68
REFERENCES.....	70

## LIST OF FIGURES

### CHAPTER 3. COMPARISONS OF THE MILK ELISA, SERUM ELISA, GAMMA-INTERFERON AND FECAL CULTURE TESTS FOR THE DETECTION OF JOHNE'S DISEASE IN AN IOWA (UNITED STATES) DAIRY HERD

Figure 1. Scatterplot comparison of MELISA vs. SELISA results .....	46
Figure 2. Scatterplot comparison of Gamma-IFN vs. MELISA results .....	47
Figure 3. Scatterplot comparison of Gamma-IFN vs. SELISA results.....	48

### CHAPTER 4. EFFECT OF A SKIN-TEST USING A CELL-FREE SONICATE OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (19698) ON THE PRODUCTION OF GAMMA-INTERFERON

Figure 1. Skin Test Lesion Diameter (mm) post skin test on day 0 .....	58
Figure 2. Daily Gamma-Interferon determinations in units of optical density.....	59
Figure 3. Daily Gamma-Interferon determinations minus Day 0 in units of optical density.....	60
Figure 4. Gamma-Interferon daily determinations from MpS-P cattle measuring Gamma-Interferon produced by 19698 MpS, Avium PPD and Bovis PPD as culture stimulagens. ....	61

**LIST OF TABLES**

## CHAPTER 2. LITERATURE REVIEW

Table 1. ELISA Test Classifications – Dr. Michael Collins <sup>60</sup> .....	26
Table 2. Johne’s Disease Test Specifications .....	31
Table 3. Positive and Negative Predictive Values for Various Testing Means .....	32
Table 4. Likelihood Ratios .....	33

CHAPTER 3. COMPARISONS OF THE MILK ELISA, SERUM ELISA, GAMMA-INTERFERON AND  
FECAL CULTURE TESTS FOR THE DETECTION OF JOHNE’S DISEASE IN AN IOWA (UNITED  
STATES) DAIRY HERD

Table 1: MELISA S/P, Grade, Exposure and Recommendations. <sup>85</sup> .....	43
Table 2: Test Results Mean Values .....	45

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## ABSTRACT

Cattle from the Iowa State University, Ames dairy herd were characterized for Johne's disease using results from the milk ELISA (MELISA) (Dairy Lab Services; Dubuque, IA), serum ELISA (SELISA) (IDEXX Laboratories, Inc., Westbrook, ME),  $\gamma$ -IFN assay (CSL Limited Parkville Victoria Australia) and fecal culture. Using the results of the initial  $\gamma$ -IFN assay and the SELISA determination, all herd members were divided into four groups consisting of: a negative SELISA and  $\gamma$ -IFN, suspect  $\gamma$ -IFN, positive  $\gamma$ -IFN and SELISA positive individuals. The first three groups were enrolled in the skin testing trial and were randomly assigned within group to one of two treatments. This consisted of an injection of either 100 $\mu$ g (0.1ml) of a *M. paratuberculosis* strain 19698 cell free sonicate (19698 MpS) or 0.1 ml of 0.9% saline solution as an intradermal injection. Blood samples were obtained on days 0, 3, 6, 9, 15, and 27 days post-injection for  $\gamma$ -IFN assay, while skin lesion measurements were made on days 0, 3, 6 and 9.

The MELISA test returned the greatest number of test positive individuals at 12.96%, The SELISA was 7.41% test positive,  $\gamma$ -IFN assay was 4.40%, and fecal culture yielded 0% positive. Further evaluation indicated the SELISA had a significantly lower age (37.2 vs. 50.5 and 48.3 months respectively) at positive test ( $P > |T| = 0.0192$ ) than did MELISA or  $\gamma$ -IFN. Due to the low number of fecal culture and  $\gamma$ -IFN positive individuals, and the significantly lower age at positive test it could be suspect that the SELISA had an inordinate number of false positive individuals.

MELISA and SELISA results were compared using an XY plot. No common individuals were identified as test positive by both tests. Analysis using kappa returned a value of  $-0.116$  indicated a very low percentage of agreement between the two testing means. With the SELISA being the regulatory US standard it is not recommended to use the MELISA for purposes of culling decisions.

Results of the skin testing portion of the study indicates that inoculation of animals with 19698 MpS significantly increased production of  $\gamma$ -IFN in cattle declared to have positive (MpS-P) baseline  $\gamma$ -IFN responses for Johne's disease. Peak production of  $\gamma$ -IFN occurred at 144 hr following skin testing and was statistically significant  $P = 0.0005$  as compared to day 0. Levels of  $\gamma$ -IFN dropped on day 9, but remained at levels that were significantly higher than day 0 ( $P = 0.0325$ ). Levels of  $\gamma$ -IFN again significantly increased on day 27 when compared to day 15 ( $P = 0.0362$ ), and was significantly higher than day 0 ( $P = 0.0004$ ). Further research is warranted to determine if the addition of skin testing to the  $\gamma$ -IFN assay could increase test sensitivity or durability.

## INTRODUCTION

*Mycobacterium avium* subsp. *paratuberculosis* was first isolated by Drs. Johne and Frothingham in 1895.<sup>1,2</sup> This is the causative agent of the disease that bears Dr. Johne's name. Johne's disease is a chronic mycobacterial infection of the lower small intestine and associated lymph nodes which affects many ruminants including cattle, sheep, goats, llama, deer and bison.<sup>1,3,4</sup> It is primarily a wasting disease, but is also evidenced by profuse, watery, untreatable diarrhea in cattle.

*Mycobacterium paratuberculosis* is closely related to *Mycobacterium avium* and it is suggested that the etiological agent of Johne's disease should be most properly classified as *Mycobacterium avium* subsp. *Paratuberculosis*.<sup>5</sup> For purposes of uniformity the causative agent will be referred to as *M. paratuberculosis* in the balance of this paper.

Webster's Second College Edition of the New World Dictionary defines the word "insidious" as, "operating in a slow or not easily apparent manner; more dangerous than seems evident." Johne's disease truly defines this term for researchers, veterinary practitioners and producers alike. Bovine oral infection generally occurs during the first 6 - 12 months of life with manifestation of the clinical disease 2 - 5 years later. Producers encountering Johne's will simply cull the affected animal and feel they have dealt with the problem. In reality, for every home-raised case of clinical Johne's there may be another 20 - 25 individuals in some form of infection present in the herd.<sup>6</sup> Removal of the clinical individual has done nothing to effectively deal with this disease if the other 20 - 25 cases are not dealt with. This is the true "insidious" nature of the disease.

Management and reduction of the incidence of Johne's disease is well worth the time and effort. Paratuberculosis is an economically devastating disease of ruminants. Recent estimates indicate the loss to dairy producers includes 640 million kg of milk, additional culling of 11,000 cows, and nearly 20,000 cow deaths (based on a total population estimate of 9,458,000 dairy cows).<sup>7,8</sup> The total amount of revenue lost is approximately \$222 million. Another method of quantifying this on a herd basis is that the loss is approximately \$227 per lactating female if 10% of the culls showed signs of infection with *M. paratuberculosis*.

The purpose of the National Animal Health Monitoring Study - United States Department of Agriculture (NAHMS) study was to begin to understand the potential individual and herd-prevalence rate occurring in the nation's dairy herds.<sup>7</sup> The study suggested a 3.4% cow-level prevalence of Johne's with approximately 21.6% of the nation's dairy herds being infected. Herds undergoing expansion, as represented by a herd size greater than 300, demonstrated a 40% herd prevalence rate.

Johne's disease has been receiving much more attention in the last few years. As Dr. Franklyn Garry outlined, there is a greater prevalence of the disease than previously assumed.<sup>9</sup> The economic impact has also been greater than previously determined. Recent litigation over the losses due to Johne's has pushed the disease to the forefront. Additionally, there is some evidence that *M. paratuberculosis* could be a zoonotic agent connected to human Crohn's disease.<sup>15, 72</sup>

Computer information management packages have made the management of thousands of cows in a single dairy operation a controllable task. Consolidation of dairy operations and the expansion of herd size have been fueled through the purchase of mature milking cows, as well as non-lactating youngstock. Current estimates suggest that a producer buying cattle on the open market has a 1:10 chance of purchasing Johne's disease in some form.<sup>10</sup> Increased levels of stress due to disease, housing, social and nutritional causes have served to shorten the presentation time of Johne's, thus pushing it into the consciousness of all dairy industry participants. The two factors of prevalence level and stress have made Johne's disease a major cause of revenue loss in the expansion herd.

Testing procedures have been criticized for their lack of sensitivity and specificity as all participants become frustrated with their inability to identify animals that have been recently, subclinically, or even clinically infected. Research has been stymied due to the inability to truly declare an animal non-infected. An additional burden is the slow 4-day generation time of *M. paratuberculosis*. Fecal and tissue culture have been considered the "gold" standard of isolation, but growth requirements of 12 – 16 weeks have taken a toll in research efforts.<sup>11</sup> Considerable work and study have been done in the 100 years since Drs. Johne and Frothingham isolated this organism, but we are no closer to a cure or even a truly effective vaccination program.<sup>1,2</sup> With the advent of new techniques in gamma-interferon detection and polymerase chain reaction amplification (PCR) of mycobacterial DNA we are starting to break down some of the barriers to identification of the early, subclinically infected individuals.

### **Gamma-Interferon Testing of Johne's Disease**

Testing for the presence of mycobacterial species first focused on detecting bovine tuberculosis caused by *M. bovis*. Historically, the test of choice was the caudal skin fold test which called for the injection of a *M. bovis* purified protein derivative (PPD) intradermally, followed by inspection of the injection site at 72 hr post-injection for the presence of skin thickening or a welt. This was a means of visualizing the effects of an activated cell-mediated immune system. While criticized for lacking sensitivity and specificity the test was successful in removing large numbers of reactors from the bovine population and was a successful element of regulatory control programs.

A recent development in cell-mediated immune system measurement has been a testing procedure to determine the amount of the cytokine gamma-interferon ( $\gamma$ -IFN) released by sensitized T lymphocytes. Gamma-interferon is produced by CD4<sup>+</sup>, CD8<sup>+</sup>, gamma/delta ( $\gamma/\delta$ ) receptor T-cells, and natural killer cells. Activation of the macrophage population through cytokine induction is responsible for the induction of tumor cell lysis, release of reactive oxygen and nitrogen intermediates, expression of major histocompatibility complex class II antigens, and secretion of arachadonic acid metabolites. Thus, there is a direct relationship between  $\gamma$ -IFN and activation of macrophages and their ability to kill intracellular pathogens.<sup>12</sup>

The detection methods of Johne's disease caused by *M. paratuberculosis* have been limited to the use of the intradermal skin test, detection of the organism through culture, and the assay of serum for the detection of

antibody production. The caudal skin fold test has come under scrutiny for poorer sensitivity and specificity in the case of Johne's disease due to the remote location (immunologically) of the lesions.<sup>12</sup> Culture and antibody detection have been very successful in identifying clinically affected individuals, but historically poor in the detection of subclinical cases. It is generally accepted that the late phase reactions of Johne's disease are to shed bacilli in large numbers, along with the production of non-neutralizing antibody. Conversely, the earliest measurable immune system response to infection of *M. paratuberculosis* is the activation and differentiation of the T cell population which is the cell-mediated phase of immunity.<sup>12</sup>

Due to the poor detection rate of the cell-mediated phase of disease by the intradermal Johne's test, research moved to adapt the  $\gamma$ -IFN assay used in bovine tuberculosis to the detection of Johne's disease. The use of this assay presents real benefits through earlier detection of Johne's disease, increased sensitivity and specificity over caudal fold skin testing and other means of testing, automated, numerical determination of  $\gamma$ -IFN levels vs. the subjective intradermal palpation technique, as well as the ability to run the test multiple times without sensitization of the animal to an intradermal PPD. While the benefits are great, this form of testing does have the drawbacks of test expense (\$16/animal) and the requirement that a heparinized whole blood sample be delivered to the laboratory within 16 hours after drawing. The latter factor can represent a great logistical hurdle for veterinary practitioners in the field.

#### **Focus of this Study**

Recent work using the  $\gamma$ -IFN assay for the detection of bovine tuberculosis has examined the effects of the intradermal injection of a PPD in an effort to "magnify" the  $\gamma$ -IFN production of already stimulated T lymphocytes. Whipple et al. demonstrated a stimulatory production ( $p < 0.01$ ) of  $\gamma$ -IFN in response to the administration of a *M. bovis* PPD intradermal injection used in a caudal fold skin test.<sup>13</sup> Optical density values of the  $\gamma$ -IFN ELISA for samples taken 72 hr post-inoculation were significantly higher than samples collected prior to skin testing, and higher than samples from cattle that were not skin tested. Likewise, Rothel et al. demonstrated a 2.5 fold increase in  $\gamma$ -IFN following caudal fold testing that peaked at 3 – 5 weeks post-inoculation.<sup>14</sup>

The  $\gamma$ -IFN assay has demonstrated real benefits in terms of improved sensitivity, specificity and standardization of technique.<sup>63,65</sup> The demonstrated "magnification" effect in  $\gamma$ -IFN production could potentially further enhance the sensitivity of the  $\gamma$ -IFN assay in determining positive reactors to Johne's disease without loss of specificity. An additional benefit could be increased longevity of the heparinized blood sample. It is possible that even though lymphocyte survival may be compromised, enough lymphocytes may still be present at an enhanced  $\gamma$ -IFN production level to make the test effective. Given the stimulatory phenomenon documented with *M. bovis*, this study was undertaken to determine if the same stimulatory activity occurred in *M. paratuberculosis*  $\gamma$ -IFN assay reactors.

### Thesis Organization

This thesis begins with a literature search examining the aspects of Johne's disease in bovines which includes: Economics and Milk Production, Transmission of *M. paratuberculosis*, Clinical and Gross Pathology, Immunology of Johne's Disease, Immunology and Transmission, Vaccination against Johne's Disease, Testing Procedures, Organism Detection, Treatment of Johne's Disease, Regulatory Concerns, Crohn's Disease, followed by a Literature Review Conclusions.

Experimental work for this thesis produced two journal articles to be submitted to the American Journal of Veterinary Research. The first article (Chapter 3) is: Comparisons of the Milk ELISA, Serum ELISA, Gamma-Interferon and Fecal Culture Tests for the Detection of Johne's Disease in an Iowa (United States) Dairy Herd. This study served as the initial classification trial to enroll individual animals in the second phase of the experiment. The second article (Chapter 4) is: Effect of a Skin test using a Cell-free Sonicate of *Mycobacterium avium* subsp. *paratuberculosis* (19698) on the Production of Gamma-Interferon. Enrolling cattle in this phase of the study required the testing procedures that were outlined in the Materials and Methods section of the first article. The reader is directed to the first article for the complete Materials and Methods. Only the procedures that were utilized in the second phase of experimentation are outlined in the Materials and Methods of the second article.

The references for both journal articles are included with the references of the literature search at the end of the thesis. A general conclusions chapter follows the journal articles.

## LITERATURE REVIEW

Paratuberculosis (Johne's disease) is a chronic, granulomatous inflammation that affects the caudal region of the small intestine, the ileocecal valve, the cecum, and the associated lymph nodes.<sup>3</sup> Johne's disease is widely distributed internationally in domesticated ruminants such as cattle, sheep, goats, deer, antelope, and bison.<sup>4</sup> Cattle become infected as calves through oral ingestion of the bacilli or through in-utero infection. Due to the slow growing nature of this pathogen disease doesn't become clinically evident until 2 to 5 years of age.<sup>4</sup>

Johne's disease is manifested by weight loss, diarrhea that lasts more than three days, and the failure to respond to therapy. Affected cattle do not have a fever, tend to eat well, and look well other than the presence of diarrhea and weight loss.<sup>15</sup> Internal tissue changes associated with this disease result in the leakage of plasma proteins across the intestinal wall, and the malabsorption of dietary amino acids from the intestinal contents<sup>11</sup>. These changes are the cause of the clinical diarrhea. With the loss of dietary amino acids the affected animal may potentially manifest a "bottle jaw" appearance (mandibular edema).<sup>16</sup> Differential diagnoses include renal amyloidosis, abdominal neoplasia, caudal vena cava thrombosis, heart failure, indigestion, salmonellosis, parasitism, internal abscess, or other disease entities that may cause chronic diarrhea.<sup>17, 18</sup>

*Mycobacterium paratuberculosis* is a gram-positive, intracellular, slow-growing, mycobactin-dependent acid-fast bacterium, closely related to *Mycobacterium avium*.<sup>19</sup> There are some phenotypic and genotypic differences between *M. avium* subsp *paratuberculosis* and the *M. avium* complex, but much more similarity exists than differences.<sup>5</sup> This is evidenced by 98% of the genome of *M. avium* being conserved in the genome of *M. avium* subsp. *Paratuberculosis*.<sup>20</sup> This conservancy represents a real diagnostic challenge due to cross-reactivity.

*M. paratuberculosis* lacks the ability to produce mycobactin, which is a siderophore produced by all other mycobacteria. Mycobactin assists in making iron available from the environment for bacillary growth, and must be supplied in the growth media for *M. paratuberculosis* isolation. Because of this siderophore deletion *M. paratuberculosis* is considered to be a parasite of the mammalian cell. As no recovery from infection is known, this organism should be considered to be an obligate parasite.<sup>21</sup>

The National Animal Health Monitoring Study – United States Department of Agriculture (NAHMS) study was initiated in 1995 to begin to understand the potential individual and herd prevalence rates occurring in the nation's dairy herds.<sup>7</sup> Using a conservative protocol, all herds that had only 1 positive serological isolation from the statistical sample were excluded from the study. Greater than 1 isolation allowed herd inclusion. The best estimate indicated a 21.6% herd prevalence rate, which excluded the 0.6% of dairy herds currently vaccinating for this disease. The adjusted cow-level prevalence was 3.4%. Other studies indicate cow-level prevalence rates at approximately 5% to 10%.<sup>22, 23</sup> Collins et al indicated that 34% of Wisconsin herds surveyed had serological evidence of Johne's with a 4.79% incidence rate among cows<sup>15, 23</sup>. Of the 79 herds with seropositive cattle, 31.6% had > 15% of the herd population test positive for paratuberculosis. Work by

Johnson-Ifearegulu and Kaneene indicated a 66% prevalence rate in Michigan dairy cattle.<sup>24</sup> Thorne and Hardin suggested that 74% of Missouri dairy herds and 40% of beef herds are infected.<sup>25</sup> These high herd prevalence rates indicate a serious threat<sup>23</sup>. With these statistics in mind it is generally accepted that a producer has a 10% chance of purchasing an animal with Johne's disease from market sources. Any producer purchasing cattle on the open market thus will be ensured of having a baseline level of Johne's disease. As most acquisition is in association with expansion efforts, this increased risk was demonstrated by the NAHMS study, which indicated approximately 40% of the herds over 300 mature cattle were infected.<sup>10</sup> It is assumed that to achieve herd sizes of 300, some animal purchasing from the open market was required.

An absorbed ELISA for detection of antibodies against *Mycobacterium paratuberculosis* was performed on serum samples obtained from the Missouri Animal Health Laboratory.<sup>25</sup> Samples from 1954 Missouri cattle representing 89 herds were randomly selected from samples submitted for Brucellosis testing. The apparent seroprevalence of paratuberculosis in dairy cattle ( $8 \pm 3\%$ ) was similar to that in beef cattle ( $5 \pm 2\%$ ). When herds were classified as dairy or beef, 74% (14 of 19) of dairy herds and 40% (27 of 68) of beef herds were positive.

As a part of the NAHMS study, participating producers were surveyed concerning their familiarity with the disease, 17.7% considered themselves to be fairly knowledgeable, 37.1% knew some basics, 35.3% recognized the name, while 9.9% had never heard of it before.<sup>7</sup> This indicates that a fairly sizable educational effort will be needed prior to the implementation of any control efforts.

### **Economics and Milk Production**

Johne's disease is economically devastating. In herds that are heavily infected there is no doubt in the owner's mind based on the perceived loss of milk production and loss of body condition at culling. These are easy parameters to see, but additional losses accrue through reductions in fertility, loss of genetic material and through the reduction of future earnings through early culling. A further loss is through higher susceptibility to concurrent disease from immune system compromise due to the lack of absorbed nutrients. Benedictus partitioned these losses as follows: increased veterinary costs (2%), loss due to idle production facilities (3%), decreased value at slaughter (20%), decreased production (32%), and unrealized future income based on age at culling and prior production (43%).<sup>26</sup>

Production losses have been demonstrated in several studies. Benedictus et al found a 16% reduction in milk yield of the final lactation of cows with clinically evident paratuberculosis. The production losses of infected animals without clinical signs were about 6%.<sup>26, 27</sup> This study also showed that infected high producers (productive potential 8% to 9% greater than herdmates) within a herd are more likely to develop clinical signs. Thus these animals have a higher risk of being culled. A study performed in 3 northern California dairy herds supported the above results by indicating 1838 lb (15%) less milk on a 305 day mature equivalent basis in subclinically infected animals.<sup>28</sup> A Netherlands study showed reduced production in culture positive cows by 12 lb per head per day. Clinical individuals demonstrated a 19.5% reduction on a mature equivalent basis.<sup>29</sup>

When examining lactation records, a New York cull cow study demonstrated the progression of milk yield losses. When compared to 2 lactations prior to culling, the lactation record the year prior to culling returned a 6% milk yield decrease. The lactation record of the year of culling was depressed by 16% when compared with the reference lactation, 2 lactations previous. The Netherlands study previously mentioned demonstrated a similar 5% decrease in milk production in the cull lactation vs. the lactation previous. Most studies indicate a greater than 15% milk yield loss for clinically infected individuals when compared to their own records, or uninfected herdmate records. Subclinical cows experienced a moderate reduction of 5% to 15%.<sup>30</sup> A New York examination of a 210 cow Holstein herd found no changes in milk yield of infected heifers as compared to non-infected herdmates.<sup>31</sup> Beginning in the second lactation, and increasing with advancing parity, milk yield losses increased. Using a pay price of \$13.00/hundredweight of milk the authors determined milk yield losses of \$80.00 to \$250.00 per lactation per individual.

Returning to the NAHMS Dairy 96 study, Johne's disease is estimated to cost US dairy producers almost 640 million kg of milk, additional culling of 11,000 cows, and nearly 20,000 cow deaths annually (based on a 1995 estimate of 9,458,000 dairy cows).<sup>7</sup> In addition, of the estimated \$60 million in lost revenue for culls in poor body condition, \$9 million can be attributed to Johne's disease. Thus, the total amount of annual revenue loss from Johne's disease is approximately \$222 million.<sup>8</sup> Realizing that Johne's is a bovine problem and not just a dairy issue, the National Cattlemen's Beef Association (NCBA) moved to incorporate producer education about Johne's disease into their Beef Quality Assurance program in 1998. It is critical to control efforts that Johne's disease be viewed as bovine problem rather than a dairy issue. Participation by the beef industry will be essential to implement control programs.<sup>9</sup>

When examining health related parameters such as mastitis incidence or infertility the overall conclusion of studies are less clear. Several studies did not indicate a correlation between Johne's and these events, while others did find a correlation. Given the lack of dietary nutrients in the advanced stage of this disease it could be assumed that there would be a logical association due to immunosuppression. A major challenge in studying these parameters is the difficulty in determining the true infective status of individuals in the early course of disease.<sup>30</sup>

A study by Elsken and Nonnecke attempted to provide information on this area.<sup>32</sup> Blood lymphocytes were obtained from control, subclinically infected and noninfected cows. Lymphocytes obtained from peripheral blood samples of infected cows demonstrated significant proliferation ( $P < 0.05$ ) while milk lymphocytes did not. Conversely, milk lymphocytes from noninfected cows demonstrated significant proliferation ( $P < 0.05$ ) while their vascular pool lymphocytes did not. Epidemiological work suggests that cows with subclinical paratuberculosis are more susceptible to mastitis and are culled for chronic mastitis more frequently than are noninfected herdmates. Possibly, cows infected with *M. paratuberculosis* may be more susceptible to mammary gland infections, or there could be an early, selective depression of mammary gland protective mechanisms.<sup>32</sup> Conversely, a study by Wilson et al. examining a 210 cow Holstein herd divided into an infected and non-infected herd demonstrated significantly lower rates of new and chronic mastitis infections

( $P < 0.05$ ,  $P = 0.05$ , respectively) in the infected herd.<sup>31</sup> Their work did verify lower mature equivalent herd production and increased culling among the members of the Johne's infected herd.

In addition to milk production one of the biggest losses sustained by the dairy industry is the premature, or involuntary, culling of affected cattle. Voluntary culling is the removal of individuals from the herd based on production reasons or for sale to another dairy for dairy purposes. Voluntary culling allows the dairy producer to improve the herd through removal of low genetic merit cows. A herd experiencing a high rate of clinical Johne's disease will have the culling program taken over by involuntary removal relating to paratuberculosis. The loss of high merit genetic individuals is an opportunity cost and is extremely difficult to quantify.<sup>30</sup> Additionally, losses continue to accrue through increased replacement animal costs due to the accelerated culling rate.

### **Transmission of *M. paratuberculosis***

Attempting to understand control of this disease requires that the veterinary practitioner and producer be well acquainted with the modes of contamination and transmission. This section will focus on the events of transmission starting at the in-utero stage and moving through the life of the bovine.

*M. paratuberculosis* is an inhabitant of the infected animal's gastrointestinal tract (GI tract) and the associated mesenteric lymph nodes. This organism is intracellular in nature and lives within macrophages in the lamina propria of the intestine, mesenteric lymph nodes, as well as the fetus, mammary gland and uterus of a heavily infected individual. *M. paratuberculosis* has been thought to be a localized phenomenon limited to the small intestine, specifically the ileum and associated lymph nodes. This location predilection is potentially due to the distribution of lymphoid tissue within the small intestine of the young calf. In calves, mucosal lymphoid tissue occupies 8.6% of the small intestine, with 1/3 of the total located in the jejunum. The remaining 2/3 of the total mass lies in the ileum.<sup>6</sup>

Upon presentation of *M. paratuberculosis* orally, a limited number of macrophages located in the subepithelial and intraepithelial regions will display the presence of this organism within 5 hr. After 20 hr many macrophages in the Peyer's patches will demonstrate the bacilli. Once present inside the macrophage the bacilli may persist for up to 5 weeks.<sup>6</sup> When *M. paratuberculosis* is mixed and administered with anti *M. paratuberculosis* serum vs. a normal bovine serum, this uptake process is significantly enhanced. The use of this antiserum mimics the oral maternal antibody present in colostrum, and thus may start to explain a potential familial or genetic link.<sup>1,6</sup>

Recent work has demonstrated the ability of this organism to move systemically, probably due to peripheral circulation of GI, non-activated macrophages containing bacilli from the gut associated lymphoid tissues (GALT). Extraintestinal sites have been documented which include the uterus, supramammary lymph nodes, udder and the sexual organs of bulls.<sup>33</sup> With the intra- and extraintestinal sites in mind the routes of bacilli shedding include feces, milk<sup>33</sup>, colostrum<sup>3</sup> and in-utero.<sup>5</sup> Sweeney et al. determined that isolation of *M.*

*paratuberculosis* from the milk and supramammary lymph nodes was possible in asymptomatic cattle. The prevalence of the organism was linked to the amount of fecal shedding.<sup>33</sup>

Realizing that some individual infections may occur prior to oral exposure to this pathogen several studies have focused on the possibility of in-utero transmission. The greatest difficulty in performing these studies is determination of the true status of enrolled individuals, thus many studies have focused on pregnant, clinically apparent dams. A number of studies have suggested that the chances of this mode of spread approaches a 20-40% transmission rate in cows with advanced stages of the disease.<sup>5</sup> Work performed in asymptomatic cows confirmed the theory of in-utero spread and documented an 8.6% rate of transmission.<sup>5</sup> As can be seen from these figures, the risk increases as the dam becomes more highly infected. Cows confirmed positive to late-phase tests such as fecal culture, agar gel immunodiffusion (AGID) and enzyme-linked immunosorbant assay (ELISA) should be considered to be at high risk of delivering an infected calf. A cut-off point with respect to fecal culture would indicate that a cow with less than 3,000 colony-forming units (CFU) per gram of feces should be considered a light shedder with in-utero transmission being unlikely. It may not be necessary to cull this offspring if a program to eliminate post-natal oral infection is successfully implemented.<sup>5</sup> As a point of reference, cattle in the clinical phase of this infection are capable of shedding bacilli at levels that exceed  $10^{10}$  organisms per gram of feces.<sup>4</sup>

Most prevention efforts have centered on the control of infection in young stock, specifically control of post-natal infection. The period of greatest transmission risk is during the first six months of life. Some studies indicate that 12 months may be required for a bovine to develop adult resistance. The theory that resistance increases with age is supported by experimental inoculation work in adults. This class of animal has proven more difficult to infect<sup>5</sup>. In addition, observational studies of infected environments have shown an increased infection rate in calves vs. adults. Horizontal transmission to mature cohorts is possible through fecal contamination of water and feed sources, but the complicating factor of adult infection is the long incubation period of *M. paratuberculosis*.<sup>34</sup> Current culling rates average 33% with most cows remaining in the dairy operation no more than 3 years. Given the incubation period associated with Johne's disease it is much more likely that the cow will be culled than manifest clinical paratuberculosis. Resistance should not be thought of as an "all-or-nothing" phenomenon, rather infection is probably an interaction between dosage and resistance. It should be emphasized that clinical disease may be observed in less than 30% of *M. paratuberculosis* infected cattle within a herd.<sup>3</sup>

As with any age group, calves are infected by ingestion of the organism through fecal, milk or colostrum contamination.<sup>11</sup> Organism exposure frequently is available through fecal contamination, thus the efficacy of a post-natal control program is judged on the ability to control this route of contamination. Control of this pathogen is highly dependent on the control of the amount of organism exposure. Goodger et al. showed through regression analysis ( $R^2 = 0.90$ ) that "high survey scores for environmental conditions, newborn calf care, grower calf care, bred heifer care, and manure handling were significantly associated with *M. paratuberculosis* prevalence in the state of Wisconsin."<sup>86</sup> The following are points that every veterinary

practitioner and producer must control to minimize risk.<sup>5</sup> Each dairy is a unique facility and relies on the expertise of the practitioner and producer to look at other risk factors beyond this list.

- Contamination of the calving stall by the dam.
- Contamination of the calving stall by the previous occupant.
- Fecal contamination of the udder.
- Fecal contamination of colostrum or milk.
- Use of the sick pen as a maternity pen with fecal contamination of potentially clinical cows.
- Failing to remove the calf soon after birth, thus allowing the calf to nurse the cow, or other cows present in the pen.
- Group housing of cows during parturition.
- Feeding bunk sweepings from adult cattle to young cattle.
- Fecal contamination of feeding utensils.
- Fecal contamination of worker clothing
- Direct manure contamination of feed (manure in the bunk).
- Contamination of feeding areas by direct manure runoff.
- Use of manure handling equipment for feed handling duties.

A cross-sectional Michigan survey by Johnson-Ifearulundu and Kaneene explored management practices as risk factors and calculated the odds associated with each significant practice.<sup>24</sup> Their work indicated three areas of significant association with increased or decreased prevalence of Johne's disease. The first factor identified was a 3-fold increase in odds of Johne's disease with the use of an exercise lot for lactating cows. As this area can be difficult to clean sufficiently, the wet and dirty surfaces may be locations where *M. paratuberculosis* can survive and be introduced to the youngstock areas of the farm. Farms that cleaned calf hutches and pens after each use demonstrated a 3-fold decrease in odds of infection with *M. paratuberculosis*.

The most interesting factor brought to light in this study was the 10-fold decrease in odds associated with the application of lime to pasture area.<sup>24</sup> Anecdotal reports exist in the literature suggesting that the application of lime to pasture and cattle housing areas seems to be associated with reduction of clinical cases of Johne's disease. Some of these reports also suggested that geographical areas with natural deposits of limestone (alkaline and calciferous) have a reduced prevalence of *M. paratuberculosis*. The potential mechanism of action lies in the ability of *M. paratuberculosis* to utilize iron. Iron is needed for bacterial growth, including mycobacterial sp. growth. With lime application iron solubility decreases as ambient pH increases, thus making iron less available.

Optimal mycobacterial growth occurs in an *in vitro* pH range of 5.5 to 6.0 and it would be expected that this is consistent *in vivo*. The pH of phagocytic vacuoles in the macrophage is 4.5 to 6.0. At this level the host iron binding compounds transferrin, lactoferrin and ferritin may provide sufficient iron for mycobacterial growth, even in the absence of mycobactin. This could be one of the most significant factors in proliferation of this intracellular pathogen in the macrophage population. Soil pH is an interesting theory with respect to

mycobacterial survival in the environment, but it may be extremely difficult to prove due to confounding factors such as soluble iron levels, calcium concentrations, organic matter levels, soil type, moisture content and bacterial as well as fungal residents. While this report is extremely interesting it must be remembered that a survey only examines associations, not a “cause and effect” relationship.<sup>24</sup>

It is well understood that a cow with clinical signs of Johne’s can shed billions of organisms each day.<sup>5</sup> While *M. paratuberculosis* does not readily multiply outside the animal host it will persist in manure, soil, manure slurry storage, and manure-contaminated water sources. Best estimates indicate that this organism may survive up to a year in manure; moisture and cold temperatures assist survival.<sup>5, 16</sup> With this in mind, all manure from Johne’s positive herds should be considered to be infected and should not be spread on ground that will be used as a forage source. The spreading of this material should be limited to row crop areas.<sup>11</sup>

Organism number reduction can be accomplished through the use of commercial disinfectants, although the common products are not effective against this organism. Control efforts should concentrate on the use of cresylic or phenyl-based products.<sup>11</sup>

One of the greatest sources of transmission to the neonate is feeding contaminated colostrum or milk.<sup>3</sup> As mentioned with in-utero transmission, the odds of dissemination of *M. paratuberculosis* increase with the progression of clinical disease.<sup>5</sup> Dissemination will directly infect the udder tissues with bacilli being liberated in both colostrum and milk. Studies of organism shed in milk indicated that 35% of infected, symptomatic cows shed through this route, as compared to 19% of asymptomatic heavy fecal shedding cows, and 3% of light fecal shedding cows. With respect to colostrum, the odds of organism shedding increase by a factor of 2 to 3, with 36% of the colostrum samples from asymptomatic heavy shedding and 9% of the light shedding individuals showing the presence of *M. paratuberculosis*.<sup>5</sup> Milk contamination is a very real risk due to the presence of the organism and milk antibody, which enhances bacilli uptake. Colostrum should be considered an even greater risk due to increased levels of antibody and mycobacterial shedding.<sup>1,6</sup>

Producers have attempted pasteurization of colostrum, but research indicates that *M. paratuberculosis* may survive pasteurization temperatures of 71.7 C for 15 sec in a water bath.<sup>15</sup> Reports indicate *M. paratuberculosis* may be more heat resistant than *M. bovis*, which is one of the reference organisms to define pasteurization time and temperature standards.<sup>15</sup> An additional risk factor of pasteurization is the reduction of passive immunity through heat deformation of immunoglobulins and loss of the attendant white blood cell population of colostrum. Commercial colostrum supplements have been tried, but insufficient immunoglobulin absorption leads to clinical failure of passive transfer to the calf. As colostrum is an essential of successful calf raising, the strategy of annual testing of the mature cow herd and timely culling of high shedding individuals is a critical step. Despite the antibody test being a late stage indicator of infection some producers will refuse to part with cattle that may be productive. In this situation the colostrum from a test positive cow should not be used in favor of utilizing banked colostrum from the oldest individual with multiple test negative results.

With dairy industry consolidation, herds of 500 - 3,000 head of mature cows have become common. Even with the contamination potential of both milk and colostrum, most producers are extremely reluctant to

discard what they perceive to be a valuable resource. An Iowa producer has purchased an old “small herd” bulk tank complete with internal refrigerant piping and agitator. The piping has been connected to a hot water source allowing uniform heating of the tank. Following loading, the milk is heated and agitated for 30 minutes with the waste milk reaching a pasteurization temperature of 150°F. Research is continuing at this time, but preliminary cultures are sterile with respect to *Escherichia coli*, *Salmonella* sp., *Staphylococcus* sp., and *Streptococcus* sp., as well as *Mycoplasma*. This has had immediate health benefits for the calf population. Additional research will be required to determine if waste milk is currently sterile to *M. paratuberculosis*, as well as following challenge doses to determine the efficacy of pasteurization.<sup>20, 75</sup>

A potential risk factor for the neonate is the phenomenon of the “open gut.” During the first 24 hours of life the newborn bovine is dependent on the absorption of immunoglobulins and white blood cells to establish a protective immune system. Failure of this absorption is a failure of passive transfer. During this time the gut is “open” to the absorption of macromolecules. It is not known if this possibly includes *M. paratuberculosis*, but it would certainly be conceivable.<sup>5</sup>

Other proposed mechanisms of spread exist. These include semen transmission, embryo transfer, transfer from wildlife sources such as deer, and veterinary procedures such as rectal examination. *M. paratuberculosis* has been isolated from semen samples and infected accessory glands of bulls. Inoculation of the uterus with infected semen has resulted in infection of the cow.<sup>5</sup> There is no evidence indicating what part this has played in the overall transmission of Johne’s disease. The use of an infected bull in a dairy operation should be considered a risk factor and mandates the annual, if not semi-annual testing of these individuals by both ELISA and cultural means to enhance testing sensitivity. The artificial insemination (AI) industry tests its donor bulls for the presence of the organism, and AI is considered a low risk.

Dissemination of *M. paratuberculosis* in advanced disease can affect most tissues and uterine washings have demonstrated the presence of the bacilli. Thus, embryo transfer could be of concern. Research has demonstrated the adherence of the bacilli to the embryo. Given the amount of organism that can adhere and the washing techniques performed, this is considered a low risk procedure. Greater risk comes from transfer of an embryo into an infected recipient dam.<sup>5</sup>

Deer and other ruminants can be infected by *M. paratuberculosis*, and could serve as a potential reservoir. Deer feces are pelleted, thus minimizing the amount of contamination that occurs over a given area of land. Deer are not considered to be a great dissemination risk to cattle, but the “cleaner” a herd becomes the greater the risk co-mingling with deer could be.<sup>5</sup>

No studies have been performed with respect to the transfer of Johne’s with routine rectal palpations. It is not known if the organisms from an individual that is shedding high amounts could penetrate the rectal mucosa of a non-infected individual.<sup>5</sup> Although this may not represent a great risk, routine sleeve changing may benefit in blocking the transmission of white blood cell entities such as bovine leukosis virus.

### Clinical and Gross Pathology

Gross lesions of Johne's disease are generally found in the terminal small intestine and associated lymph nodes. The ileum will display a uniform corrugated thickening of the mucosa. The mucosal layers are soft in consistency. Gross lesions are rarely seen in the cecum, colon, and rectum. The lymph nodes associated with the terminal ileum tend to be enlarged by a factor of 3 to 5 fold, and are edematous and soft. There are no lesions suggestive of caseous necrosis, fibrosis or evidence of tubercle formation. The subserosal lymphatics of this area tend to be affected as well, appearing dilated, beaded, thickened, and with a tortuous appearance. The presence of arteriosclerosis is another sign of advanced Johne's disease.<sup>6</sup>

Examination of tissue histopathology indicates a preponderance of epithelioid macrophages in the mucosa and submucosa. These will appear without evidence of necrosis despite gross proliferation.<sup>6</sup> The Ziehl-Neelsen technique or Immunogold Silver staining reveals large numbers of clustered bacilli in the epithelioid macrophages in the mucosa of the affected small intestine. These bacilli are readily apparent in the associated lymph nodes as well. The villar tips of the absorptive surfaces of the ileum will appear to be fused, and thus contribute to the nutrient malabsorption characteristics of this disease. The granulomatous enteritis results in a protein-losing enteropathy and hypoproteinemia.

Small intestine infection may be described in two ways. Distinct, focal lesions predominate in the paucibacillary form. The pluribacillary form is more diffuse and widespread with the lamina propria of the intestinal mucosa appearing thickened with numerous macrophage-infiltrated granulomas. Multinucleated giant cells can also be evident in the sub-mucosa layer. Similar lesions can be found in the mesenteric lymph nodes.<sup>35</sup> Using Ziehl-Neelsen staining techniques, many macrophages in the pluribacillary form will display reddish clumps and are considered positive. In paucibacillary form these positive macrophages are less evident. The multinucleated giant cells will appear unstained.

Changes in the clinical pathology of the animal are predictable, but can't be used as a diagnostic test due to individual characteristics. Most signs are related to the protein losing enteropathy and malabsorption from the small intestine. These include reductions in total protein, albumin, triglycerides and cholesterol as the disease progresses. Muscle enzymes such as creatine kinase will show elevations as muscular wastage starts to occur during the advanced stages of Johne's.<sup>6</sup>

Johne's disease can be characterized by four stages of disease.<sup>6</sup> They are as follows:

- Stage I – Silent Infection
- Stage II – Subclinical Disease
- Stage III – Clinical Disease
- Stage IV – Advanced Clinical Disease

**Stage I – Silent Infection.** These animals manifest no outward signs of disease, even though they have been exposed and infected with *M. paratuberculosis*. When compared to uninfected cohorts there are no visible differences in growth or weight gain. The only means of detecting these individuals is through tissue or fecal cultures. To obtain positive results multiple sites would have to be sampled on post mortem. Fecal cultures

need to be performed on a multiple basis, and even then affected cattle may be shedding at such a low rate as to be virtually undetectable. Detection using antibody or cell-mediated means would be impossible.<sup>6</sup>

**Stage II – Subclinical Disease.** These animals manifest few if any outward signs to distinguish them from their cohorts. Depending on the individual host response there may be a possibility to detect these cases through antibody or cell-mediated response, but the vast majority are as yet inapparent. A greater number of these individuals may start shedding sufficient bacilli to be detected on a fecal culture (15 - 25%).<sup>6</sup> Due to progressive infection and reduced immunocompetency some of the individuals in this category may be more prone to cases of mastitis or other types of infection.

**Stage III – Clinical Disease.** The first visible sign at this stage is intermittent or persistent diarrhea. This may be accompanied by some weight loss. Depending on the signs and symptoms it may be possible to see some changes in clinical chemistry, especially protein values. Animals are not expected to occupy this stage longer than 3 to 4 months before progressing to the advanced stage of disease. Physical examination of these cows will not yield any abnormalities in terms of vital signs, i.e., heart rate, breathing or ruminations. Diarrhea with evidence of blood, mucus or tenesmus should be discounted as being due to Johne's disease. Animals at this stage of infection have a good chance of testing positive for the presence of antibody or being fecal culture positive.<sup>6</sup>

**Stage IV – Advanced Clinical Disease.** At this stage the physical signs of clinical Johne's disease become readily apparent. Due to the protein-losing enteropathy, bottlejaw, weight loss and emaciation are readily apparent in addition to the copious, watery diarrhea. This is the terminal stage of the disease and usually the point at which cattle are removed from the herd due to low milk production, emaciation, or clinically apparent diarrhea and shedding. Dissemination of the bacilli throughout the animal may also occur at this time with the result that *M. paratuberculosis* may be shed through the milk and passed to a developing fetus. Affected cattle can progress through stages II to IV in a matter of weeks depending on the host's immune response.<sup>6</sup>

### **Immunology of Johne's Disease**

Infection with *M. paratuberculosis* and subsequent disease is a dynamic event. The progression of this disease is not a response to any toxins or substances liberated by *M. paratuberculosis*. It is mediated by the host's immunologic reactivity; thus the course of disease will vary from individual to individual. With this dependency on host immune system response, Johne's has the capability to fluctuate between periods of exacerbation and remission. This variability is reflected by the "paradoxical" manner in which cattle infected with *M. paratuberculosis* may respond. Depending on the stage of disease, infections may be characterized by cell-mediated response, humoral response, both or neither (anergy).<sup>12, 36, 37</sup>

Immunological variability is the result of at least two factors – the cellular level route of antigen presentation in the GI tract, and the opposition of immunological forces, i.e. cellular vs. humoral immunity. The

immunology of mycobacterium species is complex, and the experience gained in work with *M. tuberculosis* and *M. leprae* has direct bearing on the pathogenesis of infection with *M. paratuberculosis*.<sup>12,36,37</sup>

*M. paratuberculosis* is unique among mycobacteria in its ability to infect the gastrointestinal tract and associated mucosal immune system. The resulting immune system response is directly due to the nature of the gut associated lymphoid tissues (GALT). GALT is self-contained and compartmentalized, unlike the bronchus associated lymphoid tissues associated with *M. tuberculosis*. The term “self-contained” refers to the ability of the GALT to determine its own immune response through presentation of antigen and activation of various lymphoid populations. Compartmentalization refers to the resident population of lymphocytes that are derived from this area. Lymphocytes from Peyer’s patches derivation tend to circulate through the mesenteric lymph nodes, into the thoracic duct and into systemic circulation, followed by a return to the lamina propria of the intestinal tract. Other than a brief appearance in the systemic circulation these cells are compartmentalized in the digestive tract. Due to this compartmentalization, events in the intestinal tract are not necessarily reflected in the immune system testing methods available via the peripheral circulation.<sup>36</sup>

The paratuberculosis organism is presented to the ruminant through fecal, milk, and colostrum presentation of the live organism. After oral ingestion the organism comes in contact with the GALT via the microfold (M) cells.<sup>38</sup> These are antigen transport entities capable of presenting particulate matter such as viruses, bacteria (including *M. paratuberculosis*) and protozoa to the intercellular spaces in the Peyer’s patches directly below the epithelial covering. In this area macrophages and dendritic cells phagocytize these antigens with subsequent presentation to the underlying lymphocytes of the Peyer’s patches. At the time of lymphocyte presentation the macrophages are not activated and are not capable of bacteriolysis.<sup>36</sup>

The receiving lymphocyte population can be divided into two categories based on functional role and lymphokine profiles. CD4<sup>+</sup> type-1 T-helper cells (T<sub>H1</sub>) produce cytokines primarily consisting of interleukin-2 (IL-2) and gamma interferon ( $\gamma$ -IFN) and mediate macrophage activation, delayed-type hypersensitivity (DTH) and IgG2a synthesis and are thus associated with cell-mediated immunity (CMI). CD4<sup>+</sup> type-2 T-helper cells (T<sub>H2</sub>) produce large amounts of IL-4, IL-5, IL-6, and IL-10 and mediate antibody production (IgG1 and IgE) and eosinophilia.<sup>12, 36</sup> Effective defenses against mycobacteria species rely on the active participation of the cell-mediated immune system. Production of IL-4 by T<sub>H2</sub> cells acts as a potent antagonist of macrophage function, IL-2 receptor sensitivity, IL-2 dependent macrophage proliferation, and production of  $\gamma$ -IFN by mononuclear cells. Conversely, IL-4 enhances the production of antibody, which is an enhancement of the humoral immune system. Activity of the humoral immune system comes at the expense, or down-regulation, of the cellular immune system.<sup>12, 36, 38</sup>

In addition to these populations of lymphocytes another major group of T cells has been identified as cytotoxic/suppressor T cells (T<sub>C/s</sub>). This population bears the CD8<sup>+</sup> marker. T<sub>C/s</sub> cells mediate cytotoxic activity and are required for effective immune responses to mycobacteria. The suppressive nature of these cells is controversial, and their precise role in mycobacterial infections is not yet clearly understood.<sup>36</sup>

Early antigen presentation and lesion management is accomplished solely through the GALT with little or no stimulation of the peripheral immune system. Two types of T-helper lymphocytes are present. In the GALT  $T_{H1}$  predominates, thus the response is primarily cell-mediated. The normal route of presentation involves the M cells presenting particulate antigen into the intercellular spaces of the GALT where the macrophage population will have access. The macrophages act as accessory cells and process (prime) the antigen for presentation to the lymphocyte population. Priming consists of reduction of the antigen to peptide bases and presentation of these bases on the cell surface in conjunction with major histocompatibility complexes (MHC). Many cells can present antigen, but the macrophages are uniquely qualified for presentation in a productive manner. The result of this presentation is the activation of a primary  $T_{H1}$  population with the attendant production of the multiple cytokines listed above.<sup>12, 36</sup>

Mycobacterial antigen can also be presented in a paracellular route consisting of movement through the tight junctions of the intestinal villi with presentation to the lamina propria of the intestine below. Presentation in this manner induces the  $CD4^+$  population and with it a sub-population of  $CD8^+$  suppresser cells. The  $CD4^+$  helper response will predominate. Antigen can also be presented through the transcellular route via cellular endocytosis. Antigen processing in this manner will stimulate a  $CD8^+$  induction with the result that T-suppresser cells will dampen immune system activity or create a tolerant state.<sup>36, 38</sup>

Upon recruitment, the  $CD4^+$   $T_{H1}$  population mediates activation of the macrophage population. As mentioned earlier, active immunity to mycobacterial infection is dependent on cell-mediated immunity. Cell-mediated immunity is based on the accumulation and activation of the macrophage population.<sup>12</sup> Unactivated macrophages are incapable of bacterial phagocytosis and have reduced antimicrobial activity. Enhanced macrophage activities alone are not enough to contain the mycobacterial threat. The T-helper cells must also elaborate cytokines which stimulate production of a granuloma site. A granuloma can contain the infection site while maximizing bactericidal activity. Critical to the production of a granuloma is the presence of an insoluble antigen. The insoluble and complex mycobacterial lipids may be granulomagenic without being immunogenic, and persistency is not dependent on the antigens being a living source.<sup>36</sup> Cell fractions or cell-wall preparations may be sufficient to initiate a granulomatous response.<sup>36</sup>

Another manifestation of cell-mediated immunity are DTH reactions as evidenced by skin inflammation and swelling following injection of a PPD. It was previously thought there was a direct link between active immunity and presentation of a DTH reaction. Study of *M. tuberculosis* has documented cases in which certain fragments of this pathogen are capable of stimulating a strong degree of resistance without the production of a demonstrable DTH response. Conversely, injection of bacille Calmette-Guerin (BCG) in humans showed no protective influence while demonstrating a vigorous DTH response.<sup>36</sup> Many of the clinical symptoms seen in paratuberculosis and leprosy may in fact be an allergic manifestation. Immunity related to the GALT is well compartmentalized and may have very little peripheral immune system exposure. It may be possible to have vigorous cell-mediated immunity with little DTH response. Correlation of infectious status with skin testing has been poor.<sup>12, 36, 38</sup>

Discussion of immune system response focuses on the active intervention of this body system. Johne's is well known to have periods of time when no measurable immune system activity can be determined by testing. Generally this is a late disease phase phenomenon called suppression. Anergy is a form of suppression that is a loss of responsiveness or desensitization to a preexisting sensitivity to an antigen.<sup>36</sup> The possible mechanisms could include suppression by host serum factors (e.g., antibody or immune complexes), host cell factors (e.g., CD8<sup>+</sup> suppressor cells or cytokines), or bacterium derived factors.<sup>37</sup> The onset of suppression is associated with the bacteremia of the late phases of this disease. An example of anergy associated with bacteremia is the inability to detect serum antibody. This may be due to immunocomplexing of antibody and free bacteria. Another concept of anergy is that it can occur selectively with respect to individual immune system responses, such as CMI and DTH. These systems may become unresponsive while the humoral system remains active and measurable. Cell-mediated immunity appears to be the only means of actively checking the course of disease. Loss, or anergy, of this response allows the bacilli to proliferate at an unchecked rate and frequently signals the end stages of clinical Johne's disease. A second form of suppression is tolerance, which is the inability to respond to initial and subsequent antigen challenges.<sup>36</sup> This form has not been demonstrated in Johne's disease.

One of the last measurable responses in clinical Johne's disease is the presence of antibody in the peripheral circulation. Antibody has no protective value in the face of a mycobacterial disease, yet tends to be a common occurrence in the late stages of this disease. There tends to be an inverse relationship between the presence of CMI and the humoral response. This could be explained by antagonistic activity between T<sub>H1</sub> and T<sub>H2</sub> type helper cells. Generally, increasing levels of humoral immunity signal increased levels of bacteria in the affected animal. High levels of circulating antibody should be considered a poor prognosis and grounds for removal of the animal from the premises. As mentioned earlier, clinical signs may have an allergic component. Immune system complexing with deposition in the kidneys has been demonstrated in humans with infection of *M. leprae*. Similar complexing has been demonstrated in the GI tract of ruminants. This could result in local, delayed or immediate hypersensitivity reactions that could contribute to the clinical signs of Johne's disease.<sup>36</sup>

### **Immunology and Transmission**

It is well documented that calves ages 0 to 6 months are the most susceptible to mycobacterial infections. In an environment where the prevalence rate is high it can almost be assured that calves will come in continual contact with this organism. Examination of early lesions tends to demonstrate multifocal characteristics vs. single lesions. This would tend to lend credibility to the theory of multiple exposures and high organism loads during the young calf's life. The desire to suck, lick and browse virtually ensures this level of exposure.<sup>36</sup> The direct form of inoculation is termed exogenous infection.

Normal means of exposure suggest that the antigen (the bacilli) will come in contact with the Peyer's patches of the GALT in the GI tract. M cells then attach the antigen and present it to the population of unactivated macrophages present below the epithelium for phagocytosis. GALT macrophages are a compartmentalized population. They may circulate briefly in the peripheral vascular system, but they return to

the GI tract. Movement of the macrophages carrying intracellular mycobacterium progresses from the Peyer's patches to the mesenteric lymph nodes, the thoracic duct, the systemic circulation, and back to the lamina propria of the small intestine.<sup>36</sup> There the live bacilli are released to the deep tissues allowing the infection mechanism to proceed. This form of hematogenous spread is referred to as endogenous infection. Support for this suspected route is the brief seroconversion of young animals early in the course of experimental infection. Presumably this is due to the release of bacteria from the unactivated macrophages into the systemic circulation. The demonstration of multifocal lesions deep within the lamina propria suggests the only route for initiation of these lesions would be through hematogenous spread.

### **Vaccination against Johne's Disease**

Vaccination for *M. paratuberculosis* was first described by Vallee and Ringjard in 1926.<sup>42</sup> Their observations demonstrated a lack of clinical disease in animals vaccinated with *M. paratuberculosis*. A product is currently licensed for sale in the US that consists of a heat-killed strain-18 *M. paratuberculosis* suspended in mineral oil. Use of this product is generally regulated by individual states. In the state of Iowa a positive diagnosis of Johne's disease is determined by an organism based test through the Iowa State University Diagnostic Laboratory. A regulatory letter will follow to the submitting veterinarian asking them to contact their client about Johne's disease and the use of a vaccination program. If the client chooses to go ahead with this technique, the producer, practitioner and State Department of Agriculture will enter into a Johne's control program, which will mandate one of the choices below.<sup>77</sup> At this time there is no formal inclusion of other management techniques pertaining to reduction of *M. paratuberculosis* exposure to young animals.

1. Culture and cull alone for very lightly infected or recently infected herds.
2. Culture and cull of adult cattle, with vaccination of replacement heifer calves with a killed whole-cell vaccine. For herds with a medium infection rate (2%-10%).
3. Removal of culture positive animals only at first clinical signs, and vaccination of replacement heifer calves with a killed, whole-cell vaccine. For herds with a heavy infection rate (>10%), where culling all culture positive animals would create a severe economic hardship.

With permission of the state veterinarian a control agreement is signed and vaccination can begin with following provisions.<sup>78</sup>

#### **The Department of Agriculture Agrees To:**

1. Permit use of Johne's disease vaccine only if herd has clinical history of paratuberculosis and *M. paratuberculosis* has been isolated.
2. Test entire herd for tuberculosis by the caudal fold intradermal method prior to start of vaccination program
3. Supply vaccine to veterinarian for administration to calves in owner's herd.
4. Receive and file vaccination and test records.
5. Supervise program as necessary.

**The Veterinarian Agrees To:**

1. Administer vaccine when calf is from 1 to 35 days of age.
2. Identify calf by official ear tag, tattoo, or registry number and further identify calf with a tattoo in the left ear which includes a number representing quarter of year when vaccinated followed by letter "J" followed by a number representing the year when vaccinated.
3. Furnish reports to the Department each time calves are vaccinated, including identification of dam.
4. Collect and submit samples for fecal culture as requested.
5. Compensate Department for cost of vaccine.
6. Issue shipping permits when requested by owner.

**The Owner Agrees To:**

1. Submit no claims against Department, veterinarian, or vaccine producer for losses or adverse affects of the vaccine.
2. Compensate veterinarian for administration of vaccine, collection of fecal samples and laboratory culture charges.
3. Move no cattle from vaccinated herd without a shipping permit or health certificate.
4. Remove fecal-shedder cattle and clinical cases for slaughter within a time frame mutually agreed upon with the State.

Vaccination produces demonstrable amounts of immune system activity in both cellular and humoral areas, yet there is very little functional immunity.<sup>36,42</sup> The vaccine does decrease mycobacterial shedding, reduce the number of animals with the appearance of clinical disease, and the number of instances of detectable intestinal infection. The vaccine does not, however, prevent infection.<sup>36,39,42</sup> Additionally, vaccination nodules are a common occurrence with the administration of this vaccine. In one study the injection site lesion rate was 28%.<sup>40</sup> Administering the vaccine is considered a health risk, and it must be handled with care by the veterinary practitioner. Accidental auto-administration has been known to cause granulomatous reactions. A study by Patterson et al. examined accidental self-inoculation by veterinary practitioners in the state of Wisconsin.<sup>41</sup> Their work identified an incidence rate of 1 needle-stick per 1,000 doses administered, or 5.5 needle-sticks per 100 veterinarians/year of bacterin use. Adverse reactions ranged from small nodules that persisted for 4 to 6 months, to painful inflammation of a finger that persisted for 24 months. Despite the human risks the vaccine may have a place in herds with high infection rates to reduce culling frequencies.<sup>39,40,42</sup> The producer must bear in mind that the presence of the vaccination program will not be felt for a minimum of two years; the time needed for young vaccinates to enter lactation.

The vaccine benefits listed can most logically be explained using the wealth of knowledge generated from the vaccination of humans using the BCG vaccine against tuberculosis. Work in this area suggests BCG vaccine inhibits the bacillemic phase of primary infection.<sup>36</sup> This would be the internal bacilli distribution

phase. Thus it could be expected that the vaccine provides protection against the endogenous (hematogenous) means of infection as compared to the exogenous routes of infection mentioned above.<sup>36</sup>

Current technology has focused on the use of whole-cell products, purified antigens and extracts of *M. paratuberculosis*. The most effective protection has been elicited using whole-cell products, which yielded the observation that adequate immunity is possibly a result of a host-parasite relationship. This theory has tended to be disproven as the antigen and extract products, if properly adjuvanted, have achieved demonstrable immune system responses (cellular and humoral). The presence of a CMI response is not the equivalent of protective immunity. It has been demonstrated that the cellular cytokines,  $\gamma$ -IFN and tumor necrosis factor- $\alpha$  tend to be produced as a response to the pathology (the granuloma) vs. the actual organism.<sup>36</sup> This would suggest that an as yet undescribed antigen may be required to stimulate T<sub>H1</sub> lymphocytes that would result in activation of the macrophage population and subsequent organism inhibition and bacteriolysis.<sup>43, 42</sup> An optimum vaccine would also be able to prevent binding at the small intestine and would thus prevent both means of mycobacterial infection (exogenous and endogenous).

Research in the area of vaccines continues today, targeting further refinement of the vaccine and the potential to produce a gene-deleted product, which could prove beneficial in differential testing of vaccinates.<sup>43, 42</sup> Other research efforts center on moving the vaccine from youngstock to application in adults of highly infected herds to reduce total herd shedding at a faster rate than has been accomplished with the youngstock program. Rates of culling need to be determined to see if any advantage is present with the later application of this product.

### Testing Procedures

Immune system response measurements can be extremely variable due to the manner in which the antigen is presented to the immune system as well as the interaction of individual host responses. One typical feature of this disease, if anything can be called typical, is that animals in early infection stages rarely mount a measurable immune system response. There is a period of anergy prior to the time that any response, cellular or humoral, can be measured. Some transient measurements are possible as *M. paratuberculosis* passes through the digestive tract (cellular) or exposure to the peripheral circulation occurs (humoral), but these are not repeatable. At the first stages of repeatable, measurable immunity, bacterial counts are likely to be greater than 10<sup>4</sup> CFU within each of the multifocal lesions.<sup>36</sup>

Patterns of measurable response will vary from animal to animal, but in the early stages of infection the cell-mediated phase tends to predominate due to active proliferation and differentiation of T cells. As the onset of clinical signs approaches, the measurable systemic reaction will shift to a predominantly humoral (antibody) response. The cell-mediated phase will decline as the humoral response increases. In the final stages of disease the animal may lapse again into a period of anergy as both cellular and humoral responses diminish.<sup>12</sup>

With this response variability in mind, a rule of thumb known as the "Iceberg Effect" can be applied to determine the effects of Johne's disease on a particular operation. The "tip of the iceberg" is the advanced case.

For every “home-raised” (reflects young stock management) advanced clinical case there are potentially another 25 cases at some level of infection. Only 15 - 20% of these cases may be detected using current testing procedures. Using the staging procedure listed above, the following is the possible breakout distribution of these cases.<sup>6</sup>

- Stage IV – Advanced Clinical Disease..... 1 animal
- Stage III – Clinical Disease ..... 1 - 2 cattle
- Stage II – Subclinical disease: carrier adults ..... 4 – 8 cattle
- Stage I – Silent infection (calves, young stock and adults) ..... 10 – 14 cattle
- Total ..... 15 – 25 cattle

Multiple testing means are available to the practitioner for determining the presence of Johne’s. These include: four means of organism detection and/or isolation, four means of antibody assay, and two assays for the presence of a cell-mediated response in the form of  $\gamma$ -IFN determinations, and a DTH skin reaction.<sup>21</sup> Most testing procedures are most sensitive in detecting the presence of Johne’s in animals that are in Stages III to IV.<sup>44</sup>

Current testing means have frequently been criticized by the cattle industry as being too insensitive to be of any value. The intracellular location and slow, progressive nature of the disease is most responsible for this criticism.<sup>17</sup> While the goal of the veterinary profession is to find a means of increased sensitivity there is often great producer reluctance to use test information to remove identified individuals due to milk production, genetic potential or the lack of any apparent disease. This is true even in the face of testing means that demonstrate a false positive rate of less than 1%. Given the variability in disease presentation due to immune system unpredictability, sensitivity rates approaching 50% may be very good. Another factor for consideration is that once an animal starts to move to late phase clinical disease the tests available perform at levels very similar to available tests for other infectious diseases.<sup>21</sup>

### Organism Detection

Bacterial culture is one of the oldest, most reliable forms of testing available. It is often considered the “gold-standard” in detection and can be performed on both feces and tissue cultures from biopsy samples or post mortems. The advantage of this test is its specificity, which is 99.9%. This method of testing should not be used as the sole diagnostic means due to the intermittent shedding of *M. paratuberculosis*. With this in mind, sensitivity of this test runs 45% to 50%.<sup>4</sup> Bacilli isolation is a true indicator of animal shedding and is good evidence for removal of identified individuals. The disadvantage lies in the slow bacillus generation time of 4 days for *M. paratuberculosis*. Typically these cultures take up to 16 weeks for completion during which time the practitioner risks losing producer interest. Additionally, cultures tend to cost \$16 to \$20 and are subject to fungal contamination which renders them unreadable.

While this method of testing is widely used, the proficiency of laboratories can vary widely. Techniques in test sensitivity improvements have included organism concentration and growth media. All samples for

culture must be decontaminated prior to culture. Most commonly this is performed using hexadecylpyridinium chloride (HPC), a disinfectant considered to be the least harmful to *M. paratuberculosis* while rapidly killing other species.<sup>21</sup> HPC has no effect on fungal contamination. The samples are then concentrated using centrifugation, sedimentation or filtration to increase the amount of *M. paratuberculosis* present for inoculation. Paradoxically these concentration efforts may also result in increased amounts of contamination. Typically this is addressed through pre-incubation in broth media incorporating antibiotics (Cornell method, and NADC method) or the inoculation of Herrold's egg yolk media containing antibiotics.<sup>45</sup> Most laboratories use Herrold's media, but there is no commercial source, again hampering standardization. Typically 4 slants of Herrold's are inoculated to maximize the chances of having a readable culture if fungal contamination is present.

A study by Whitlock et al. examined the current practice of using single or limited numbers of fecal and tissue cultures as compared to the odds of finding *M. paratuberculosis* growth from an infected animal.<sup>46</sup> The authors widened their scope of tissue cultures to include the pulmonary, mandibular, retropharyngeal, hepatic, supramammary, ileal-cecal and mesenteric lymph nodes, as well as multiple sites through the jejunum and ileum. Their conclusions indicated that fecal culture remains one of the most sensitive testing means available, but most infected cattle will only be detected after multiple, serial cultures. Some cattle will remain undetected until examination at slaughter; 30% of the cattle with repeated negative fecal cultures were tissue culture positive at post mortem examination.

The authors also indicated that tissue culture methods were a more sensitive means of detection as compared to histopathological examination looking for macrophages, giant cells and epithelioid cells with acid-fast bacilli in a granulomatous response.<sup>46</sup> Current diagnostic practice suggests that tissue culture of the ileal-cecal lymph nodes and ileum should be performed as an adjunct to histopathological examination. Given the results of multiple tissue samples, the authors also suggested that current culture practice may misdiagnose approximately 20% of the cases currently declared negative. The study found that disseminated infection occurs frequently in cattle with Johne's disease, especially in the later stages of infection, but it can also occur as early as the subclinical phase.

A Netherlands study by Kalis et al. examined a test and removal program utilizing fecal cultures every 6 months.<sup>47</sup> Their work demonstrated that the second 6-month test period was less successful in the identification of positive individuals. The majority of infected cows identified in the third period took greater than 6 months to excrete a detectable number of CFU. This work could be an argument for fecal culture test intervals of not less than 1 year in length. As a part of this study, fecal culturing was extended to younger members of the enrolled herds. No heifers <11 months of age cultured positive, while a significant increase in culture positive results was recorded in heifers 13 to 14 months of age (5.9% positive). Like the heifers < 11 months of age, heifers > 14 months demonstrated significantly fewer culture positive individuals as compared to the 13 to 14 month old population. This would support the observation that fecal shedding can occur in an early phase of

bovine paratuberculosis, followed by a phase of silent infection not detectable by fecal culture.<sup>47</sup> These culture results also raise the question of horizontal transmission among heifers housed in less than sanitary conditions.

A modification to the fecal culture technique is the commercial culture kit known as the BACTEC (Bentson Dickson Laboratories, Inc., Sparks, MD). Inoculations are made into a liquid media (BACTEC 12 B) that contains a radioisotope-labeled nutrient source (<sup>14</sup>CO<sub>2</sub>-labeled palmitate). Growth of the organism is detected using an analytical device that measures <sup>14</sup>CO<sub>2</sub> released by bacterial metabolism. This technique has been combined with a filter concentration technique of *M. paratuberculosis* to yield more rapid isolations (4 to 7 weeks) along with increased sensitivity. Growth may be detected as early as 9 days post-inoculation on severely infected animals.<sup>48</sup> This testing method is not currently in widespread use.<sup>21</sup>

The specificity for this culturing technique is estimated at 99.9%. While this specificity is very high there does exist the detection risk of “pass-through” isolations.<sup>49</sup> In heavily contaminated environments, bacilli may be presented to the animal with the result that organisms are “passed-through” or shed. These individuals could possibly show a false positive isolation, but given the sensitivity of this testing method at 45% to 50%, the risk is very low. This risk could become a greater concern with the development of increased sensitivities in fecal testing.

The third form of organism detection is performed using the DNA probe method. This is a very rapid detection technique and depends on the test identification of the insertion sequence 900 (IS900) that is present in 15 to 20 copies per organism.<sup>50</sup> This insertion sequence is thought to be unique to *M. paratuberculosis*. Detection of the IS900 is dependent on gene amplification techniques such as PCR. Even with amplification this test remains somewhat less sensitive than culture since the concentrations of *M. paratuberculosis* must approach 10<sup>4</sup> organisms per gram of feces for detection. Current culture techniques can detect as low as 10 to 100 bacteria per gram of feces.<sup>51</sup>

Van Der Giessen et al. suggest that the majority of animals shedding *M. paratuberculosis* will be missed by the PCR technique.<sup>51,52</sup> Their work has demonstrated that PCR can detect chromosomal DNA down to a level of 2 to 3 bacteria, which is the equivalent of 50 bacteria per gram of feces. The potential reason this testing method is not performing at this level could be due to the presence of inhibitors in the fecal samples. Removal of these inhibitors may hold the key to routine DNA probe use.

A recent concept being tested to remove contamination is the use of glass or latex immunomagnetic beads. The beads are coated with antibody specific for *M. paratuberculosis*. The fecal sample and water are placed in a glass vial containing the beads. The contents are shaken, and a magnet is applied to the outside of the vial to hold the beads in place. The feces are removed and the beads are washed to remove fecal inhibition factors. The PCR technique can then proceed. This concept is currently used to detect *E. coli* O157:H7 from fecal samples.<sup>53</sup>

In studies by JR Stabel’s laboratory (National Animal Disease Center – Ames) the commercial DNA probe was found to identify only 60% of the isolates that were determined as positives by fecal cultures. Recent work has focused on combining two amplification steps in the PCR, thus increasing the sensitivity to 50

organisms/gram of feces vs.  $10^4$  organisms/gram. The number of manipulations currently required for the second amplification step limits the utility of the two-amplification probe.<sup>50</sup>

The power of the DNA probe lies in its speed. From start to finish this organism-based test requires only 3 days to complete, and, no confirmatory testing is required for organism identification as IS900 is considered to be diagnostic at this time.<sup>50,51</sup> Sensitivity for this procedure is approximately 35%, while specificity is 99.9%. An additional benefit over culture based testing is that no samples are lost to fungal contamination. This form of testing can be difficult to run, and the accuracy of the technique varies with the laboratory. False positive results can occur if gene products produced in a lab are allowed to contaminate samples. Due to current sensitivity constraints, some labs are limiting this test to use as a rapid means of cultural identification.

The fourth method of organism detection is the use of histopathology and immunohistochemistry techniques of prepared tissues. Tissues are acquired from post mortem samples or from surgical biopsies of genetically high merit animals. Tissue changes and bacteria are detectable in the intestinal lining and adjacent lymph nodes. Generally animals in the early stage of infection, such as Stages I or II, are not detectable by this method, with the result being a false negative.<sup>44</sup> The reader is referred to the section on Clinical and Gross Pathology for a more detailed explanation of the tissue characteristics of Johne's disease.

Work by Coetsier et al. focused on the creation of polyclonal and monoclonal antibodies to a recombinant polypeptide (a362), which carries *M. paratuberculosis* species-specific epitopes.<sup>35</sup> These epitopes are not cross-reactive with any other mycobacterial species, and would be extremely useful for tissue staining techniques. This procedure demonstrates advantages over the routine Ziehl-Neelsen staining technique. These include: 1) increased sensitivity, 2) detection of *M. paratuberculosis* infection in both paucibacillary and pluribacillary forms of the disease, 3) Ziehl-Neelsen staining only detects bacilli with intact cell walls, while immunohistochemistry can detect fragments, and 4) the technique can be performed on fixed tissues. An additional benefit is evident when compared to PCR identification which is not capable of detecting intracellular *M. paratuberculosis*. This method of immunohistochemistry can localize these organisms.<sup>35</sup>

### **Antibody Assays**

Antibody assays for the detection of Johne's disease have been an area of serious work starting first with the complement fixation (CF) and agar gel immunodiffusion (AGID or Rapid Johne's Test, ImmuCell, Portland, ME) techniques. Development and improvement of the ELISA has made it the standard for antibody detection in bovines.<sup>21</sup> Unfortunately most antibody production occurs during the late phase of disease, and due to the slow, chronic, and progressive nature of Johne's most animals are in a pre-clinical phase of disease when tested. This makes antibody detection a later phase test than the industry would like.<sup>54, 55</sup>

As *M. paratuberculosis* enters the peripheral circulation during dissemination of the organism, B-lymphocytes become activated to produce antibody. Antibody does not exert a protective influence, but can serve as a marker element for the presence of this disease. As this is a circumstance of dissemination, antibody is a late phase marker of this disease, as is the liberation of significant amounts of organism in the feces

allowing cultural detection. Although a late phase response, the presence of antibody may be the first signs of clinical disease, thus allowing the practitioner and producer to cull animals while they still have market value.<sup>21</sup>

Sensitivity in these types of tests is dependent on the analytical sensitivity or concentration of antibody needed to turn the test positive. It is also a measure of the percentage of *M. paratuberculosis* infected cattle within a herd with positive test results.<sup>55</sup> Currently the ELISA test is the most sensitive with an aggregate sensitivity of  $45.5\% \pm 6.7\%$ , with a specificity of  $99.7\% \pm 0.3\%$ .<sup>56</sup> Work by Collins et al. demonstrated that the antibody concentration in low-level shedders may allow sensitivities of only  $24.6\% \pm 10.2\%$ , while use in animals displaying clinical signs may have a sensitivity of  $88.2\% \pm 5.5\%$ .<sup>55, 56</sup> With this in mind, a practitioner performing a whole herd test should assume that the ELISA means of testing, as a general rule, will under-report the actual prevalence by 50%.

While making an estimation of herd prevalence a practitioner should keep in mind that aggressive culling following detection may cause severe under-estimation of the actual rate. AGID and CF testing method sensitivities are in the area of 18.9% - 26.6% and 10.8% - 31.1% with specificities approaching 99.4% - 99.9% and 90.3% - 97.4% respectively.<sup>57, 18</sup> The positive predictive value of the AGID is very high, but due to its low sensitivity it should not be considered as the test of choice for herd screening purposes.<sup>27</sup> The AGID test is useful for the detection of Johne's in individuals exhibiting weight loss and/or diarrhea. In these cases the sensitivity of this test is 96.9% with a specificity of 94%.

The CF test is required by many countries for import/export regulations, but with many false positives and false negatives, CF is not recommended in the US for routine diagnostic use.<sup>44</sup> Complement fixing antibodies are a relatively late phenomenon in the course of Johne's disease. It has been reported that CF reactivity is associated with persistent fecal shedding of *M. paratuberculosis*. Animals that shed few or no organisms in the feces are negative to CF testing.<sup>58</sup>

Specificity is determined by the percentage of noninfected cattle having negative test results for the presence of *M. paratuberculosis*. One of the major criticisms of the ELISA technique has been the lack of specificity. This has been addressed with the refinement of antigens and the removal of cross-reacting, non-specific antibodies. The ELISA test currently utilizes an extract of *M. phlei* as a preabsorption agent to increase specificity.<sup>16, 55</sup> With these refinements antibody test specificities are greater than 99%.<sup>21</sup> Determination of specificity can be elusive with Johne's disease. Due to the long incubation time some *M. paratuberculosis* infected cattle may be enrolled in the presumptive noninfected group.<sup>55</sup> Additional criticism was leveled at antibody tests suggesting that positive results were false since fecal culture testing of the individual was negative. With the determination that antibody production can precede fecal shedding, this criticism has been removed.

Due to the sensitivity and specificity of the ELISA this procedure has become one of the regulatory standards in the US. The major breakthrough in this testing procedure came with the preabsorption treatment of sera with *Mycobacterium phlei* which removes cross-reacting mycobacterial antibodies.<sup>55</sup> The USDA licensed kit is the absorbed ELISA (IDEXX Laboratories, Inc., Westbrook, ME). The arrival of this test has been

advantageous in the standardization of procedures and results among laboratories. In addition to these benefits test results are now reportable in a quantitative manner. The use of optical density values to calculate a sample to positive ratio (S/P ratio) provides real advantages for the practitioner and producer in identifying and prioritizing animals with the greatest response.<sup>59</sup> Increased antibody response generally means that the animal is in greater danger of becoming a clinical case. With that in mind a list can be developed of tested animals within a herd that should potentially be culled or immediately retested. Work by Sweeney et al. suggested that reference negative cows they tested were consistently below the recommended cutoff S/P ratio of 0.10, while infected cows were broadly distributed on both sides of the 0.10 cutoff value.<sup>56</sup> To maximize specificity, while retaining reasonable sensitivity, the IDEXX test utilizes a cutoff S/P ratio of 0.25. Recognizing the gray zone between 0.25 and 0.10, Dr. Michael Collins developed a grading procedure to help the practitioner and producer in assembling a prioritized action list (Table 1).<sup>60</sup> The graded test allows identification and inclusion of animals that just missed the positive designation. The practitioner may then elect to target these animals for additional testing at a later date.

**Table 1. ELISA Test Classifications – Dr. Michael Collins<sup>60</sup>**

Classification	IDEXX ELISA S/P Ratio	Recommendation
Negative	0.0 – 0.099	Do nothing
Suspect	0.1 – 0.25	Retest in 6 mo.
Low Positive	0.26 – 0.39	Infected – early stage
Positive	0.4 – 0.9	Infected & shedding
Strong Positive	>1.0	Soon to go clinical

Sweeney noted that disease progression is linked to diminished response of the cell-mediated immune system. The onset of the humoral response and increased fecal shedding makes clinical cases more easily detectable. The risk of fecal shedding thus increases as the humoral response increases. Serodiagnosis with the ELISA method is not useful in detecting animals prior to shedding detectable *M. paratuberculosis* in the feces.<sup>56</sup> A test procedure that would detect a positive animal preshedding would be of great utility.

When used to test the same serum samples, the ELISA test agrees very well with the CF and AGID testing methods ( $\kappa > 0.5$ ). When compared with tests that detect *M. paratuberculosis* in feces the level of agreement is lower. The low  $\kappa$  statistic of 0.36 suggests that the ELISA test is selecting a different population of infected cattle than the fecal culture test. This stands to reason as fecal culture is detecting the organism in the feces, while the ELISA is detecting the event of antibody production.<sup>57</sup> With this in mind the ELISA test can be used in a parallel manner with fecal culture to increase the detection sensitivity. This sensitivity increase would come with the premise that a positive on either or both testing means would be considered a positive designation. Collins et al. suggest combination sensitivities may approach 69%.<sup>55</sup>

A relatively new advance in ELISA testing is the introduction of the Tip-Test<sup>®</sup> (ImmuCell, Portland, ME), a rapid, simple Johne's diagnostic test designed to be run in the veterinary practitioner's office.<sup>18,84</sup> The basis of the test is a pipette tip packed with cellulose in a 3-stage filter. A plasma or serum sample is run

through a series of solutions that are provided in the test kit, and then aspirated into the test tip. The two upper sections of the cellulose media are positive and negative control areas. The lowest section is for sample color determination. The cellulose capture area is a unique development. The large amount of surface area available maximizes the test area to sample volume ratio, thus improving the test sensitivity. The test can be performed as a single animal test or up to 12 tests can be run simultaneously using a 12-channel pipetter. The total time required to perform this test is 25 minutes. The sensitivity of the Tip-Test<sup>®</sup> is similar to the commercial ELISA test available to diagnostic laboratories (45%).<sup>18,84</sup> Of 40 animals with clinical signs of Johne's the test detected 95.2% of the positives. Of 28 individuals that were fecal and tissue culture positive while having no signs of clinical paratuberculosis the test identified 57.1% of the positive animals. Further testing indicated an 86.7% specificity, or a 13.3% false positive rate.

While the Tip-Test<sup>®</sup> brings Johne's testing directly to the practitioners office there are some concerns. The Tip-Test<sup>®</sup> is licensed as a test for clinical cattle only. Some states may elect to ban the use of this test all together to eliminate any regulatory confusion within state boundaries. The Tip-Test<sup>®</sup> is a color change test, which leaves some gray zone for individual interpretation by the practitioner or technician performing the procedure. Additionally, the lack of an optical density reading and no calculation of an S/P ratio will limit the practitioner and producer in following the course of disease of any individual animal or allowing comparison with herd mates. Conversely, the Tip-Test<sup>®</sup> may have great utility in obtaining "same-day" results for making individual cow culling decisions.<sup>18,84</sup>

Serum is not the only location where antibody is present; milk is another readily available source. Most of the IgG in milk originates from serum, although some may be produced locally in the mammary gland. Thus the concentration of IgG in milk is proportionate to serum IgG.<sup>61</sup> The ELISA test can be adapted to process milk as a sample and this means of testing is readily available through some dairy milk laboratories. The advantages of this procedure are convenience of collection and the minimization of stress to the cow.<sup>61</sup>

Initial reports of milk ELISA results have not been encouraging. Hardin demonstrated that the ELISA test procedure for milk did not agree with the results from serum ELISA determinations ( $\kappa = 0.076$ ).<sup>61</sup> Additionally there was little agreement between the two tests in the determination of the herd test prevalence rate or test positive percentage ( $R^2 = 0.1077$ ). Due to these discrepancies determination of individual culling decisions or herd test prevalence rates should be performed using the serum ELISA. An additional unanswered question Hardin raised was how the factors of milk quality and quantity impacted the correlation between the serum and milk ELISA tests.

Sweeney et al. used a milk ELISA containing lipoarabinomannan (LAM) antigen.<sup>62</sup> Their work determined a specificity of  $87\% \pm 8.1\%$  when the sensitivity cutoff was set at 50%. When the cutoff was raised to 60% the specificity dropped to  $83\% \pm 9.1\%$ . The predictive value of a positive test using these values for sensitivity and specificity offers only a 29% chance that a positive test is correct. This value is too low to be considered for culling purposes.

### Determinations of Cell-mediated Immunity (CMI)

The first response by the infected individual is mediated by activated T-lymphocytes. This is reflected by DTH reactions, and the release of cytokines such as  $\gamma$ -IFN. Skin testing, or DTH is the traditional means of tuberculosis detection in both humans and cattle and has been successful. Thickness of the skin is measured at injection and again 72 hr post-inoculation, with any change  $>5$ mm considered to be a positive reaction. Unfortunately, the close association of *M. paratuberculosis* and *M. avium* and the presence of environmental mycobacterial species may preclude a definitive diagnosis of *M. paratuberculosis* infection. This testing procedure has been criticized for the high number of potential false positive reactions.<sup>12</sup>

If poor sensitivity and specificity weren't enough to create murky results, a study by Wentink et al demonstrated strong hypersensitivity reactions in the face of lower levels of intestinal infection of *M. paratuberculosis* and evidence of degenerated bacilli in the macrophages.<sup>40</sup> Conversely, a weak reaction was associated with high levels of gut infection and viable *M. paratuberculosis* in the macrophages. Other researchers suggest that DTH reactions may not be a good representation of cell-mediated activity located in the distant gut area, again due to poor sensitivity and specificity.<sup>12</sup> Due to their long history, the johnin (paratuberculosis antigen) skin test and IV johnin tests still have applications in some international trading procedures. Given the efficacy of some of the testing methods previously mentioned the use of these testing means should be limited to mandatory regulated uses.<sup>21</sup>

A testing means that merits mention as a research tool, but not as a practical animal test, is lymphocyte blastogenesis measures of lymphocyte proliferation after incubation with *M. paratuberculosis* antigens. Radiolabeled nucleic acids are incorporated and included in the DNA following mitotic division of stimulated lymphocytes. The degree of incorporation is measured to indicate the amount of lymphocyte reactivity encountered. Like the DTH test, this measure lacks sensitivity and mycobacterial species specificity. Additionally isolating lymphocytes is labor-intensive and the procedure requires long incubation times.<sup>12, 14</sup>

The most recent work in CMI determination uses the  $\gamma$ -IFN enzyme-linked immunoassay (EIA).<sup>12</sup> By testing an earlier phase response this procedure holds promise of detecting earlier infections of *M. paratuberculosis*, identifying infected individuals prior to, or during early shedding, and potentially allowing detection in younger cattle. ELISA testing is not valid in sheep or goats. Testing methods for these species have centered on the use of the AGID test with its attendant lack of sensitivity. The  $\gamma$ -IFN EIA can be used in sheep and goats and may represent a step forward in combating Johne's in these species.

Activation of the macrophage population to become antimycobacterial is highly dependent on the T cells and their secreted products. Gamma-interferon is secreted by  $CD4^+$ ,  $CD8^+$  and  $\gamma/\delta$  receptor T cells and natural killer cells, and is responsible for inducing macrophage tumor cell lysis, release of reactive oxygen and nitrogen intermediates, expression of major histocompatibility complex class II antigens, and secretion of arachidonic acid metabolites. With this pattern of activation there is a direct link between  $\gamma$ -IFN production and activation of the antimycobacterial macrophage population.<sup>12</sup>

The logistics of the  $\gamma$ -IFN EIA can be challenging as the test must be performed on live lymphocytes from a fresh blood sample collected in sodium heparin. Collection in sodium citrate or EDTA will negate the production of  $\gamma$ -IFN.<sup>14</sup> The sample must be delivered to the lab within 16 hours after collection to ensure a live lymphocyte population. For protection of the lymphocyte population the samples should be stored at 25 C. The samples should not be refrigerated.<sup>14</sup> The usual procedure is to divide the blood sample into 1-ml aliquots incubated with stimuligens. One sample remains unstimulated to serve as a negative control. The viability of the lymphocytes is determined through stimulation with a mitogen as a positive control. Other aliquots are stimulated with antigens to *M. avium* and *M. paratuberculosis*. After incubation the plasma is harvested and processed using an ELISA technique specific for the detection of  $\gamma$ -IFN. Samples that display a positive optical density value  $>0.1$  units over the non-stimulated control are considered to be positive. If the *M. avium* optical density is greater than or equal to the *M. paratuberculosis* response, the animal is considered positive due to environmental exposure. Wood et al. suggested that *M. avium* sensitivity in cattle is often transient, whereas reaction to *M. paratuberculosis* infections tends to be persistent.<sup>63</sup> Repeated testing would allow construction of a profile on individual animals to distinguish this cross-reactivity. Once plasma is harvested from the stimulated sample it may be stored for up to 1 year at -20 C or up to 2 months at 4 C under sterile conditions.<sup>14</sup>

An aspect of the current dairy expansion is an increase in individual stress and its impact on  $\gamma$ -IFN testing. Decreased lymphocyte response to mitogens has been demonstrated under conditions of stress. A trial was performed to determine if dexamethasone administration could suppress the  $\gamma$ -IFN response of cattle tested for reactivity to *M. bovis* antigen. As early as 24 hr following the first dexamethasone injection a decline in  $\gamma$ -IFN was recorded. Parturition, shipping- and production-related stresses are associated with the release of endogenous cortisol. While dexamethasone is a more immunosuppressive glucocorticoid than is cortisol, stress may well be a factor that practitioners will want to keep in mind with respect to cell-mediated testing procedures.<sup>64</sup>

A study by Thoen and Waite demonstrated decreased delayed type hypersensitivity reactions to *M. paratuberculosis* for 7 days following exposure to modified-live Bovine Viral Diarrhea vaccine (ML-BVDV).<sup>29</sup> Decreased lymphocyte blastogenesis was observed during this period. Previous work also suggests that lymphocyte responses to phytohemagglutinin, pokeweed mitogen, and concanavalin A were altered, which could have a direct bearing on the efficacy of the  $\gamma$ -IFN EIA. Simultaneous testing showed no decrease in ELISA responses by affected cattle. Information from field investigations also suggests that increased levels of clinical Johne's disease have been observed in herds that have experienced clinical BVD. Given the frequency of BVD outbreaks in cattle concentrated in expansion herds, the practitioner will want to keep this in mind if  $\gamma$ -IFN EIA comes into widespread use.<sup>29</sup>

A study by Wood et al. 1991 tested 6 herds infected with Johne's disease.<sup>63</sup> The testing methods included the  $\gamma$ -IFN and absorbed ELISA assays. Verification of test results was performed on diseased cattle removed from the herd through the use of fecal culture and histopathological examination of the tissues collected at post mortem. Gamma-interferon was equally sensitive in detecting subclinical (71.8% to 93.3%)

and clinical animals (100%) whereas the absorbed ELISA was more sensitive in detecting cattle with advanced infections (80%) than those with subclinical infections (20.5% to 26.6%).<sup>63, 65</sup> Work by Bilman-Jacobe et al. suggested that the specificity was 97.6% for the  $\gamma$ -IFN EIA.<sup>65</sup>

### Testing Summary

Current arguments surrounding the testing, culling and control of Johne's disease suggest that organized control efforts should wait until a better test is found. Test sensitivities and specificities are approaching 50% and 99%, respectively. Different modes of testing are available, such as detection for antibody, organism and cell-mediated responses. Using more than one test at any given sampling period is considered to be parallel testing which increases the sensitivity.<sup>66</sup> Collins et al. suggests that use of the ELISA, Bactec and  $\gamma$ -IFN tests together will increase sensitivity levels to 97%, while specificity approaches 99.9%.<sup>67, 34</sup> Increasing the frequency of testing within a herd will increase specificity, again to levels approaching 99.9%. The real question that remains is, do we as practitioners and producers have the will and financial reserve to cull the animals that are indicated as positive by these testing means.

Collins and Morgan designed a spreadsheet program to perform a decision tree analysis for the control of paratuberculosis.<sup>68</sup> Their findings indicated that a test and cull program was profitable if Johne's disease caused a 6% or greater decrease in milk production. A herd prevalence of greater than 5% suggested test and cull profitability. This strategy was based on 50% sensitivity and 98% specificity, with test cost being \$8 or less per cow. Parameters considered also included \$1,000 for a replacement animal, average cull weight of 556 kg and a cull price of \$40 to \$45.5 per kg.<sup>68</sup>

Test selection depends on several factors which include test cost, time to run, sensitivity, specificity and stage of disease that can be detected. Table 2 shows a summary of test specifics.<sup>44, 55</sup>

### Predictive Values of a Positive or Negative Test

Veterinarians are frequently questioned concerning their confidence in test results. The client can be given a more precise evaluation through calculation of the predictive value of a positive test and the predictive value of a negative test. This information can be gained through the use of three statistics, which include the sensitivity (SE), specificity (SP), and best estimate of herd prevalence rate (P).<sup>69</sup>

**Estimated prevalence (EP).** The first step is estimation of herd prevalence. The estimated prevalence is based on the veterinarian's knowledge of the herd history of confirmed cases of Johne's disease. This confirmation would be on the basis of fecal culture, histopathological examination, or evidence of Johne's-like lesions on post mortem. A rule of thumb is, for every cow out of a hundred confirmed per year with Johne's disease the prevalence rate may be between 5% and 15%. This is a very rough estimation, but it provides a starting point.<sup>69</sup> An alternative method would be to use the NAHMS survey overall prevalence rate value of 3.4% and adjust up or down based on history, and/or new data.<sup>7</sup>

**Table 2. Johne's Disease Test Specifications**

TEST	Cost	Time	Sensitivity	Specificity	Stage Detected
Fecal Culture	\$16 - \$25	12 - 16 weeks	40% ± 10%	99.9%	Late II - IV
Fecal Bactec	\$16 - \$25	7 weeks ± 2	54.5% ± 7.3%	99.9%	Late II - IV
DNA Probe	\$25 - \$45	3 days	33.5% ± 6.9%	99.9%	III - IV
Histology	\$6 - \$12	3 days	20% ?	99.9%	III - IV
CF Test	\$3 - \$6	2 days	26.6% ± 6.5%	97.4% - 90.3%	Late III - IV
AGID	\$3 - \$6	2 days	18.9% - 26.6%	99.4% - 99.7%	Late III - IV
Serum ELISA subclinical to clinical	\$3 - \$6	2 days	18% - 85%	96% - 99%	Late II - IV
Serum ELISA Aggregate	\$3 - \$6	2 days	45.5% ± 6.7%	98% - 99.7%	III - IV
Serum ELISA & Fecal Culture	\$19 - \$31 Parallel Testing	12 - 16 weeks	69%	99.5%+	Late II - IV
Milk ELISA	\$5 - \$8	2 days	50%	87% ± 8.1%	III - IV
Milk ELISA	\$5 - \$8	2 days	60%	83% ± 9.1%	III - IV
γ-IFN subclinical	\$16 - \$25	3 days	71.8% - 93.3%	97.6%	II - IV
γ-IFN clinical	\$16 - \$25	3 days	100%	97.6%	III - IV
Johne's Skin Test	\$2 - \$5	3 days	54%	79%	II ? - IV

**Apparent prevalence (AP).** This statistic is based on the application of a whole herd test and is the number of positive animals divided by the total number within the herd. This figure alone will be a better measure of prevalence, but it is not adjusted for the sensitivity of the testing method. As the aggregate sensitivity of the ELISA method is approximately 45%, the apparent prevalence rate could legitimately be doubled to give a more accurate assessment of the true prevalence rate. If the test in question has a lower sensitivity, such as 35%, the AP could realistically be multiplied by a factor of 2.5 to 3.0.<sup>69</sup>

**True prevalence (TP).** Following determination of the apparent prevalence the TP can be calculated using the sensitivity and specificity of the whole herd test utilized to generate the herd data. The formula is as follows:  $TP = [AP + (SP - 1)] / [SE + (SP - 1)]$ . An example would be a herd that has an apparent prevalence rate of 8% following a whole herd fecal culture test. Using a sensitivity of 40% and a specificity of 99.9%,  $TP = [0.08 + (.999 - 1)] / [.40 + (.999 - 1)]$ . TP is equal to 0.20 or 20%.<sup>69</sup>

The **sensitivity (SE)** of a test is the ability to detect a positive animal with a positive test result, while the **specificity (SP)** is the determination of a negative animal with a negative test result. If test sensitivity is low, there will be a larger number of false negative responses. Conversely, a high test specificity will yield fewer false positive determinations.<sup>17,69</sup> Most Johne's testing procedures tend to have high specificity in an effort to reduce the number of animals falsely incriminated.

Using an ELISA test as an example, there are two possible outcomes for any individual test, either positive or negative. This does not reflect all possibilities, as the result could also be a false positive or a false negative. To be properly informed the producer needs to be aware of the chances of a positive or negative determination being truly positive or negative. Having gained an estimate of prevalence above, the predictive value of a positive test (PVP) can be generated. Following is the formula for this determination:  $PVP = (SE \times TP) / ((SE \times TP) + ((1-SP) \times (1-TP)))$ . Using a sensitivity = 45%, specificity = 98% and a true prevalence = 10%, the formula would be as follows:  $PVP = (0.45 \times 0.10) / ((0.45 \times 0.10) + ((1-0.98) \times (1-0.10)))$ .<sup>69</sup> The PVP would equal 71.4%, meaning that a positive test had a 71.4% chance of being a true positive or a 29.6% chance of being a false positive.

Conversely, an estimate can be made evaluating a negative test being truly negative. This is the negative predictive value (PVN) and is determined through this formula:  $PVN = (SP \times (1-TP)) / ((SP \times (1-TP)) + ((1-SE) \times TP))$ . Using the same test statistics above for determining the PVP, the result would look like this:  $PVN = (0.98 \times (1-0.10)) / ((0.98 \times (1-0.10)) + ((1-0.45) \times 0.10))$ . The PVN would equal 94.1%, indicating that a negative test had a 94.1% chance of being a true negative or a 5.9% chance of being a false negative.<sup>69, 17</sup>

Table 3 demonstrates the positive and negative predictive values for various testing means, sensitivities and specificities.<sup>69, 17</sup> The ELISA 25 test is calculated using a sensitivity of 25% and a specificity of 98%. The ELISA 60 has a sensitivity and specificity of 60% and 97%, while the culture is at 40% and 99.9%, respectively.

**Table 3. Positive and Negative Predictive Values for Various Testing Means**

Estimated Prevalence	ELISA 25		ELISA 60		Fecal Culture	
	PVP	PVN	PVP	PVN	PVP	PVN
1%	15%	99%	16%	99%	99%	99%
5%	48%	96%	51%	97%	100%	97%
10%	67%	92%	69%	95%	100%	94%
20%	82%	84%	83%	91%	100%	87%
30%	88%	75%	90%	85%	100%	80%
40%	92%	66%	93%	78%	100%	71%
50%	95%	57%	95%	71%	100%	63%

### Likelihood Ratios

Likelihood ratios are the true-positive rate divided by the false-positive rate. This can also be expressed as the test sensitivity divided by (1 – test specificity). This can be a useful statistic as it removes the influence of prevalence rate from the calculation of risk. It adds value to producer information by providing odds ratios vs. a pure positive/negative classification. According to manufacturer instructions, samples that are 0.1 optical density (OD) units above the test negative well should be considered positive. Table 4 is a likelihood table calculated for various OD readings for the serum ELISA test.<sup>55</sup>

**Table 4. Likelihood Ratios**

Difference between ELISA result and negative control (OD units)	Likelihood ratio for infection	Likelihood ratio for isolation of <i>M. paratuberculosis</i> from fecal samples
0.0	1	1.0
0.050 to 0.099	15	1.2
0.100 to 0.149	29	1.5
0.150 to 0.199	78	1.7
0.200 to 0.249	64	2.0
0.250 to 0.299	55	2.4
0.300 to 0.349	51	2.4
0.350 to 0.399	246	2.4
0.400 to 0.449	217	2.5
0.450 to 0.499	194	2.8
> 0.500	177	3.0

For any particular ELISA OD reading a likelihood ratio can be determined. This is the equivalent of the number of times the result is more likely to be from a *M. paratuberculosis* infected cow vs. a noninfected animal. For example, an animal with a reading of 0.11 would be 29 times more likely to be infected with *M. paratuberculosis* and would be 1.5 times as likely to be shedding the organism in its feces.<sup>55</sup> These ratios would be useful tools in assisting producers in making culling choices.

#### Treatment of Johne's disease

Historically, treatment of clinical Johne's disease has been limited to animals of very high genetic merit. *M. paratuberculosis* can be isolated from the accessory glands and semen of the bull, as well as the uterine tract of the cow. A cow that is heavily infected has a 20 - 40% chance of delivering a calf that is infected in-utero.<sup>5</sup> With this in mind treatment efforts have been pursued with the goal to preserve genetic lines through further embryo retrievals or semen collection.<sup>19</sup>

Prior to the decision to treat, several factors must be considered in consultation between the veterinary practitioner and the producer. The first of these factors center on satisfying the terms of the regulations codifying the Animal Drug Use Clarification Act of 1994 (AMDUCA) enacted by the Food and Drug Administration (FDA). This act allows the veterinary practitioner to make use of human pharmaceuticals only after the following considerations: Within the terms of a valid client/patient relationship a definitive diagnosis of Johne's must be made; determination must be made that no labeled food animal drug exists to treat the patient; a substantially extended withdrawal period must be determined for both milk and meat that is appropriate to the treatment choice; the animal must be clearly identified by both animal ID and through the use of treatment records; and appropriate measures must be taken to ensure that the extended withdrawal times are met and that no illegal residues enter the human food chain.

Given the long-term, if not lifetime, therapy required and the class of chemotherapeutics that have to be administered, the treated animal will in reality never be in a position to move through the human food chain.

An additional consideration is the cost of human antituberculin and antilepreptic drugs given at effective dosage levels and for a very long period of time. The final reality of treatment is that chemotherapy is not a definitive cure, rather it effects remission/palliation.<sup>19</sup> It is entirely possible that the removal of antibiotic therapy may result in the exacerbation and fatal reoccurrence of the disease symptoms. With this clearly in mind, these antibiotics could be considered for treatment purposes: isoniazid, rifampin, clofazimine, dapsone, streptomycin, kanamycin, amikacin and ethambutol.<sup>19</sup>

In the face of concerns about antimicrobial resistance, research efforts have moved toward the identification of immune system modulators that may prove effective in heightening immune system responses to specific pathogens. Work by Kreeger et al. examined the effects of leukocyte lysates containing transfer factor on animals infected with chronic Johne's disease.<sup>70</sup> Transfer factor is so named due to the transfer of delayed hypersensitivity to animals devoid of this response. It is theorized that the persistency of paratuberculosis (failure to clear) in cattle is due to the individual inadequacies of the cell-mediated immune system. Blood monocytes and monocyte-derived macrophages from noninfected cattle have been shown to be unable to effectively kill the organism, thus allowing intracellular proliferation. A deficient T cell response (e.g., decreased killer T cell and cytotoxic activity and interferon production) could explain why infected cattle cannot eliminate the organism.

Treatment efforts in humans using transfer factor have been attempted in the areas of cancer immunotherapy, internal parasitism, lupus erythematosus, sarcoidosis, lepromatous leprosy, and intestinal cryptosporidiosis in patients with human immunodeficiency virus infections. Treatment results have been variable, but have shown some promise as indicated by an increase in IL-2 activity. At this point, transfer factor is a crude product and contains both suppresser and induction agents, thus explaining the wide range of applications listed above. Treatment of cattle chronically infected with *M. paratuberculosis* showed little effect and no increase in lymphocyte blastogenesis in this study, but more research effort is indicated by increased IL-2 activity in the enrolled cattle.<sup>70</sup>

### **Regulatory Concerns**

Current regulations in the United States are a patchwork affair with rules governing Johne's disease varying from state to state. The federal regulations do not provide a much better model. The Code of Federal Regulations (CFR) Parts 80 and 71 are the current governing statutes.<sup>22</sup> Part 80 directly concerns the interstate movement and identification of animals that are Johne's disease reactors by the Secretary of Agriculture. As this rule was "outdated, not enforced, and not supported by the livestock industry" a change was made to remove the designated "reactor" status.<sup>22</sup> This in effect removed any restrictions on the interstate movement of Johne's positive cattle. The only potentially enforceable regulation is Part 71 which restricts the interstate movement of "diseased" animals. With the lack of federal guidelines it must be expected that the regulations between states would be highly variable and a patchwork effort at best.

A concern for the practicing veterinarian is the creation of health papers. Most will contain a general statement that the animal has been examined by a licensed, accredited veterinarian, and is not exhibiting any sign of contagious disease. In a case where the veterinarian has knowledge of Johne's disease within a herd, this may present some very real ethical concerns about the signing of a health paper, especially given the insidious nature of this disease. A legal brief published in the *Journal of the American Veterinary Medical Association* warned of the possibility of a judgment being rendered against a veterinarian that "fails to warn a seller or a buyer of the risk to other animals" when an interstate health paper is written. It may be prudent for the veterinarian writing the certificate to state that the signing veterinarian gives no guarantee that the animals listed are free of *M. paratuberculosis* infection.<sup>22</sup>

An attempt has recently been made to offer a solution to the patchwork nature of Johne's regulations in the United States. The United States Animal Health Association (USAHA) was created more than 100 years ago by the sanitary boards of Texas and Missouri to control Texas fever. The primary objective of this organization was to "prevent, control and eliminate livestock diseases that cost ranchers, farmers, and consumers approximately \$1 billion per year." From this mission statement a National Johne's Working Group (NJWG) was formed in 1997 to make recommendations to facilitate control and eventual eradication of paratuberculosis.<sup>71</sup>

A program was designed that would be voluntary in participation, but provide the framework for individual herd owners to be certified by their state of origin as having completed various levels of the program. The assignment of these levels would provide a rough guideline to potential buyers of the risk of purchasing a Johne's infected animal from the participant. The program consists of five levels with the first, undeclared level being the untested herd. From that point a producer can ascend through the four program levels at his/her own pace choosing to remain at a level with an annual repeat of testing procedure, or deciding to ascend to the next level with the completion of the testing requirements unique to that stage.<sup>83</sup>

The initial two stages of testing focus on the ELISA test and cattle that are second lactation or greater. The third stage mandates a fecal culture of all individuals first lactation or greater. The fourth and final test returns to the ELISA method and sampling a statistical population of all second lactation and greater cows. With the completion of each test a percentage value is assigned estimating the probability of the herd being free of Johne's. Unknown herd status is assigned a 70% chance of being free, while level 1 is 85%, level 2 is 95%, level 3 is 98% and level 4 is 99%. While serial testing increases the likelihood that a herd may be free of Johne's this program will never declare a herd 100% free due to the insidious nature of the organism.<sup>83</sup>

Confidentiality of testing has been a major issue in the adoption of this framework by individual states. Producers desire that the results remain entirely confidential, while most state laws demand open access to testing records. It is hoped that implementation of this framework will prove a desirable advertising feature and increase herd value for those that successfully complete individual level testing. This would be an incentive for producers to forge ahead with herd clean-up efforts vs. the current attitude of not testing or hiding the results.

For further details on this program the reader is directed to the USAHA website <http://www.usaha.org/reports98/r98js2.html> for the full text and description of this program.<sup>83</sup>

### **Crohn's disease – Is *M. paratuberculosis* a Zoonotic Agent?**

Crohn's disease (Crohn's colitis, Crohn's ileitis or regional ileitis) is a chronic granulomatous ileocolitis of humans whose etiological agent is unknown.<sup>72,73</sup> The trans-mural granulomatous inflammatory response is considered diagnostic for the presence of Crohn's disease and has great similarity to the histopathology of cattle with Johne's disease.<sup>15</sup> The symptomology of Crohn's includes chronic weight loss, abdominal pain, diarrhea or constipation, vomiting and lethargy. It generally strikes during young adulthood (teens to twenties) and has a slight predisposition towards females vs. males. Treatment efforts focus on the surgical resection of the affected bowel, with 70 – 80% of those suffering this condition undergoing surgery. Further control efforts are symptomatic and centered on the use of steroidal anti-inflammatories, immunosuppressives and antidiarrheal agents.

Current investigative efforts have centered in the areas of allergic reactions, autoimmune dysfunction and infectious agents. None of these investigations has been able to identify the etiological agent. Epidemiological evidence to date supports a multi-factorial etiology with both environmental and inherited factors involved.<sup>73</sup> The local intestinal response to the unknown stimulus has been characterized as a hyper-responsiveness of the internal immune system to a persistent environmental stimulus.<sup>72</sup> This could be an over exuberant response to an exogenous source (*M. paratuberculosis*?) or an attack on an endogenous source such as the intestinal tissue itself. The common thread in both possibilities is that symptoms suggest a defect in the “down regulation” of the intestinal immune response.

Previous efforts to characterize this disorder centered on the description of regional ileitis. As work progressed it was found that regional ileitis could be split into two separate entities known as Crohn's disease and ulcerative colitis. These are now known as inflammatory bowel disease (IBD). The significance of these two entities is that ulcerative colitis is frequently used as a negative control in the study of Crohn's. Experimental work looking at the presence of *M. paratuberculosis* focuses on the isolation of this organism from both conditions.<sup>72</sup>

*M. paratuberculosis* was first isolated in 1984 from 3 patients suffering from Crohn's disease and not from control patients. Primary isolations of the bacilli occurred in the spheroplast, or cell wall deficient form<sup>72</sup>. True confirmation of *M. paratuberculosis* occurred with the transformation of the organism back to a bacillus. This occurred in approximately 25% of the isolations. Subsequent work showed that the isolated strains were genetically identical to the strains of *M. paratuberculosis* originating from cattle. These strains were able to cause disease in infant goats when orally inoculated<sup>15</sup>. Other labs have reported the isolation of *M. paratuberculosis* from human patients but only in a small percentage of cases. Summarization of cultural retrieval has demonstrated isolation of *M. paratuberculosis* in 7.5% of the Crohn's tissues and 0.3% of the controls.<sup>72</sup>

Due to the difficulty of organism recovery, the focus has shifted to the DNA probe method and examination for IS900. Studies examining surgical resection tissues of Crohn's and control patients have demonstrated isolations on the average of 30% in Crohn's patients compared to isolations of 0.8% in control patients. Summarization of studies has shown organism recovery rates ranging from 13% to 100% in Crohn's patients, while control isolations range from 0% to 88%.<sup>73</sup> In nearly all studies, isolations in the Crohn's patients were higher than the controls, but the presence of this organism in control tissues is consistent with it being prevalent in the normal intestine.<sup>73</sup> Work by Francois et al.<sup>74</sup> compared human and animal isolations of *M. paratuberculosis* at the DNA level, both by analysis of restriction fragment length polymorphism (RFLPP) in and around the insertion sequence IS900 and by the arbitrarily primed polymerase chain reaction (AP-PCR). Results favor a common clone origin for the 4 strains isolated from Crohn's disease and for 8 of the 11 strains isolated from cattle and sheep with Johne's disease.

The question has become, is *M. paratuberculosis* a surface opportunist of disrupted tissues or is it truly infecting the tissues themselves? Work on acid fast staining and immunohistochemistry of resected tissues has proved unrewarding. DNA:DNA hybridization performed to detect the DNA of mycobacteria within tissues indicated 53% of the Crohn's patients, 33% of the ulcerative colitis cases, and 17% of non-IBD cases demonstrated evidence of mycobacterial DNA. This tends to point towards opportunistic colonization of the disrupted mucosa in both Crohn's and IBD patients.<sup>72, 73</sup> In addition to the DNA probe studies mentioned above, further work has focused on the *M. avium* insertion sequence IS902. Results indicated an across-the-board level of isolation approaching 14.8% for all three types of tissue samples, Crohn's, ulcerative colitis and non-IBD cases. This would reflect the environmental nature of all species of mycobacteria, which can be cultured or isolated in 30% to 50% of all soil, water and even air samples.<sup>72</sup> Detection doesn't confirm an etiological agent. At this time it is not known whether the association is causal or coincidental.<sup>15</sup>

As Crohn's disease is well described as a hyper-responsive immunological event, an immunological response to *M. paratuberculosis* should be evident.<sup>72, 73</sup> Demonstration of this event would point toward an etiological relationship. Summarization of work to date indicates half the studies show a response, while half do not. Thus the work is inconclusive to demonstrate a response to either *M. paratuberculosis* or any other mycobacterium.

Johne's disease affects cattle and it would be expected that if a link existed between *M. paratuberculosis* infection and Crohn's, the incidence rate of Crohn's would be higher among rural communities, farm and animal health care workers. No such increase in incidence rate has been identified; conversely an increased Crohn's rate has been demonstrated in urban areas.<sup>72</sup> This may be due to over-representation of urban populations in these studies, but no clear link in the above mentioned rural populations has been demonstrated.

Work has also focused on identification of a reservoir of infection with milk as the source. Studies have been performed using the DNA probe detecting IS900 (Millar et al.).<sup>72</sup> The insertion sequence was found in approximately 7% of the samples (18 of 254) from cartons of milk purchased from retail outlets in central and southern England. The high levels of isolations (25%) appeared during the winter months, while no positive

results were obtained through the months of April through July. Identification using IS900 cannot differentiate between living, dead or destroyed bacilli. As a follow-up, all samples were cultured and 9 samples yielded long-term positive liquid cultures after 13 to 40 months of incubation.

Grant et al. and Hermon-Taylor found that *M. paratuberculosis* suspended in raw milk is more heat resistant than *M. bovis*, which is one of the reference organisms for current pasteurization heat and time requirements. If in high enough numbers, *M. paratuberculosis* could survive high temperature, short time pasteurization. Hermon-Taylor determined that the rate of cooling following heating and strain differences also affected survival.<sup>15</sup> Recent work by US Department of Agriculture (USDA-Agricultural Research Station) indicates that pasteurization is adequate to inactivate *M. paratuberculosis* in milk, but it will not remove the organism.<sup>10</sup> In this study the authors attempted to culturally isolate *M. paratuberculosis* from the IS900 positive samples; none were successful.

In examination of food-borne contamination two questions must be answered. The first is what is the thermal death curve of *M. paratuberculosis* and the decimal reduction time. The second is what is the expected number and possible range of *M. paratuberculosis* per milliliter of milk. The death rate of this organism can be reliably predicted, but if contamination levels greatly exceed the reference bacilli can survive. Determination of contamination levels is very difficult, and when factoring in the additional consideration of meat and cheese contamination this is a question that will remain open for some time.<sup>15</sup>

If *M. paratuberculosis* is a causal agent of Crohn's disease in humans it can be asked what type of transmission is occurring. The potential exists and can't be discounted for transmission among humans. *M. paratuberculosis* is not a free-living organism in the environment, and it is plausible that the primary reservoir (animals) could serve as a transmission source to humans. During clinical infection ruminants shed the organism through their feces and milk. Additionally, blood-borne dissemination occurs with the subsequent infection of several organ tissues such as lymph nodes, gastrointestinal tract, liver, kidney, spleen, uterus, mammary gland and epididymis. This potentially opens the door for contamination of human food through both pre-harvest and post-harvest contamination. Further work is indicated with respect to thermal inactivation to adequately judge the risk of food-borne transmission to the human population.<sup>15</sup>

As mycobacterial species may be treated with chemotherapeutics it would be expected that remission of Crohn's disease would occur with the application of these products.<sup>19</sup> Spontaneous remission of Crohn's can occur in approximately 20% of cases. Additionally, placebo effects can occur in 30% to 40% of patients with short-term therapy. This is a major complicating factor in the assessment of successful treatment. Beyond that *M. paratuberculosis* and *M. avium* appear to be very resistant to treatment when compared to *M. tuberculosis*.<sup>72</sup> Furthermore, little is known about the efficacy of these drugs on the spheroblast phase of these bacteria. Recently multi-drug resistant variants of *M. tuberculosis* have been demonstrated. Treatment failures are well described if the proper antimicrobial regimes are not employed. If treatment efforts for *M. paratuberculosis* resemble the multi-drug resistant variants it may be extremely difficult to treat. Therefore, it is very difficult to draw any conclusions about *M. paratuberculosis* as an etiological agent of Crohn's with response to therapy.<sup>72</sup>

At this time the possible relationship between *M. paratuberculosis* infection and human Crohn's disease can't be ignored, but its etiological relationship has not been proven. Food animal veterinarians are intimately involved with food safety, so this must remain an item of concern to the profession. Additional work is needed to determine that pasteurization will consistently kill this organism in the food supply at all shedding levels. The following is a summary of the components both pro and con towards the etiological role of *M. paratuberculosis* and Crohn's:<sup>72</sup>

#### **Information Supporting**

1. Pathology similarities
2. Cultivation of *M. paratuberculosis* from some patients
3. Ability of human isolates to cause disease in experimental animals
4. Detection of IS900 in some human patients with Crohn's

#### **Information Against**

1. Lack of pathological hallmark (acid-fast bacilli, as can be demonstrated in infected animals)
2. Low cultivation success
3. Variability of PCR data
4. Detection of IS900 in control patients
5. Suggestion of an environmental opportunistic role

### **Literature Review Conclusions**

The history of Johne's disease is filled with the frustration of dealing with a disease syndrome that defines the term insidious. The long-term growth pattern of *Mycobacterium paratuberculosis* defies research efforts to deal with it in an expedient manner. These same growth patterns lull producers into a false sense of security when they believe they are dealing with Johne's by culling clinical cases. Research has also been hampered by the paradoxical immune system response of the disease. Thus, the ability to define a subject bovine as truly negative for testing purposes becomes a "best guess" effort.<sup>12</sup>

Research is beginning to make some inroads into Johne's disease through development of tests with increasing amounts of sensitivity while maintaining high levels of specificity. This is occurring in all fields of testing including culture, PCR identification, antibody detection, and  $\gamma$ -IFN determinations. With increasing sensitivity and specificity the question will shift from the inadequacies of the testing procedures to how committed the producer is to removing identified *M. paratuberculosis* infected animals from the farm. This may become an even more pressing issue if a definitive link with human Crohn's disease is established.

In the following study the authors seek to add to the body of knowledge concerning increasing the sensitivity and specificity of the  $\gamma$ -IFN test. A cell-free sonicate of *M. paratuberculosis* strain 19698 was administered as a pre-treatment to a group of cattle identified as positive, suspect and negative for the production of  $\gamma$ -IFN. The enrolled animals were serially monitored for any increased or enhanced levels of  $\gamma$ -IFN as a result of the cell-free sonicate pre-treatment.

# COMPARISONS OF THE MILK ELISA, SERUM ELISA, GAMMA-INTERFERON AND FECAL CULTURE TESTS FOR THE DETECTION OF JOHNE'S DISEASE IN AN IOWA (UNITED STATES) HERD

A paper submitted to the American Journal of Veterinary Research

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## INTRODUCTION

*Mycobacterium avium* subsp. *paratuberculosis* is the causative agent of Johne's disease.

Paratuberculosis is a chronic mycobacterial infection of the lower small intestine and associated lymph nodes affecting many ruminants including cattle, sheep, goats, llama, deer and bison.<sup>1,3,4</sup> It is primarily a wasting disease, but is also evidenced by profuse, watery, untreatable diarrhea in cattle. Bovine oral infection generally occurs during the first 6 - 12 months of life with manifestation of the clinical disease 2 - 5 years later.

Paratuberculosis is an economically devastating disease of ruminants. Recent estimates indicate the loss to dairy producers includes 640 million kg of milk, additional culling of 11,000 cows, and nearly 20,000 cow deaths (based on a total population estimate of 9,458,000 dairy cows). The total amount of revenue lost is approximately \$222 million.<sup>7,8</sup>

Current testing methods have frequently been criticized by the cattle industry as being too insensitive to be of any value. The intracellular location and slow, progressive nature of the disease (4-day generation time of *M. paratuberculosis*) results in delayed or undetectable immune responses.<sup>11,17</sup> Research has been stymied due to the inability to truly declare an animal non-infected. Fecal and tissue culture have been considered the "gold" standard of isolation, but 12 - 16 weeks growth requirements have taken a toll on research efforts and producer motivation.<sup>11</sup>

Multiple testing methods are available to the practitioner for determining the presence of Johne's disease. These include: four means of organism detection and/or isolation, four means of antibody assay, and two assays for the presence of a cell-mediated response in the form of gamma-interferon ( $\gamma$ -IFN) determinations, and a DTH skin reaction.<sup>21</sup> The available testing procedures are most sensitive in detecting Johne's in animals in Clinical Disease or Advanced Clinical Disease.<sup>44</sup>

Patterns of measurable immune response vary from animal to animal, with the early stages of infection being immunologically indicated by cell-mediated reactions from active proliferation and differentiation of T cells. As the onset of clinical signs approaches, the measurable systemic reaction shifts to a predominantly humoral (antibody) response and organism shedding. The shift to a humoral response comes at the expense of

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the cell-mediated phase. In the final stages of disease the animal may lapse into a period of anergy (no immune response) as both cellular and humoral responses diminish.<sup>12</sup>

Multiple testing means allow identification of the differing immunological signalments of Johne's disease.<sup>57</sup> This can be considered an advantage as these responses are from differing populations within Johne's disease progression. With this in mind the ELISA test can be used in a parallel manner with fecal culture to increase detection sensitivity. This sensitivity increase would come with the premise that a positive on either or both testing means would be considered a positive designation. Collins et al. suggest combination sensitivities may approach 69%.<sup>55</sup>

The objectives in this trial were to compare and contrast multiple means of testing in an Iowa herd, to examine the possibility of increased sensitivity with multiple tests, and to characterize this population for selective enrollment of individuals for further serial testing utilizing the gamma-interferon ( $\gamma$ -IFN) assay.

## MATERIALS AND METHODS

**Classification Trial.** Cattle used in the study were obtained from the Ames Dairy facility at Iowa State University,. This teaching herd represents the six major dairy breeds and consists of 102 Holsteins, 26 Jerseys, 8 Guernseys, 17 Ayrshires, 17 Brown Swiss, and 9 Milking Shorthorns. While no clinical cases of Johne's disease were observed in this herd, initial fecal sampling 1 year previous indicated a true prevalence rate approaching 10 to 12%.

The study focused on testing procedures with the exception of the  $\gamma$ -IFN assay, that are readily available to the US veterinary practitioner. Samples from the herd were submitted during a 10-day period utilizing milk ELISA (MELISA) (Dairy Lab Services; Dubuque, IA), serum ELISA (SELISA) (IDEXX Laboratories; Inc.; Westbrook, ME),  $\gamma$ -IFN assay (CSL Limited; Parkville, Victoria, Australia) and fecal culture (National Animal Disease Center; Ames Iowa). Sampling consisted of obtaining 2 oz of fluid milk for MELISA assay at the regular dairy records processing center (DRPC) monthly check. Five days later the cattle were restrained and 60 ml of blood was obtained for partitioning between a 10-ml sodium heparin tube and three 15-ml serum separator tubes. The heparinized whole blood sample was delivered to the laboratory within 6 hours for  $\gamma$ -IFN assay. Serum from the separator tubes was used to obtain a serum sample for ELISA determination, with the balance being subdivided for serum banking purposes. A fecal sample was also submitted for *M. paratuberculosis* culture.

**Milk ELISA.**<sup>85</sup> Milk was harvested into a 2 oz snap-cap vial containing 0.5 ml of Bronolab-W® (2,-Bromo-2, Nitro Propane-L; D&F Control Systems; San Ramon, Ca.) by the DRPC technician performing the herd test. The sample was placed in a Styrofoam shipping container, and shipped unrefrigerated to Dairy Lab Services, Inc. Sample requirements include the following:

1. Samples must be run in less than 4 days from the sampling date.
2. Cows must be at least 10 days into lactation to avoid false positives.
3. Samples should not be refrigerated.

The following are test reagents used in the processing of the milk samples: Phosphate Buffered Saline Tween 80 Gelatin solution (PBSTG) is 1,250 ml of a 0.20 % solution of Sodium dihydrogenphosphate monohydrate ( $\text{NaH}_2\text{PO}_4$ ; Sigma; St. Louis MO), with 21 drops Tween 80 (T80 - Polyoxyethylene-sorbitan mono-oleate; Sigma; St. Louis MO), 10.60 g NaCl, 15.45 g Sodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ; Sigma; St. Louis MO) and 1.25 g EIA grade reagent Gelatin (Biorad; Hercules, CA). The PBSTG must be buffered to pH 7.6 using NaOH. Tween saline solution consists of 500 ml of 0.85% NaCl solution and 9 drops of Tween 80 (Polyoxyethylene-sorbitan mono-oleate; Sigma; St. Louis MO).

Test controls were prepared by adding 25  $\mu\text{l}$  of *M. paratuberculosis* positive control (Allied Monitor; Fayette, MO) to 2 ml of PBSTG and by adding 10-15  $\mu\text{l}$  of *M. paratuberculosis* negative control (Allied Monitor; Fayette, MO) to a second 2-ml of PBSTG. Immulon<sup>®</sup> 96-well plates (Dynex Technologies; Chantilly, VA) were sensitized by adding 100  $\mu\text{l}$  to each well of a solution of 20 ml, 0.6%  $\text{Na}_2\text{CO}_3$  (Sigma, St. Louis, MO) and 144  $\mu\text{l}$  of Paratuberculosis Protoplasmic Antigen (PPA) (Allied Monitor; Fayette, MO). The plate was placed in a re-usable ziplock bag and refrigerated at 6 C for 18-48 hr (plates must be used within 48 hr following sensitization). Milk samples were warmed to 40 C and once cool were rolled to remove the cream line. A culture tube (12x75 mm, Kimball, Raleigh NC) was filled approximately one-third full with the test sample milk. The sensitized plate was then “slung” and filled with Tween saline. The plate was immediately slung and refilled with Tween saline, allowed to stand 2 min and the procedure repeated, followed by another slinging after an additional 2 min period. Slinging consists of:

1. Dumping plate contents 3 times.
2. Tapping plate on towel 3 times.
3. Shaking plate in the air 3 times.

The milk sample was vortexed and 100  $\mu\text{l}$  was pipetted into each of 3 consecutive wells. The filled plate was incubated at 12-15 C for 2 hr. Immunoglobulin G Conjugate (IgG) (Allied Monitor; Fayette, MO) is thawed 5 min prior to the end of incubation to create an IgG PBSTG solution. Actual product mixing rates vary with the manufacturers IgG lot number. Following incubation the plates were slung, washed with PBSTG 4 times and 100  $\mu\text{l}$  of IgG was added to each well. The plates were re-incubated at 12-15 C for an additional 90 min. Following incubation, the plates were slung and rinsed 3 times with PBSTG, followed by the addition of 100  $\mu\text{l}$  of substrate solution. The substrate solution consisted of 20 ml of pH 4.0, 1% citrate buffer, with 100  $\mu\text{l}$  of 2.3% Diammonium salt solution (ABTS; Sigma; St. Louis, MO) and 4.4 ml of hydrogen peroxide solution ( $\text{H}_2\text{O}_2$ ). The  $\text{H}_2\text{O}_2$  solution consisted of 3.0 ml distilled water and 1.4 ml  $\text{H}_2\text{O}_2$  (30 wt %  $\text{H}_2\text{O}_2$  solution in water 34.02 fw; Aldridge; Milwaukee, WI). Using an optical density plate reader set at  $A_{405\text{nm}}$  (Model # ELX800; Biotech Instruments; Winooski, VT) the plate was read until the threshold value for the positive control exceeded 240. The positive control value was recorded and the balance of the sample wells were read for optical densities.

The results were interpreted by averaging the 3 results of positive control (P). Outlying values were discarded prior to averaging. There were 2 sets of positive control wells per plate. The 3 results from each

**Table 1: MELISA S/P, Grade, Exposure and Recommendations.**<sup>85</sup>

Numerical S/P Range	Assigned Value	Antibody Level & Probable Exposure	Recommendations
<b>Individual cow samples</b>			
1.0 – 2.0	A	Low	Continue routine Milk ELISA testing
2.1 – 3.5	B	Moderate	Retest by Serum ELISA/ Milk ELISA in 6 mo.
3.6 – 5.0	C	High	Retest by Serum ELISA
5.1 – 10.0	D	Very High	Retest by Serum ELISA: Cull as advised by veterinarian
<b>Bulk tank samples</b>			
1.0 – 1.2	A	Low	Routine annual Milk ELISA testing of bulk tank or individual cows
1.3 – 2.0	B	Moderate	Test individual cows by Milk ELISA or Serum ELISA: consult veterinarian
2.1 – 4.5	C	High	Test individual cows by Serum ELISA: consult with veterinarian

sample (S) were averaged following outlier discard. A Sample/Positive (S/P) ratio was calculated. Table 1 describes the recommendations associated with the S/P ratios.

**$\gamma$ -IFN Assay.** All blood samples for  $\gamma$ -IFN assay were maintained and transported at room temperature (20 C to 30 C) and processed within 12 hr of collection. The whole heparinized blood was dispensed in 6 - 1.0-ml aliquots in 24-well tissue culture plates (Falcon; Becton-Dickinson; Franklin Lakes NJ). Blood samples were cultured alone, nonstimulated (NS), or with concanavalin A (ConA) and pokeweed mitogen (PWM) at a concentration of 10  $\mu$ g/ml to act as positive controls within the assay to evaluate cell function and test for anergic responses. The remaining 3 samples were plated with avium purified protein derivative (AvPPD), bovis purified protein derivative (BoPPD) or a whole cell sonicate of *M. paratuberculosis* (19698 MpS).

Whole blood cultures were incubated for 18 hr at 39 C in a humidified atmosphere of 5% CO<sub>2</sub>. Plates were centrifuged at 500 x g for 15 min and plasma was harvested from each well. Plasma samples were frozen at -20 C until analyzed for  $\gamma$ -IFN concentration by ELISA using a commercial kit. Plasma samples were incubated in 96-well plates precoated with an anti-bovine  $\gamma$ -IFN antibody for 1 hr at room temperature. After washing with phosphate buffered saline (PBS), plates were incubated at room temperature for 30 min with anti-bovine  $\gamma$ -IFN-horseradish peroxidase conjugate. Plates were washed again and incubated with substrate solution (tetramethylbenzidine, TMB) for 30 min at room temperature. Dilute hydrochloric acid was added to each well as a stop solution and plates were read on a MR7000 microplate reader at A<sub>650nm</sub>. A sample was considered positive if the absorbance of the stimulated sample (19698 MpS) was 0.100 absorbency (abs) units greater than the absorbance achieved for the nonstimulated control well for that animal.<sup>20</sup>

**19698 MpS Production.** Cell-free soluble protein 19698 MpS was prepared by sonication of 1-ml volumes of *M. paratuberculosis* (1 x 10<sup>9</sup>/ml) in phosphate buffered saline (PBS) at 25 W for 25 min, followed by centrifugation at 1,400 x g for 5 min to pellet the cellular debris. The cell-free supernatant was removed and a protein concentrate was determined. Final concentration of each PPD or MpS in culture was 10  $\mu$ g/ml.<sup>20</sup>

**Absorbed ELISA.** Serum was harvested from the blood samples and presented to the Iowa State University Veterinary Diagnostic Laboratory for antibody determination using a commercial ELISA kit. The samples were processed according to manufacturer's instructions.<sup>59</sup> Samples were placed in diluent containing *M. phlei* to remove cross-reacting antibodies and dispensed into 96-well plates coated with *M. paratuberculosis* antigen. Samples were incubated for 30 min at room temperature, plates were washed 4 times and horseradish peroxidase-conjugate was added to each well. After incubation plates were washed again and TMB substrate was added. Plates were incubated for another 15 min at room temperature and a stop solution (0.125% hydrofluoric acid) was added to each well. Plates were read at  $A_{650nm}$ .

**Fecal and Tissue Culture.** Fecal samples (1 gram) were added to 35 ml sterile deionized water, shaken, and allowed to settle at room temperature for 30 min. After settling, the entire supernatant fraction (25-30 ml) was removed and placed in a clean 50 ml tube. Samples were then centrifuged at 1,700 rpm for 20 min, the supernatant was decanted, and a pellet was resuspended in 30 ml 0.75% final concentration hexadecylpyridinium chloride (HPC) and half strength brain heart infusion broth (BHI). After overnight decontamination at 37 C, samples were centrifuged at 1,700 rpm for 20 min. After supernatants were removed, pellets were resuspended in 1 ml antibiotic solution (100 µg/ml naladixic acid, 100 µg/ml vancomycin, 50 µg/ml amphotericin B) and incubated overnight at 37 C. Sample suspensions (0.2 ml) were pipetted onto 4 tubes of Herrold's egg yolk media (HEYM) containing naladixic acid (50 µg/ml) and vancomycin (50 µg/ml) and incubated. Inoculated tubes were incubated in a horizontal position with loose caps for 1 wk at 37 C to permit evaporation of residual moisture from the surface of the medium. Caps were then tightened, tubes returned to an upright position, and incubation of samples continued for a total of 12 wk. Tubes were examined every 4 wk and colonies were enumerated using a dissecting microscope at 25X magnification.<sup>20,45</sup>

**Statistics.** Johne's test results were gathered on each individual and placed into an Excel spreadsheet (Microsoft Corporation, Redmond WA), along with individual cow production data from PC-Dart (Raleigh Dairy Records Processing Center, Raleigh NC). The spreadsheet was sorted and ranked on each individual test parameter. Individuals with no test results were removed from consideration and a percentage positive for each individual test was then calculated.

For comparison of 2 test procedures all individuals with 1 or more missing tests were removed from consideration. Animals were identified as negative to both tests, positive to 1 test and negative to the other, or positive for both tests. Percentages were calculated for each of the four possibilities, and the data was plotted on an XY graph. The kappa statistic was calculated as per instructions.<sup>82</sup> Comparison of ages at positive test was performed using pairwise t-tests (The SAS System; Software Release 6.12; SAS Institute; Cary NC).

## RESULTS

The MELISA test returned the greatest number of test positive individuals at 12.96% of the herd (21 of 162). All positives were assigned a "B" grade. No "C" or "D" grade positives were found (Table 1.). The SELISA followed with 7.41% test positive (14 of 189). These individuals showed a S/P ratio of  $\geq 0.250$ . The  $\gamma$ -IFN assay found 4.40% (8 of 182) had an optical density of 0.100 absolute units above the non-stimulated

standard. The fecal culture yielded 0% positive. For the entire herd the percentage of individuals negative to all testing was 74.19% (115 of 155).

All results were reported numerically as either S/P ratios or absolute optical densities. Average values were determined for test positive and negative individuals as well as an overall test average value for each testing method. These values shown in Table 2. Individual cow data was obtained using PC-Dart records (Dairy Records Processing Center, Raleigh, NC). Average values were determined for age in months for both test positive and negative cattle. The SELISA test ( $P > |T| = 0.0192$ ) had a significantly lower age at test positive than the MELISA and  $\gamma$ -IFN. Within the SELISA assay there was a significant difference between test negative and positive ( $P > |T| = 0.0296$ ).

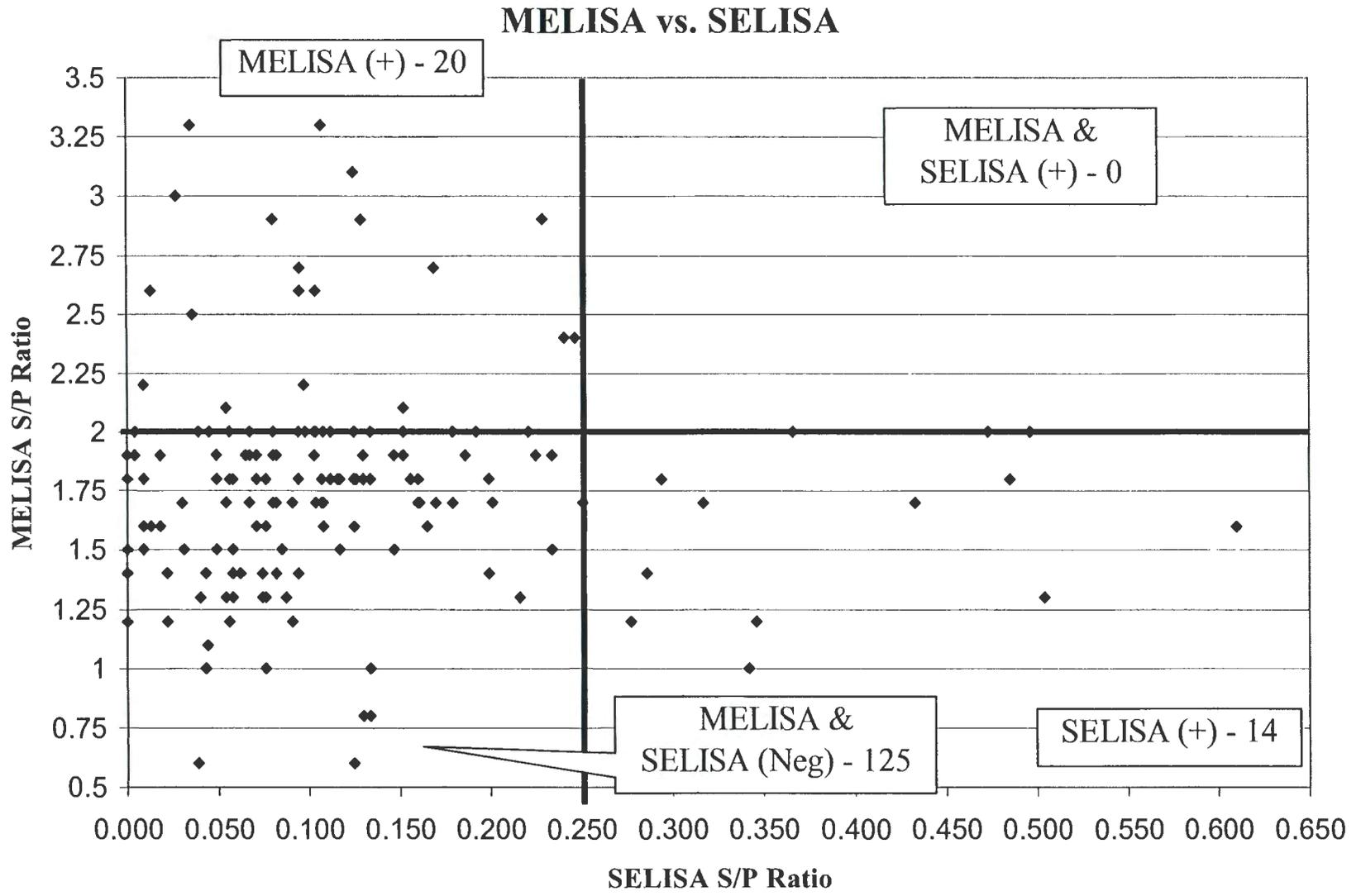
**Table 2: Test Results Mean Values**

	MELISA	SELISA	$\gamma$ -IFN
Mean Positive Value	2.690	0.391	0.263
Mean Negative Value	1.647	0.084	0.020
Mean Test Value	1.779	0.118	0.032
Standard Deviation of Positive Values	0.392	0.108	0.239
Mean Age (mon.) Positive Animals	50.52	<b>37.21</b>	48.25
Mean Age (mon.) Negative Animals	46.47	47.74	46.88
Prob >  T  - mean age of positive test	0.3588	<b>0.0192</b>	0.8252

The results of each of the three diagnostic tests were cross-examined using an XY scatter plot to allow visualization of test agreement. The comparison of the MELISA vs. the SELISA test (Figure 1) yielded 125 individuals negative by both tests, 20 positive MELISA, 14 positive SELISA, and 0 individuals both tests agreed were positive. Test agreement was indicated by a kappa value of  $-0.116$ . Comparison of the  $\gamma$ -IFN test vs. MELISA (Figure 2) yielded 126 individuals negative by both tests, 20 positive MELISA, 8 positive  $\gamma$ -IFN, and 1 individual both tests agreed was positive. The kappa value for test agreement was 0.0005. Comparison of the  $\gamma$ -IFN test vs. SELISA (Figure 3) yielded 159 individuals negative by both tests, 14 positive SELISA, 8 positive  $\gamma$ -IFN, and 1 individual both tests agreed was positive. The kappa value for test agreement was 0.0287. Values for kappa  $< 0.40$  should be considered an indicator of poor agreement.

## DISCUSSION

The MELISA test has recently been offered in Iowa by Iowa Dairy Lab Services Inc., for use in determining potential herd prevalence rates and individual determinations of Johne's disease.<sup>85</sup> This method offers convenient sample collection compared to serum and can be obtained through routine milking procedures, thus limiting stress to cattle.<sup>61</sup> The majority of IgG in milk originates from serum, although some local production occurs in the mammary gland itself. Thus the concentration of IgG in milk is proportionate to serum IgG.<sup>61</sup> Milk could represent an ideal sample to detect the presence of *M. paratuberculosis* antibody and the ELISA test can be adapted to process this sample medium.



**Figure 1.** Scatterplot comparison of MELISA vs. SELISA results (bars represent positive thresholds).



### Gamma-IFN vs. SELISA

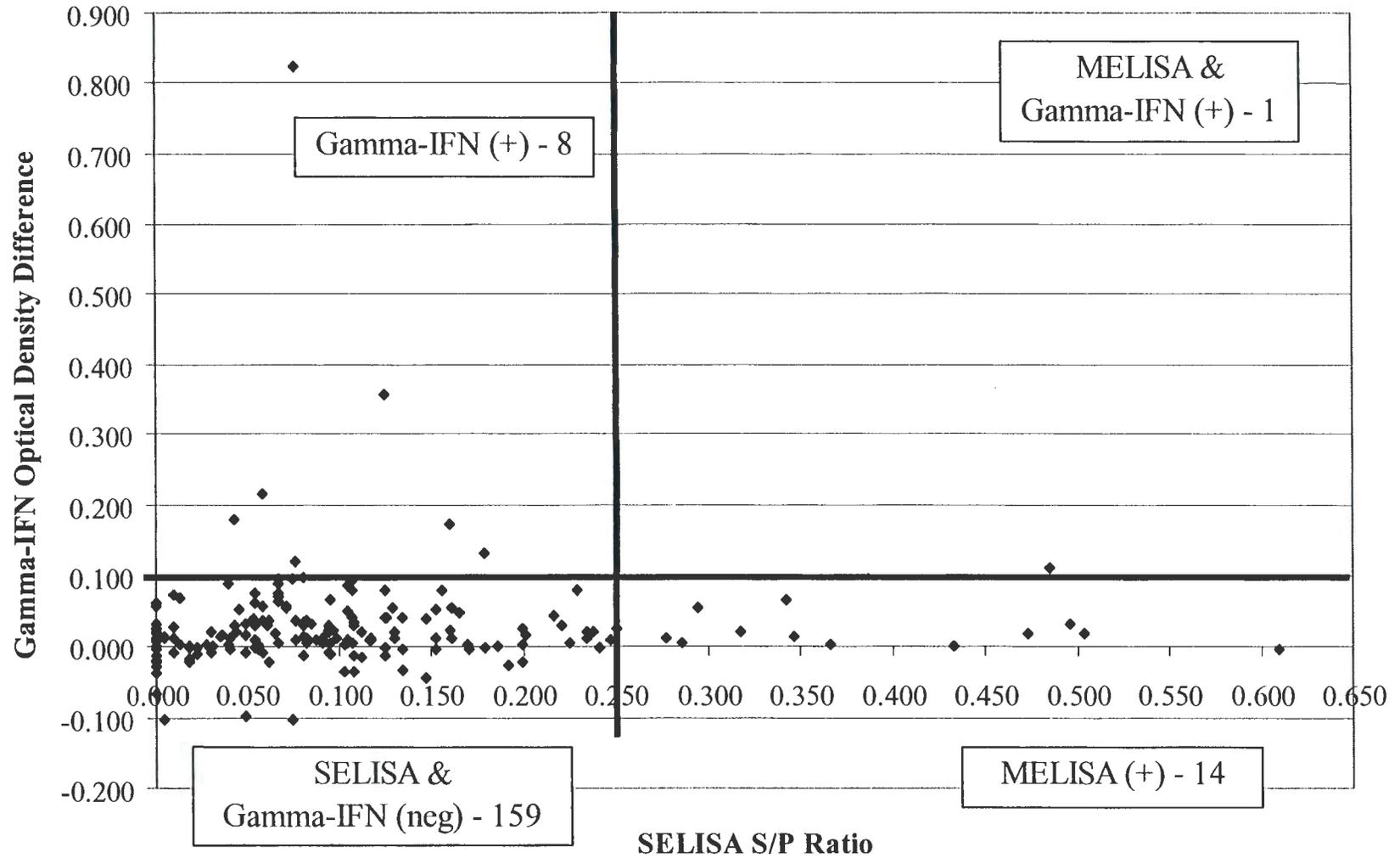


Figure 3. Scatterplot comparison of Gamma-IFN vs. SELISA results (bars represent positive thresholds).

Our study indicated the MELISA determined the highest percentage of test positive animals at 12.96%. While this high rate of sensitivity looks encouraging, comparison with the results of the regulatory standard SELISA test casts some doubt. When compared using an XY plot (Figure 1) no common individuals were identified as test positive by both tests. Analysis of the proportions in each quadrant with the kappa statistic at 0.116 indicated a very low percentage of agreement between the two testing means. The amount of agreement present could simply be due to chance.

Other reports of MELISA results have not been encouraging. Hardin and Thorne demonstrated that the ELISA test procedure for milk did not agree with results from serum ELISA determinations ( $\kappa = 0.076$ ).<sup>61</sup> Additionally, there was little agreement between the two tests in the determination of herd test prevalence rate or test positive percentage ( $R^2 = 0.1077$ ). Due to these discrepancies, and use of the SELISA as a regulatory standard, individual culling decisions or herd test prevalence rates determinations should not be made using the MELISA.

Factors affecting the performance of the MELISA related to milk samples have not yet been identified. Hardin and Thorne raised the issues of milk quality and quantity possibly impacting the agreement between the serum and milk ELISA tests.<sup>61</sup> As can be seen from the MELISA materials and methods, cows less than 10 days fresh should not be checked due to the risk of false positives. Perhaps high somatic cell counts or the presence of clinical mastitis may adversely affect the outcome of the test.

An additional factor is the sensitivity and specificity of the MELISA test. Sweeney et al. used a MELISA containing lipoarabinomannan (LAM) antigen.<sup>62</sup> Their work determined a specificity of  $87\% \pm 8.1\%$  when the sensitivity cutoff was set at 50%. When the cutoff was raised to 60% the specificity dropped to  $83\% \pm 9.1\%$ . The predictive value of a positive test using these values for sensitivity and specificity offers only a 29% chance that a positive test is correct. This value is too low to be considered useful for culling purposes.

Evaluation of the data for mean values at positive test indicated that the SELISA had a significantly lower age at positive test ( $P > |T| = 0.0192$ ) than did the MELISA or  $\gamma$ -IFN. There was also a significant difference within the SELISA assay between test negative and positive status ( $P > |T| = 0.0296$ ). Conversely, there was no significant difference in the mean age of negative animals between the three testing methods. This raises potentially serious questions. Patterns of measurable response will vary from animal to animal, but active proliferation and differentiation of T cells is generally the first measurable immune system response through DTH reactions or assay of  $\gamma$ -IFN.<sup>12</sup> It would be expected that the  $\gamma$ -IFN assay would have the lowest mean positive age vs. the antibody determinations. Furthermore, as the majority of IgG in milk originates from serum there should be little difference between the mean ages of the MELISA and SELISA methods.<sup>61</sup> The results of the MELISA test can be criticized for the lack of agreement with the regulatory standard, but the results of the SELISA in this herd could be questioned for the presence of false positives due to the significantly lower mean age at positive test.

Testing results can have great impact on examination and implementation of management practices. Examination of the SELISA positive results indicated a preponderance of test positive individuals as first

lactation cows. If this is correct, it would call into question the young calf management program in place at this herd. The Iowa State University Ames Dairy Herd has made an effort to control the spread of Bovine Leukosis Virus (BLV) within the herd, including serum BLV AGID testing of all lactating females. Any individual with a positive titer was considered contaminated and their colostrum was discarded to prevent transmission. Colostrum from negative cows was banked, frozen and retrieved for use when a BLV positive dam calved. At the time of implementation of this practice no Johne's disease determinations had been carried out due to lack of clinical cases. It is conceivable that a BLV negative, Johne's positive cow may have colostrum present in the bank that could infect calves coming in contact with it. This could represent a major Johne's biosecurity breach.

The fecal samples submitted failed to yield any culture positive individuals. No clinical cases of Johne's disease have been observed in this herd, but initial fecal culturing 1 year previous indicated a true prevalence rate approaching 10 to 12%. Fecal shedding of *M. paratuberculosis* can be an intermittent event, and in a stable, well-managed herd stress levels may be low enough to not greatly advance organism dissemination and subsequent shedding. Conversely, the lack of any fecal positives may call into question the results of the MELISA and SELISA as potentially having too many false positives. The combination of a significantly lower mean age of SELISA test positive individuals and no fecal culture positives would lend further credence to the false positive issue.

A recent development in Johne's disease testing has been the introduction of the  $\gamma$ -IFN enzyme-linked immunoassay (EIA).<sup>12,63,65</sup> This test of the cell-mediated immune system has the advantages of being rapid, numerically quantifiable, and not affecting the outcome of further testing through the injection of a PPD. By testing an earlier phase response this procedure holds promise of detecting earlier infections of *M. paratuberculosis*, identifying infected individuals prior to or during, early shedding and potentially allowing detection in younger cattle.<sup>12</sup> This study indicated a 4.40% positive rate, with a mean age of detection at 48.25 months. As this is an earlier phase test, one would expect it to verify the age results seen using the SELISA and further identify positive individuals among the younger herd members.<sup>12</sup> The  $\gamma$ -IFN assay did not verify the results of the SELISA.

Cross-examinations of  $\gamma$ -IFN results were conducted against the SELISA (Figure 2) and MELISA (Figure 3). Both antibody tests had similar responses with only 1 individual showing a positive agreement between the two types of testing procedures (antibody vs. interferon). Kappa statistics again indicated poor agreement between the tests with values of 0.0287 and 0.0005 respectively for the SELISA vs.  $\gamma$ -IFN and MELISA vs.  $\gamma$ -IFN. These kappa values would indicate poor agreement between the  $\gamma$ -IFN assay and antibody determinations. In this case the lack of agreement would be expected. As seen in the kappa values, the difference between the two testing means indicates that two entirely different populations are being selected. Identification of separate populations would "widen the area of capture" and could result in increased sensitivity when used in a parallel manner. The MELISA and SELISA assays don't detect differing populations and as a result no increase in sensitivity should be expected with parallel testing using these two diagnostic modalities.

Additional evidence that the antibody ELISA and  $\gamma$ -IFN detect differing populations is indicated by the physiology of the immune system. CD4<sup>+</sup> type-1 T-helper cells (T<sub>H1</sub>) produce cytokines primarily consisting of interleukin-2 (IL-2) and gamma interferon ( $\gamma$ -IFN) and mediate macrophage activation, delayed-type hypersensitivity (DTH) and are thus associated with cell-mediated immunity (CMI). CD4<sup>+</sup> type-2 T-helper cells (T<sub>H2</sub>) produce large amounts of IL-4, IL-5, IL-6, and IL-10 and mediate antibody production (IgG1 and IgE) and eosinophilia.<sup>12, 36</sup> Production of IL-4 by T<sub>H2</sub> cells acts as a potent antagonist of macrophage function, IL-2 receptor sensitivity, IL-2 dependent macrophage proliferation, and production of  $\gamma$ -IFN by mononuclear cells while enhancing the production of antibody, which is a product of the humoral immune system. Activity of the humoral immune system comes at the expense, or down-regulation, of the cellular immune system.<sup>12, 36, 38</sup>

Examining the population using all testing means yielded a test positive rate of 25.81% which indicated an apparent increase in detection sensitivity. Collins et al. determined a potential 69% sensitivity when SELISA and fecal culture were combined compared to approximately 45% sensitivity when each was used separately.<sup>4, 55, 56</sup> Collins et al. suggested that in a rapid eradication program the combination of fecal culture, SELISA and  $\gamma$ -IFN could increase sensitivity to 97%.<sup>67</sup> Due to lack of organism verification we could not speculate on the precise increase in sensitivity with the utilization of three testing means. Additionally the lack of confidence in the MELISA testing means would diminish any perceived increase in detection.

Results of this study indicated poor agreement between the three testing methods. As the  $\gamma$ -IFN assay sampled a completely different immunological response the lack of agreement between this assay and the determination of antibody would be expected. What was not expected was the lack of agreement between the ELISA determinations. Due to the acceptance of the SELISA as a regulatory standard this study does not support the use of the MELISA for determination of herd prevalence or detection of individuals to cull. While the SELISA is a regulatory standard, the significantly lower age at positive test compared to the  $\gamma$ -IFN assay should raise concerns that the SELISA in this instance may be operating at a higher sensitivity and lower specificity yielding an excess number of false positives. Further supporting evidence of this observation would be indicated by the lack of fecal culture positive animals in the sampled individuals.

# EFFECT OF A SKIN-TEST USING A CELL-FREE SONICATE OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* (19698) ON THE PRODUCTION OF GAMMA-INTERFERON

A paper submitted to the American Journal of Veterinary Research

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## Introduction

*Mycobacterium avium* subsp. *paratuberculosis* is the causative agent of a chronic enteritis called paratuberculosis (Johne's disease). Infection produces a granulomatous inflammation that affects the caudal region of the small intestine, the ileocecal valve, the cecum, and associated lymph nodes.<sup>3</sup> Johne's disease is manifested by rapid weight loss, diarrhea, and failure to respond to therapy. Infection with *M. paratuberculosis* causes a leakage of plasma proteins to occur across the intestinal wall, along with malabsorption of dietary amino acids from the intestinal contents.<sup>11</sup> Affected animals may manifest a "bottle jaw" appearance (mandibular edema) due to the loss of vascular proteins.<sup>16</sup>

Paratuberculosis affects all ruminants including cattle, sheep, goats, deer, antelope, and bison.<sup>1,3,4</sup> Cattle become infected as calves through oral ingestion of the bacilli through fecal, colostrum or milk contamination, or through in-utero infection.<sup>4</sup> Due to the pathogen's slow growth patterns, clinical disease doesn't become evident until two to five years of age.<sup>4</sup> This economically devastating disease is estimated to cost \$222 million in annual losses in the US alone.<sup>7,8</sup>

A new development in Johne's testing procedures is the evaluation of cell-mediated immunity through measurement of gamma-interferon ( $\gamma$ -IFN) produced by stimulated T-cells. The first measurable immunological response of an infected individual is mediated by activated T-lymphocytes.<sup>12</sup> This is reflected by delayed type hypersensitivity (DTH) reactions and the release of cytokines such as  $\gamma$ -IFN.<sup>12</sup> Anti-mycobacterial action is accomplished through macrophage activation mediated by CD4<sup>+</sup> T<sub>H1</sub> cells and its secreted cytokines,  $\gamma$ -IFN and IL-2. Gamma-interferon is produced by CD4<sup>+</sup>, CD8<sup>+</sup>, and gamma/delta ( $\gamma/\delta$ ) receptor T-cells and natural killer cells. Activation of the macrophage population through cytokine induction is responsible for the initiation of tumor cell lysis, release of reactive oxygen and nitrogen intermediates, expression of major histocompatibility complex class II antigens, and secretion of arachidonic acid metabolites. There is a direct relationship between  $\gamma$ -IFN presence and activation of macrophages and their ability to kill intracellular pathogens.<sup>12</sup>

The  $\gamma$ -IFN assay promises improvement in Johne's disease diagnosis. A study by Wood et al. (1991) tested six herds infected with Johne's disease.<sup>63</sup> Testing methods included the  $\gamma$ -IFN and absorbed ELISA

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assays. Verification of test results was performed on diseased cattle removed from the herd through the use of fecal culture and histopathological examination of the tissues collected at post mortem. Gamma-interferon was equally sensitive in detecting subclinical (71.8% to 93.3%) and clinical animals (100%) whereas the absorbed ELISA was more sensitive in detecting cattle with advanced infections (80%) than those with subclinical infections (20.5% to 26.6%).<sup>63, 65</sup> Work by Bilman-Jacobe et al. suggested that the specificity was 97.6% for the  $\gamma$ -IFN enzyme-linked immunosorbent assay (EIA).<sup>65</sup>

Bovine tuberculosis eradication programs have a longer history and have been in place in the US since 1917 when overall animal-level prevalence was 5%.<sup>13</sup> The caudal fold skin test has been the primary means of detection and is conducted using an intradermal injection of a *M. bovis* purified protein derivative (PPD). In 72 hr the injection site is examined for the presence of a palpable nodule. Some countries have adopted the  $\gamma$ -IFN assay as a supplementary procedure. For example, cattle in New Zealand are initially tested using the intradermal skin test with reactors being subsequently examined with the  $\gamma$ -IFN assay. Because of this testing sequence a natural research question is what effect does an intradermal PPD injection have on the production of  $\gamma$ -IFN in a skin test positive animal.

Whipple et al. demonstrated increased production ( $p < 0.01$ ) of  $\gamma$ -IFN by stimulated cells after administration of a *M. bovis* PPD intradermal injection used in a caudal fold skin test.<sup>13</sup> Optical density (OD) values of the  $\gamma$ -IFN EIA for samples taken 72-hr post-sensitization were significantly higher than for samples collected prior to skin testing, and higher than samples from cattle that were not skin tested. Rothel et al. demonstrated a 2.5-fold increase in  $\gamma$ -IFN production following skin testing which peaked at a period of 3 to 5 weeks post-skin testing.<sup>14</sup> This increase in  $\gamma$ -IFN production could potentially enhance the sensitivity of the  $\gamma$ -IFN assay in determining positive reactors to bovine tuberculosis.

Given the stimulatory responses documented with *M. bovis*, we hypothesized that the same enhanced response may be evident during the cell-mediated phase of infection with *M. paratuberculosis*. This study was undertaken to determine if enhancement of  $\gamma$ -IFN levels would occur following skin testing using a cell-free sonicate of *M. paratuberculosis* strain 19698 (19698 MpS) and to determine if this response was present given the more immunologically distant nature of the Johne's infection site in the gut.<sup>12</sup>

Skin testing for Johne's disease is performed using a PPD of *M. paratuberculosis*. This product is derived from precipitated proteins secreted by the bacilli into the culture filtrate.<sup>20</sup> 19698 MpS is a sonication of the whole cell containing internal and external, as well as secreted proteins of *M. paratuberculosis*. The investigators chose to use 19698 MpS as the inclusion of cellular proteins could prove more immunogenic in both skin testing and stimulation of the  $\gamma$ -IFN assay cell cultures than the standard *M. paratuberculosis* PPD. Additionally, whole cell cultures of *M. paratuberculosis* strain 19698 for sonication are relatively easier to produce than a PPD.

## Materials and Methods

**Classification trial.** The cattle used in the study were obtained from the Ames Dairy facility at Iowa State University. This teaching herd representing the six major dairy breeds consists of 102 Holsteins, 26

Jerseys, 8 Guernseys, 17 Ayrshires, 17 Brown Swiss, and 9 Milking Shorthorns. While no clinical cases of Johne's disease have been observed in this herd, initial fecal sampling one year previous indicated a true prevalence rate approaching 10 to 12%.

This study focused on testing procedures that are readily available to the US veterinary practitioner with the exception of the  $\gamma$ -IFN assay. The herd was sampled in a ten-day period utilizing milk ELISA (MELISA) (Dairy Lab Services, Dubuque, IA), serum ELISA (SELISA) (IDEXX Laboratories, Inc., Westbrook, ME),  $\gamma$ -IFN (CSL Limited, Parkville, Victoria, Australia) and fecal culture (National Animal Disease Center, Ames, IA). Sampling consisted of obtaining 2 oz of fluid milk for MELISA assay at the regular dairy records processing center (DRPC) monthly check. Five days later sampling consisted of obtaining 60 ml of blood that was partitioned between a 10-ml sodium heparin tube and three 15-ml serum separator tubes. The heparinized whole blood sample was delivered within 6 hours for  $\gamma$ -IFN assay. Serum from the separator tubes was used to obtain a serum sample for SELISA determination, with the balance being subdivided for serum banking purposes. A fecal sample was also submitted for *M. paratuberculosis* culture. The herd was divided into 3 groups to facilitate sampling. This sampling process was completed within a 10-day period.

**Milk ELISA,<sup>85</sup> Serum ELISA<sup>59</sup> Fecal and Tissue Culture<sup>20</sup> and  $\gamma$ -IFN assay.<sup>20</sup>** These assays were performed as previously described in the Materials and Methods section of the article, Comparisons of the Milk ELISA, Serum ELISA, Gamma-Interferon and Fecal Culture Tests for the Detection of Johne's Disease in an Iowa Herd.<sup>79</sup>

**19698 MpS Production.** Cell-free soluble protein 19698 MpS was prepared by sonication of 1-ml volumes of *M. paratuberculosis* ( $1 \times 10^9$ /ml) in phosphate buffered saline (PBS) at 25 W for 25 min. This was followed by centrifugation at  $1,400 \times g$  for 5 min to pellet the cellular debris. The cell-free supernatant was removed and a protein concentrate determined. Final concentration of each PPD or MpS in culture was 10  $\mu\text{g/ml}$ .<sup>20</sup>

**Treatment Assignment.** Using the results of the initial  $\gamma$ -IFN assay and the ELISA determination the herd was divided into 4 groups, consisting of cattle with:

- **No response** – negative to all testing.
- **$\gamma$ -IFN suspect** individuals – optical density (OD) of 0.075 – 0.099 above the negative stimulation (NS) cell line.
- **$\gamma$ -IFN positive** individuals – OD  $\geq 0.10$  or greater.
- **Serum ELISA positive** individuals – sample/positive (s/p) ratio  $\geq 0.250$ .

To be enrolled for further testing a cow had to be a member of the first three groups. These three groups were considered to be either negative response or early phase *M. paratuberculosis* reactors. All  $\gamma$ -IFN assay positive ( $n = 7$ ) and suspect ( $n = 8$ ) cattle were enrolled in the skin testing portion of the trial. Cattle testing negative to both  $\gamma$ -IFN assay and serum ELISA were randomized (The SAS System, Software Release 6.12, SAS Institute, Cary NC) and ( $n = 8$ ) were selected for enrollment.

Enrolled cattle were randomly assigned to one of 2 treatments within the negative, suspect and positive groups, consisting of an injection of either 100 µg (0.1ml) of 19698 MpS or 0.1 ml of 0.9% saline solution. These products were given as an intradermal injection using a tuberculin 1-ml syringe and a 26-gauge, 0.5-inch needle. The initial determinations for enrollment and balancing of treatments were performed using data obtained during the initial sampling period in October 1998. Baseline  $\gamma$ -IFN samples were acquired at the start of the skin testing project in January 1999 and the final test status (negative, suspect or positive) for statistical analysis was determined at the end of the project within the 2 treatment groups. The final assignments yielded “n” as follows: MpS-N = 5, MpS-S = 3, MpS-P = 4, Sal-N = 4, Sal-S = 3 and Sal-P = 3. During the project, the researchers were blinded to the official status of individual cows. The following are the treatment and status designations:

- **MpS-N** – Cattle **negative** to all testing and treated with 100 µg 19698 MpS.
- **MpS-S** – Cattle declared **suspect** at initial  $\gamma$ -IFN assay and treated with 100 µg 19698 MpS.
- **MpS-P** – Cattle declared **positive** at initial  $\gamma$ -IFN assay and treated with 100 µg 19698 MpS.
- **Sal-N** – Cattle **negative** to all testing and treated with 0.1 ml 0.9% saline.
- **Sal-S** – Cattle declared **suspect** at initial  $\gamma$ -IFN assay and treated with 0.1 ml 0.9% saline.
- **Sal-P** – Cattle declared **positive** at initial  $\gamma$ -IFN assay and treated with 0.1 ml 0.9% saline.

On day 0, each cow’s head was deviated and secured to the right in a head chute. A blood sample from the jugular vein was taken in a 12-ml sodium heparin tube for  $\gamma$ -IFN assay determination and delivered for processing within 6 hr. This was to determine a pre-skin testing baseline  $\gamma$ -IFN level. A mid-cervical area was then selected and prepared with a #40 clipper blade to make a single pass 3 in long. The area was prepped using a single 3x3 gauze pad soaked in alcohol and the intradermal injection made.

On subsequent examinations (Day 3, 6 and 9) the skin over the injection site was palpated for the presence of a delayed-type hypersensitivity reaction. If present, the diameter of the reaction was measured in mm. The investigators elected to measure the diameter of the skin lesion vs. the thickness as it was deemed to be a more sensitive measure of activity in the cervical location. To avoid masking of any lesion by skin stretching, animals heads were not deviated during acquisition of measurements. Repeat blood samples to monitor  $\gamma$ -IFN levels were obtained on days 3, 6, 9, 15, and 27 post-injection.

**Exploratory laparotomies.** For purposes of test verification an abdominal lymph node and cecal biopsy were obtained from all enrolled animals. During a 5 day period a flank laparotomy was performed on each enrolled cow. Starting on day 27 post-injection, 4 to 6 individuals per day were transported 2 miles from the Iowa State University Ames Dairy facility to the Veterinary Teaching Hospital at the Iowa State College of Veterinary Medicine. On the same day following surgery, the individuals were moved back to their home facility, stalls and rations.

For the surgical procedure each animal was placed in a surgical headgate and chute. The right paralumbar fossa and the area over the lumbar vertebra were clipped with a large animal clippers followed by clippers using a #40 blade. The surgical site and vertebral area were washed and prepped for surgery using a 3-

pass technique of disinfectant soaping with a 4x4 gauze pad, followed by wiping using an alcohol soaked 4x4 gauze pad. A Kakala vertebral block was administered to provide regional anesthesia followed by an additional 3-pass disinfection routine as described above. An 8 in. incision was then made in the paralumbar fossa. The cecum was located and exteriorized to determine the ileal/cecal junction and the first available lymph node was harvested. A site between blood vessels on the cecum was then selected to obtain a 1.0-1.5 cm diameter biopsy. The biopsy site was closed using #2-0 Chromic Gut in a 2-layer inverting closure pattern. The abdominal muscular layers were closed in a 3-layer closure pattern using #3 Vicryl. The final skin closure was made using #3 Vetafil. All animals recovered uneventfully, and the skin closures were removed 14-21 days post-surgery. Tissue samples were submitted for *M. paratuberculosis* culture at the National Animal Disease Center (NADC), Ames, Iowa.

Two of the enrolled cows died prior to surgery due to post calving complications. Cow #1168 became a paralysis case due to electrolyte imbalances, and it was decided to euthanize this animal on the last day of the study. Cow #1079 was treated for hypocalcemia 6 hr post-calving and died 2 hr after treatment due to peracute *Clostridial* enteritis. Both cows were submitted to the Iowa State University Veterinary Pathology department for post-mortem examination and diagnosis. Tissues were harvested, cultured and examined using histopathology for the presence of *M. paratuberculosis* consistent with the established surgical protocol.

**Histopathology.** Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4  $\mu$ m. Tissues were then stained with hematoxylin and eosin (H&E), acid fast by the Ziehl Neelsen method, and immunohistochemistry with primary antibodies for *Mycobacterium bovis* for light microscopy. Tissues were also stained with auramine O and acridine orange for fluorescent microscopy using an Olympus BX-60 microscope with a WIBA filter cube.<sup>80</sup> The H&E stain was used for general tissue morphology. Acid fast staining was employed as the classical mycobacterial stain. Fluorescent staining was used to facilitate finding low numbers of mycobacterium in sections, and the immunostain was utilized as it is more sensitive and specific than acid fast staining.<sup>81</sup>

**Statistics.** The relative difference in the  $\gamma$ -IFN determinations from day 0 was computed for each of the following days: 3, 6, 9, 15, and 27. For each day, these relative differences and the actual measurements for  $\gamma$ -IFN values were analyzed with analysis of variance methods (ANOVA - The SAS System, Software Release 6.12, SAS Institute, Cary NC) to examine the average effect of the various  $\gamma$ -IFN/stimulation combinations on the  $\gamma$ -IFN values and the relative change in  $\gamma$ -IFN values from day 0. Both one-way and two-factor classification models were fit on each day. Pairwise t-tests between the various  $\gamma$ -IFN/stimulation groups were performed. Paired t-tests were used to compare the mean responses of 19698 MpS, Avium, and Bovis for the  $\gamma$ -IFN positive, MpS stimulated cows for each day. Standard error of the means were calculated for each group, on each day and are presented in the relevant figures. Relative differences were calculated between days.

## Results

Sampling periods were modeled after the protocol of a standard Johne's delayed type hypersensitivity (DTH) skin test, with sampling and measurement periods being conducted at 72 hr (3-day) intervals for the first

9 days post-injection (Figure 1). At 72 hr minimal skin reactions were apparent, although a mean reaction diameter of 13.8 mm ( $P < 0.0012$ ) was determined for the MpS-P individuals compared to the day 0 value. Contrary to expectations of diminishing skin reactions, the 144 hr (6-day) sampling period yielded visually apparent and distinct cervical area skin lesions (Figure 1). These skin reactions on day 6 displayed mean diameters of 34.9 and 23.5 mm in the MpS-P and MpS-S populations, with a  $P = 0.0018$  and  $0.0459$  compared to day 0, respectively. While not significant, MpS-N individuals on day 6 displayed mean diameters of 17.48 mm with a  $P = 0.0545$ . By 9 days post-skin test the lesion sites had regressed to undetectable levels. When comparing skin measurements of day 6 vs. day 3 there were no areas of statistically significant change.

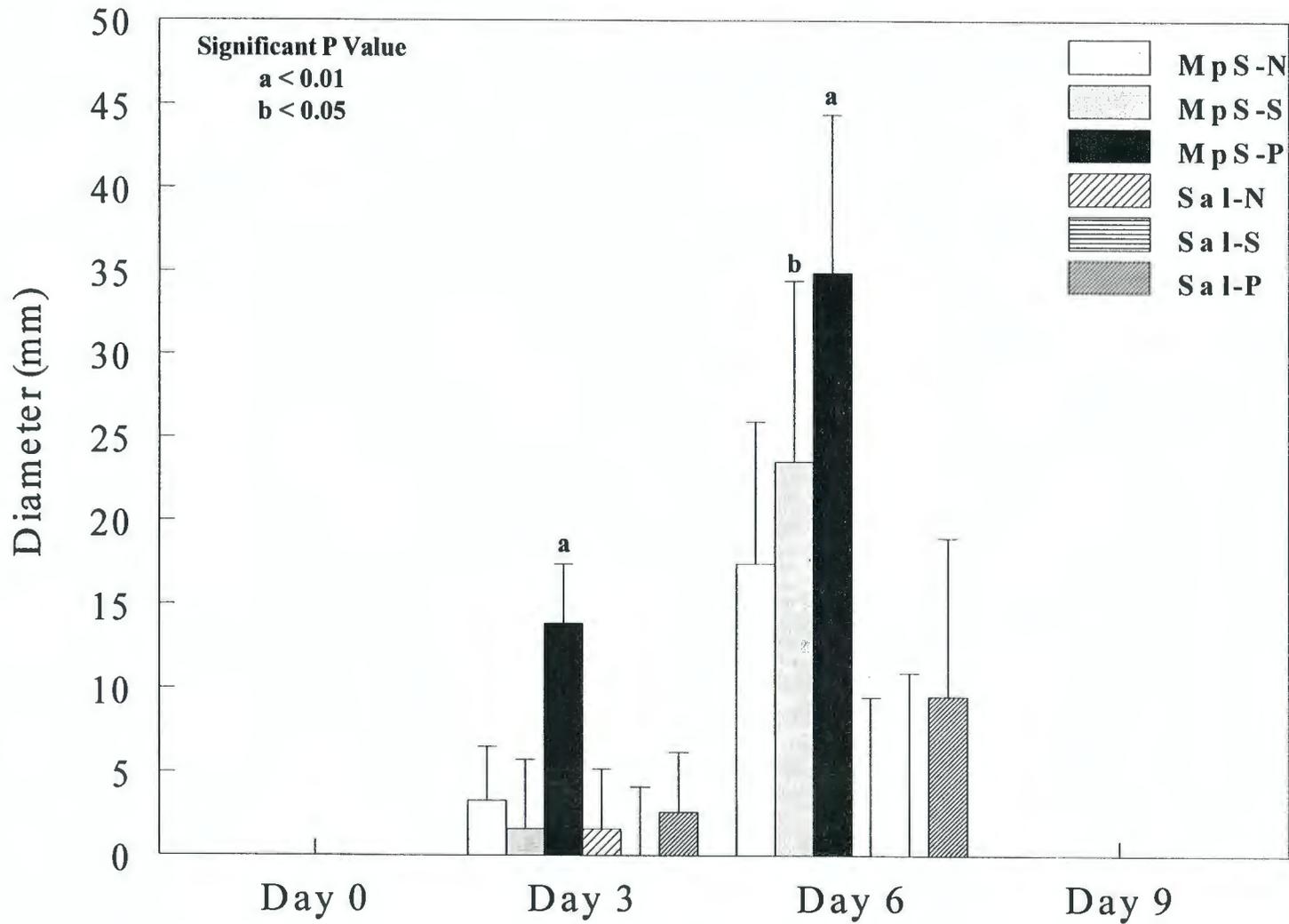
Cell-mediated immune system activity was indicated by DTH reactions. Similarly to the skin lesion activity,  $\gamma$ -IFN levels peaked at day 6 (Figure 2). Significant elevations were noted in the MpS-P population on day 6 (mean = 0.944), compared to day 0 (mean = 0.198) with  $P = 0.0005$ . Compared to day 0,  $\gamma$ -IFN values remained significantly elevated on days 9, 15 and 27 with optical densities and  $P$  values of 0.467 ( $P = 0.0325$ ), 0.455 ( $P = 0.0293$ ) and 0.619 ( $P = 0.0004$ ), respectively. The MpS-P values dropped significantly from day 6 to day 9, optical density of 0.944 to 0.467 ( $P = 0.0001$ ), while no significant change was evident between day 9 and 15. On day 27 following the skin test there was a significant elevation in the  $\gamma$ -IFN optical density. The values increased from 0.455 to 0.619 with  $P = 0.0362$ .

The  $\gamma$ -IFN levels were also examined utilizing daily mean differences (Figure 3). Values were obtained by using days 3 through 27 samples and subtracting day 0 baseline values from each. The same trends existed in this study as in the daily value analysis, with the exception that the 19698 MpS stimulated elevations became visually more evident as the “steady-state” saline reactions were removed from consideration through the subtraction of day 0 baselines. Statistical significance was the same as documented in the daily  $\gamma$ -IFN analysis. On day 3 Sal-S was considered significantly lower ( $P = 0.0131$ ) than MpS-P, Sal-P, and MpS-S. The drop in  $\gamma$ -IFN optical density on day 9 vs day 6 was more visually apparent after removal of the “baseline” day 0 values, as was the elevation on day 27.

Cattle injected with saline exhibited no increase in  $\gamma$ -IFN levels at the 3 or 6-day interval, nor was there a significant increase in skin lesion diameters in these individuals. The exception was the Sal-P group, which exhibited skin lesions diameters of 2.6 mm and 9.5 mm on days 3 and 6 respectively. These were not considered to be significant elevations in DTH response. This same group also, exhibited higher overall levels of  $\gamma$ -IFN during the pre-injection and post-injection phases than the Johne’s positive animals skin tested with MpS. While the overall  $\gamma$ -IFN response was elevated it remained flat at the MpS 144 hr peak. The high overall Sal-P reaction was due primarily to the high readings exhibited by one individual (#1278), thus increasing the overall level of the group.

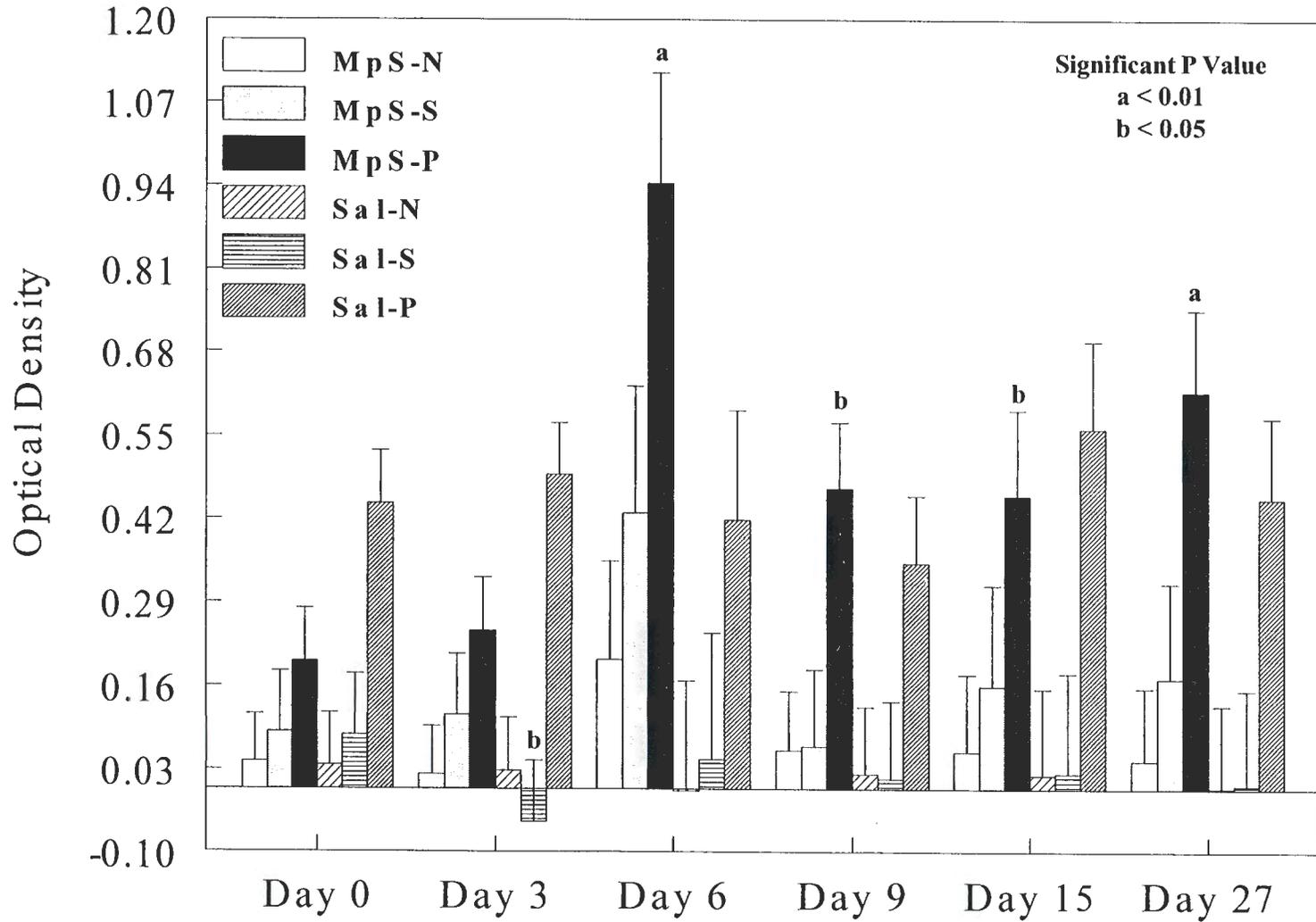
In addition to stimulation using 19698 MpS, the cell samples of the  $\gamma$ -IFN assay were stimulated using *M. avium* PPD (AvPPD) and *M. bovis* PPD (BoPPD) (Figure 4). The administration of the skin test using 19698 MpS stimulated a 4.76-fold increase in  $\gamma$ -IFN produced by the MpS-P individuals on day 6 compared to a 2.45 and 2.13-fold increase for the AvPPD and BoPPD, respectively (Figure 4). Post-skin testing  $\gamma$ -IFN

# Skin Test Lesion Diameter (mm)



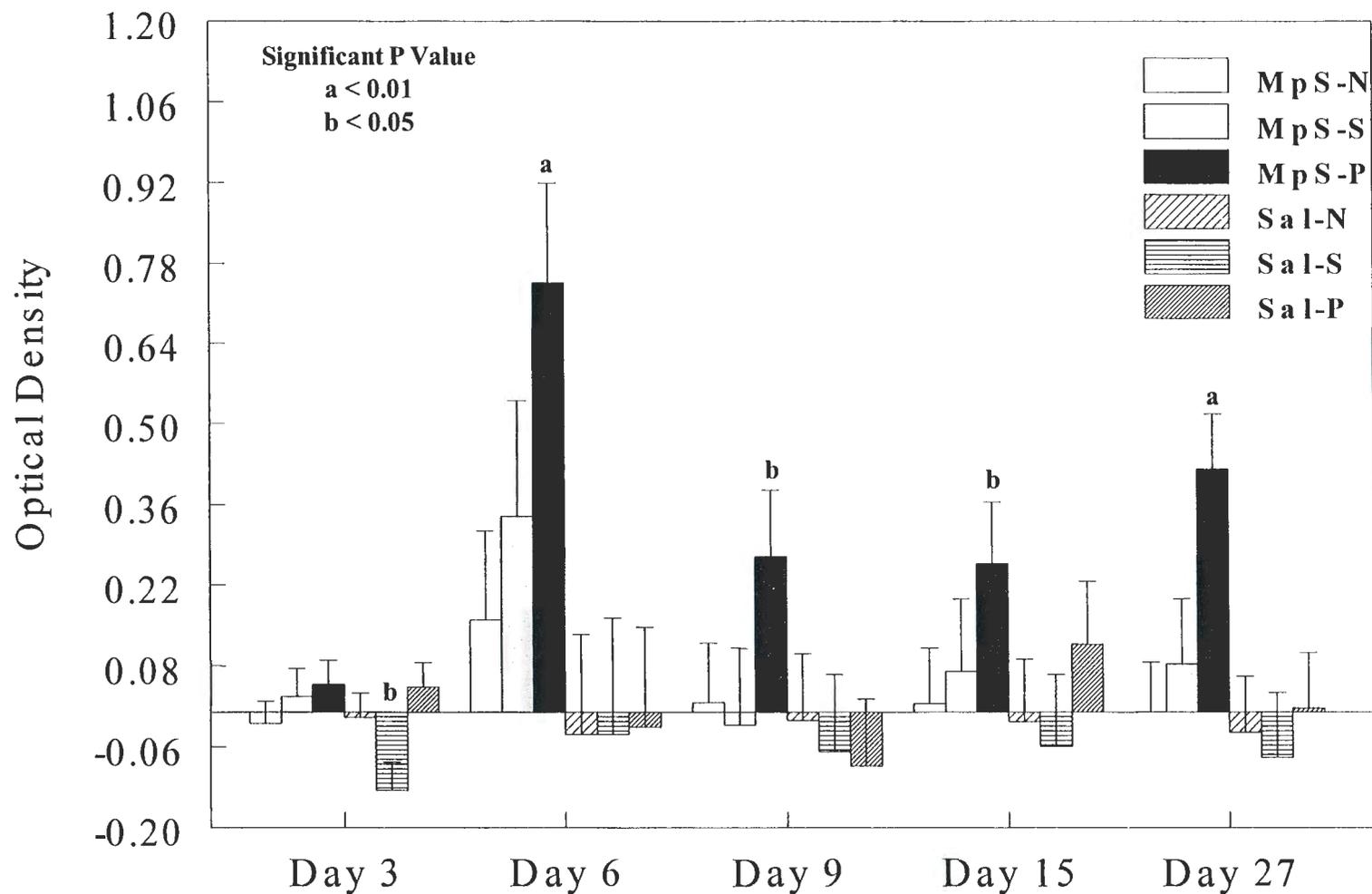
**Figure 1.** Skin Lesion Diameter (mm) post skin test on day 0. Error bars are standard error of the mean. Values above the error bars represent significant P-values.

# Gamma-Interferon Daily Determinations



**Figure 2.** Daily Gamma-Interferon determinations in units of optical density. Error bars are standard error of the mean. Values above the error bars represent significant P-values.

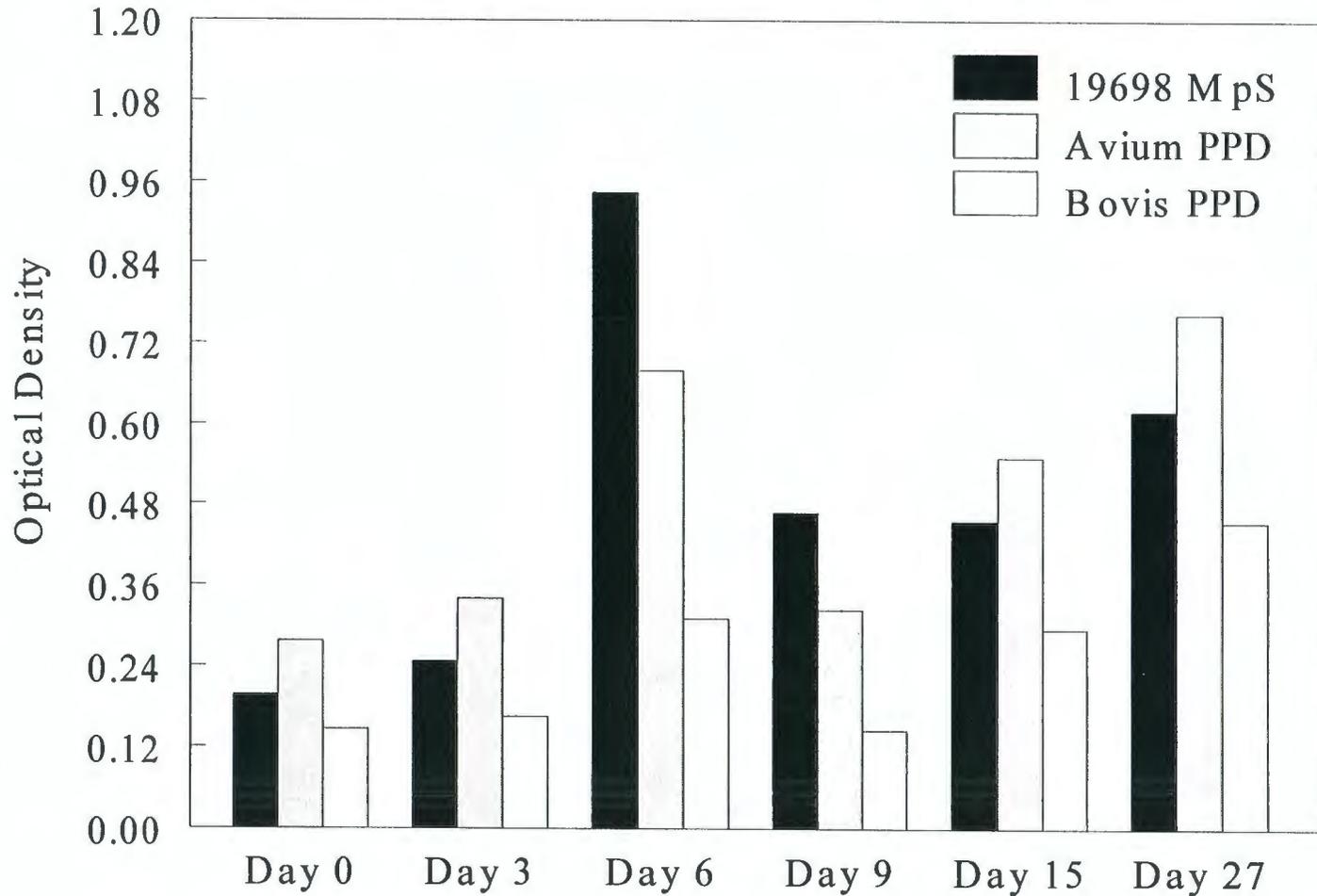
## Differences - Gamma-Interferon Determinations (Current Day - Day 0)



**Figure 3.** Daily Gamma-Interferon determinations minus Day 0 values in units of optical density. Error bars are standard error of the mean. Values above the error bars represent significant P-values.

# Gamma-Interferon - MpS-P Cattle

## 19698 MpS, Avium PPD and Bovis PPD



**Figure 4.** Gamma-Interferon daily determinations from MpS-P cattle (n = 4) measuring Gamma-Interferon produced by 19698 MpS, Avium PPD and Bovis PPD as culture stimulagens. Expressed in Optical Density units. Numbers were too low for meaningful statistical analysis.

elevations diminished by day 9 to levels that were 2.36, 1.17 and 0.995-fold increases over day-0 measurements for 19698 MpS, AvPPD and BoPPD, respectively. At day 15 AvPPD levels exceeded the  $\gamma$ -IFN levels for the 19698 MpS. This trend continued through day 27. Statistical significance for comparison between the stimulants was difficult to ascertain due to the small number of individuals occupying the MpS-P category ( $n = 4$ ).

Tissue samples were obtained for verification of Johne's disease status via flank laparotomies and post-mortem sampling. Following culture and staining procedures no granulomatous lesions consistent with *M. paratuberculosis* were seen in any of the sections, nor were any bacteria consistent with *M. paratuberculosis* observed. All culture attempts on harvested samples were negative.

### Discussion

Results of this study indicate that pre-inoculation of animals with 19698 MpS significantly increased production of  $\gamma$ -IFN in cattle declared to have positive (MpS-P) baseline  $\gamma$ -IFN responses for Johne's disease. Peak production of  $\gamma$ -IFN occurred at 144 hr (day 6) following skin testing. This elevation was statistically significant at the P level of 0.0005 for MpS-P (Figure 2). Examination of the daily means minus day 0 baseline values verified the significance and lent further visual evidence to the significance of the elevation of MpS-P ( $P = 0.0005$ ).

Work by Whipple et al.<sup>13</sup> and Rothel et al.<sup>14</sup> focusing on use of the  $\gamma$ -IFN assay in detection of *M. bovis* infection demonstrated enhanced production of  $\gamma$ -IFN following an intradermal injection of *M. bovis* PPD. Rothel demonstrated peak levels at 28 days post-injection, while Whipple's study showed peak stimulatory effect at 4 days. These increases persisted and did not return to pre-injection levels at 28 (Whipple) or 59 days (Rothel) post-skin testing in either study. With the introduction of  $\gamma$ -IFN testing for *M. paratuberculosis* it could be postulated that a similar stimulatory response would occur. As demonstrated in Whipple and Rothel's *M. bovis* work, this study showed a significant elevation ( $P = 0.0005$ ) of  $\gamma$ -IFN in individuals declared  $\gamma$ -IFN positive (MpS-P) and stimulated with 19698 MpS. Peak concentrations occurred at 6 days post-injection. Unlike Whipple and Rothel's *M. bovis* studies,  $\gamma$ -IFN levels dropped significantly by day 9 compared to day 6 ( $P = 0.0001$ ) to a level that still remained significantly above the pre-injection baseline (day 0) for the duration of the study ( $P = 0.0325$ ). At day 27 though,  $\gamma$ -IFN again increased significantly ( $P = 0.0039$ ) compared to day 15. This follows the trend of Whipple and Rothel of elevations that hadn't returned to baseline levels by 28 or 59 days, respectively. Injection with the 19698 MpS would appear to have more than a transient stimulatory effect to the cell-mediated immune system.

Cattle treated with 19698 MpS and previously designated as  $\gamma$ -IFN suspect (MpS-S) and negative (MpS-N) also demonstrated increases in  $\gamma$ -IFN production (Figure 2 and 3). Both elevations were not statistically significant. Following peak at day 6, MpS-S and MpS-N cattle returned to previously measured baseline levels of  $\gamma$ -IFN. As  $\gamma$ -IFN elevations were deemed significant only for the MpS-P individuals, it could be suggested

that the non-stimulated cut-off level of  $\geq 0.100$  OD above NS for determination of positive status could be valid and should be retained.

As part of the  $\gamma$ -IFN testing protocol all blood samples were cross-examined using AvPPD to determine if a positive  $\gamma$ -IFN reaction was that of *M. avium* subsp. *paratuberculosis* or a cross-reaction with *M. avium*. Examination of the Johne's  $\gamma$ -IFN positive individuals (Figure 4) demonstrated an increase in  $\gamma$ -IFN produced as a response to stimulation of the cell cultures using 19698 MpS, AvPPD and BoPPD. The dynamics of the AvPPD and BoPPD responses were similar to 19698 MpS. While 19698 MpS had the highest response, BoPPD had the lowest. All  $\gamma$ -IFN levels declined following the peak at 6 days with *M. avium* overtaking the  $\gamma$ -IFN levels stimulated by 19698 MpS at 15 and 27 days.

An explanation for the documented 19698 MpS and PPD stratification is that the highest response should be to the 19698 MpS as this product was used for the skin test procedure. The high response by AvPPD vs. 19698 MpS is potentially due to high genetic similarity. The genome of *M. avium* is approximately 98% conserved within *M. avium* subsp. *paratuberculosis*.<sup>20</sup> As *M. bovis* is less closely related, its  $\gamma$ -IFN response is less. While cross-stimulation is evident as seen by the responses of the AvPPD and BoPPD, this did not negate the ability of the test to determine 19698 MpS positive reactions at day 6. Due to the low population numbers of the MpS-P group ( $n = 4$ ) statistical differences were inapparent. Further evaluation of the cross-stimulation effect will have to be examined using higher numbers of positive individuals. Trends indicated in Figure 4 would suggest that use of skin testing procedures with a  $\gamma$ -IFN assay would need to be a timed event to avoid confusing cross-reactions with *M. avium* at 15 and 27 days post-skin test.

The development of the  $\gamma$ -IFN assay has opened an additional door in Johne's disease determinations through evaluation of cell-mediated activity.<sup>12,36,63,65</sup> While the response to *M. paratuberculosis* infection can be termed paradoxical, it is generally accepted that the first and most effective response to the presence of this organism is through cell-mediated means.<sup>12,36</sup> The determination of  $\gamma$ -IFN is not in widespread use in the US due to test cost, lack of United States Department of Agriculture approval and the requirement that the whole blood sample be delivered to the laboratory by 16-hr post-sampling. It does, however, represent a great advance by providing a quantitative numerical value indicative of cell-mediated activity. Skin testing procedures have the advantage of cost and speed, but suffer from an inherent lack of sensitivity and specificity due to the vagaries of individual injection techniques and determinations by testing personnel of a true reaction.<sup>12</sup> Historically, DTH reactions have been a successful form of testing for the presence of *M. bovis*.<sup>13,14</sup> This may not be the case when applied to detection of *M. paratuberculosis* due to the distant location of the gut.<sup>12,36</sup> Testing using the  $\gamma$ -IFN assay should be considered advantageous over lymphocyte blastogenesis, another cell-mediated testing means, due to the long incubation time and labor-intensive nature of the latter procedure.<sup>12</sup>

Skin testing using 19698 MpS could represent a further enhancement for the  $\gamma$ -IFN assay and may have merit in two areas. The first advantage is derived from an increase in test sensitivity due to the enhanced nature of the  $\gamma$ -IFN response. A "magnification" effect was clearly demonstrated when positive individuals manifested a 4.76-fold increase over baseline  $\gamma$ -IFN levels. However it must be remembered that while not statistically

significant, all individuals injected with 19698 MpS demonstrated some increase in  $\gamma$ -IFN levels. Determination of a cutoff “magnification” level would be appropriate so as not to lose specificity with the use of this technique.

A second benefit is related to the current test time limitations of the  $\gamma$ -IFN procedure. Since  $\gamma$ -IFN production is significantly increased using this method, it may be possible to have a valid test with transportation times exceeding 16 hr. Further work would be required to validate increased test durability.

While injection of the 19698 MpS successfully increased the expressed amounts of  $\gamma$ -IFN, use of this product for skin testing purposes only would not be advisable. Skin reactions were maximal at 144 hr. Evaluation of skin lesion data suggests that animals designated as positive and suspect both mounted significant skin wheals compared to day 0 (34.93 and 23.54 mm) with P values of 0.0018 and 0.0459, respectively. Likewise, MpS-N cattle displayed palpable lesions of 17.48 mm with a P value of 0.0545. The lack of differences between 19698 MpS injected individuals suggests that clear skin lesion determinations between negative and positive may be blurred with the application of this product.

Rothel et al. demonstrated an enhanced effect remaining in place at 59 days.<sup>14</sup> Current recommendations in the European Union for administration of the DTH skin test for *M. bovis* suggest this form of testing should not be repeated for a minimum of 60 days to allow the animal sufficient time to clear the stimulatory effect.<sup>87</sup> This may be a potential negative aspect to skin testing as all individuals, whether negative, suspect or positive, injected with the sonicate mounted an increased response. Even so, a clear, significant difference emerged between animals designated positive vs. those declared suspect or negative. While skin testing with either a PPD or sonicate has a lasting effect,  $\gamma$ -IFN determinations can be repeated at any time without compromising Johne’s status. Injecting a sonicate or PPD should not be taken lightly and application of this type of stimulation should be repeated only on a semi-annual or annual basis.

Current skin testing protocols require examination of the injection site at 72-hr post-injection to declare a positive reaction. Very little response was noted at 72 hr compared to  $\gamma$ -IFN levels and skin lesion dimensions seen at 144 hr. By the 216 hr (9 day) check, the  $\gamma$ -IFN and skin lesions had diminished to baseline levels. The delayed response of 144 hr vs. 72 hr is puzzling. This could possibly be explained by the difference in skin testing products; traditional PPD vs. 19698 MpS. A PPD is made from precipitating proteins secreted by *M. paratuberculosis* into the culture filtrate, while a sonicate is a whole cell product which contains intracellular proteins.<sup>20</sup> These two products differ in bacterial protein types and this may account for the delayed 144-hr response, as well as the increased diameter skin lesions seen in cattle injected with 19698 MpS. Given the observed delayed response in DTH activity, this observation may call into question evaluating DTH responses at only the 72-hr sampling period. A second period of examination at 144 hr post-injection may be advantageous.

As a verification step, biopsy samples were obtained for tissue culture purposes. No positive animals were found among the enrolled individuals. The objective of this study was to examine the early, cell-mediated phase of infection by *M. paratuberculosis*. As culture methods are more accurate during the later phase of

infection it may be that no animals were in a stage of disease to allow effective culturing. Furthermore, culture and the  $\gamma$ -IFN assay are determining two entirely different populations as  $\gamma$ -IFN examines early cell-mediated immune system activity, while culture detects later phase shedding. While culturing has a very high specificity (99.9%) it must be kept in mind that the sensitivity of this test is 45% to 50% due to intermittent shedding which limits test positive yield.<sup>4</sup>

Previous work has indicated that the serum ELISA prescreening examination may have had an inordinately high number of false positives in this herd.<sup>79</sup> The test positive rate was 7.41% with the age at positive test of 37.21 months. Examination of the herd using  $\gamma$ -IFN indicated a test positive rate of 4.40% and an age at positive test of 48.25 months. The age of serum ELISA positive cattle was significantly lower ( $P = 0.0192$ ) than the  $\gamma$ -IFN and milk ELISA testing means. These results contrast with the current knowledge of the progression of immune system responses to *M. paratuberculosis* infection. It is generally accepted that the first and most effective response to the presence of this organism is through cell-mediated means.<sup>12, 36</sup> Given the low number of  $\gamma$ -IFN positive tests and finding no culture positive animals in the screening examination, this herd may be in the very early stages of Johne's disease. A low prevalence rate and being in the early stages of disease would tend to complicate the ability to find biopsy culture and histopathology positive animals.

Whitlock et al. examined the relationship between fecal and multiple tissue cultures at post-mortem.<sup>46</sup> In this study the authors widened their scope from the traditional single tissue samples of ileum, jejunum, ileal/cecal valve and associated mesenteric lymph nodes to include multiple samples within the mesenteric lymph node chain as well as pulmonary, mandibular, retropharyngeal, hepatic, and supramammary tissue samples. Their conclusions indicated that some of the 55 cattle with negative fecal and tissue cultures had infected tissues that were not detected with the limited tissue samples obtained. The proportion of these infected, previously undetected, animals is difficult to estimate but could be as high as 20%. Additional samples were not obtained in this study because this would have required the sacrifice of the enrolled animals. The subjects were active production individuals and sacrifice was not possible. An alternative would be conducting long-term herd monitoring using annual serum ELISA and fecal cultures along with post-mortems following animal removal to determine the accuracy of various forms of testing procedures.

Results of this study indicate that skin testing of  $\gamma$ -IFN positive individuals using a *M. paratuberculosis* cell-free sonicate yielded significantly increased amounts of  $\gamma$ -IFN produced from sensitized lymphocytes. Further research is warranted to determine if the addition of skin testing to the  $\gamma$ -IFN testing method could increase test sensitivity or durability.

## GENERAL CONCLUSIONS

The history of Johne's disease is filled with the frustration of dealing with a disease syndrome that defines the term insidious. The long-term growth pattern of *Mycobacterium paratuberculosis* defies research efforts to deal with it in an expedient manner. These same growth patterns lull producers into a false sense of security when they believe they are dealing with Johne's by culling clinical cases. Research has also been hampered by the paradoxical immune system response of the disease.<sup>12</sup> Thus, the ability to define a subject bovine as truly negative for testing purposes becomes a "best guess" effort.

Research is beginning to make some inroads into Johne's disease through development of tests with increasing amounts of sensitivity while maintaining high levels of specificity. This is occurring in all fields of testing including culture, PCR identification, antibody detection, and  $\gamma$ -IFN determinations. With increasing sensitivity and specificity the question will shift from the inadequacies of the testing procedures to how committed the producer is to removing identified *M. paratuberculosis* infected animals from the premises. This may become an even more pressing issue if a definitive link with human Crohn's disease is established.

### Comparisons of Testing Procedures for the Detection of Johne's Disease

In performing the experimentation that produced the Chapter 3 journal article, the authors experienced the frustration of determining the true status of individual cows. Evaluation of the data for mean values at positive test indicated that the serum enzyme-linked immunosorbant assay (SELISA) had a significantly lower age (37.2 vs. 50.5 and 48.3 months respectively) at positive test ( $P > |T| = 0.0192$ ) than did the milk enzyme-linked immunosorbant assay (MELISA) or gamma-interferon assay ( $\gamma$ -IFN). This raises potentially serious questions. Patterns of measurable response will vary from animal to animal, but active proliferation and differentiation of T cells is generally the first measurable immune system response through DTH reactions or assay of  $\gamma$ -IFN.<sup>12</sup> It would be expected that the  $\gamma$ -IFN assay would have the lowest mean positive age vs. the antibody determinations. Furthermore, as the majority of IgG in milk originates from serum there should be little difference between the mean ages of the MELISA and SELISA methods.<sup>61</sup> Additionally, no fecal culture positive animals were found in this herd. This would raise serious concerns that the SELISA and MELISA methods are overly sensitive in determining a positive designation. Due to these concerns, the results of the regulatory standard SELISA test in this herd could be questioned for the presence of false positives due to the significantly lower mean age at positive test.

The MELISA test has recently been offered in Iowa by Iowa Dairy Lab Services Inc., for use in determining potential herd prevalence rates and individual determinations of Johne's disease.<sup>85</sup> This method offers convenient sample collection compared to serum and can be obtained through routine milking procedures, thus limiting stress to cattle.<sup>61</sup> This study indicates the MELISA determined the highest percentage of test positive animals at 12.96%. While this high rate of sensitivity looks encouraging, comparison with the results of the regulatory standard SELISA test casts some doubt. When compared using an XY plot (Figure 1, Chapter 3) no common individuals were identified as test positive by both tests. Analysis of the proportions in

each quadrant with the kappa statistic at  $-0.116$  indicated a very low percentage of agreement between the two testing means. The amount of agreement present could simply be due to chance. Sweeney et al. used a MELISA containing lipoarabinomannan (LAM) antigen.<sup>62</sup> Their work determined a specificity of  $87\% \pm 8.1\%$  when the sensitivity cutoff was set at 50%. When the cutoff was raised to 60% the specificity dropped to  $83\% \pm 9.1\%$ . The predictive value of a positive test using these values for sensitivity and specificity offers only a 29% chance that a positive test is correct. This value is too low to be considered useful for culling purposes.

A recent development in Johne's disease testing has been the introduction of the  $\gamma$ -IFN enzyme-linked immunoassay (EIA).<sup>12,63,65</sup> This test of the cell-mediated immune system has the advantages of being rapid, numerically quantifiable, and not affecting the outcome of further testing through the injection of a PPD. By testing an earlier phase response this procedure holds promise of detecting earlier infections of *M. paratuberculosis*, identifying infected individuals prior to or during, early shedding and potentially allowing detection in younger cattle.<sup>12,63,65</sup> This study indicated a 4.40% positive rate, with a mean age of detection at 48.25 months. As this is an earlier phase test, one would expect it to verify the age results seen using the SELISA and further identify positive individuals among the younger herd members.<sup>12,63,65</sup> The  $\gamma$ -IFN assay did not verify the results of the SELISA.

Cross-examinations of  $\gamma$ -IFN results were conducted against the SELISA (Figure 2, Chapter 3) and MELISA (Figure 3, Chapter 3). Both antibody tests had similar responses with only 1 individual showing a positive agreement between the two types of testing procedures (antibody vs. interferon). Kappa statistics indicated poor agreement between the tests with values of 0.0287 and 0.0005 respectively for the SELISA vs.  $\gamma$ -IFN and MELISA vs.  $\gamma$ -IFN. These kappa values would indicate poor agreement between the  $\gamma$ -IFN assay and antibody determinations. In this case the lack of agreement would be expected. As seen in the kappa values,<sup>61</sup> the difference between the two testing means indicates that two entirely different populations are being selected. Identification of separate populations would "widen the area of capture" and could result in increased sensitivity when used in a parallel manner. The MELISA and SELISA assays don't detect differing populations and as a result no increase in sensitivity should be expected with parallel testing using these two diagnostic modalities.

Examining the population using all testing means yielded a test positive rate of 25.81% which indicated an apparent increase in detection sensitivity. Collins et al. determined a potential 69% sensitivity when SELISA and fecal culture were combined compared to approximately 45% sensitivity when each was used separately.<sup>4,55,56</sup> Collins et al. suggested that in a rapid eradication program the combination of fecal culture, SELISA and  $\gamma$ -IFN could increase sensitivity to 97%.<sup>67</sup> Due to lack of organism verification we could not speculate on the precise increase in sensitivity with the utilization of three testing means. Additionally the lack of confidence in the MELISA testing means would diminish any perceived increase in detection.

Results of this study indicated poor agreement between the three testing methods. As the  $\gamma$ -IFN assay sampled a completely different immunological response the lack of agreement between this assay and the determination of antibody would be expected. What was not expected was the lack of agreement between the ELISA determinations. Due to the acceptance of the SELISA as a regulatory standard this study does not

support the use of the MELISA for determination of herd prevalence or detection of individuals to cull. While the SELISA is a regulatory standard, the significantly lower age at positive test compared to the  $\gamma$ -IFN assay should raise concerns that the SELISA in this instance may be operating at a higher sensitivity and lower specificity yielding an excess number of false positives. Further supporting evidence of this observation would be indicated by the lack of fecal culture positive animals in the sampled individuals.

#### Effect of a Skin-test on the Production of Gamma-Interferon

Results of this study indicate that pre-inoculation of animals with 19698 MpS significantly increased ( $P = 0.0005$ ) production of  $\gamma$ -IFN in cattle declared to have positive (MpS-P) baseline  $\gamma$ -IFN responses for Johne's disease. Peak production of  $\gamma$ -IFN occurred at 144 hr (day 6) following skin testing (Figure 2 Chapter 4). Examination of the daily means minus day 0 baseline values lent further visual evidence to the significance of the elevation of MpS-P ( $P=0.0005$ ) through the enhancement of positive increases by the removal of day 0 baseline levels (Figure 3 Chapter 4).

Cattle treated with 19698 MpS and previously designated as  $\gamma$ -IFN suspect (MpS-S) and negative (MpS-N) also demonstrated increases in  $\gamma$ -IFN production (Figure 2 and 3). Both elevations were not statistically significant. Following peak at day 6, MpS-S and MpS-N cattle returned to previously measured baseline levels of  $\gamma$ -IFN. As  $\gamma$ -IFN elevations were deemed significant only for the MpS-P individuals, it could be suggested that the cut-off level of  $\geq 0.100$  OD over the negative stimulation culture for determination of positive status could be valid and should be retained.

The development of the  $\gamma$ -IFN assay has opened an additional door in Johne's disease determinations through evaluation of cell-mediated activity. While the response to *M. paratuberculosis* infection can be termed paradoxical, it is generally accepted that the first and most effective response to the presence of this organism is through cell-mediated means<sup>12,36,63,65</sup>. The determination of  $\gamma$ -IFN is not in widespread use in the US due to test cost, lack of United States Department of Agriculture approval and the requirement that the whole blood sample be delivered to the laboratory by 16-hr post-sampling. It does however, represent a great advance by providing a refereed numerical value indicative of cell-mediated activity. Skin testing procedures have an advantage of cost and speed, but suffer from an inherent lack of sensitivity and specificity due to the vagaries of individual injection techniques and determinations by testing personnel of a true reaction.<sup>12</sup> Historically, DTH reactions have been a successful form of testing for the presence of *M. bovis*.<sup>13, 14</sup> This may not be the case when applied to detection of *M. paratuberculosis* due to the distant location of the gut.<sup>12, 36</sup>

Skin testing using 19698 MpS could represent a further enhancement for the  $\gamma$ -IFN assay and may have merit in two areas. The first advantage could come from an increase in test sensitivity due to the enhanced nature of the  $\gamma$ -IFN response. A "magnification" effect was clearly demonstrated when positive individuals manifested a 4.76-fold increase over baseline  $\gamma$ -IFN levels. However it must be remembered that while not statistically significant, all individuals injected with 19698 MpS demonstrated some increase in  $\gamma$ -IFN levels.

Determination of a cutoff “magnification” level would be appropriate so as not to lose specificity with the use of this technique.

A second possible benefit would be related to the current test time limitations of the  $\gamma$ -IFN procedure. Since  $\gamma$ -IFN production is significantly increased using this method, it may be possible to have a valid test with transportation times exceeding 16 hr. Further work would be required to validate increased time durability.

While injection of the 19698 MpS successfully increased the expressed amounts of  $\gamma$ -IFN, use of this product for skin testing purposes only, would not be advisable. Skin reactions were maximal at 144 hr. Evaluation of skin lesion data suggests that animals designated as positive and suspect both mounted significant skin wheals (34.93 and 23.54 mm) with P values of 0.0018 and 0.0459 respectively. Likewise, MpS-N cattle displayed palpable lesions of 17.48 mm with a P value of 0.0545. The lack of differences between 19698 MpS injected individuals suggests that clear skin lesion determinations between negative and positive may be blurred with the application of this product.

Current skin testing protocols require examination of the injection site at 72-hr post-injection to declare a positive reaction. Very little response was noted at 72 hr compared to  $\gamma$ -IFN levels and skin lesion dimensions seen at 144 hr. By the 216 hr (nine day) check, the  $\gamma$ -IFN and skin lesions had diminished to baseline levels. The delayed response of 144 hr vs. 72 hr is puzzling. This could possibly be explained by the difference in skin testing products; traditional PPD vs. 19698 MpS. A PPD is made from precipitating proteins secreted by *M. paratuberculosis* into the culture filtrate, while a sonicate is a whole cell product which contains intracellular proteins<sup>20</sup>. As these two products differ in bacterial protein types this may account for the delayed 144-hr response, as well as the increased diameter skin lesions seen in cattle injected with 19698 MpS. Given the observed delayed response in DTH activity this observation may call into question evaluating DTH responses at only the 72-hr sampling period. A second period of examination at 144 hr post-injection may be advantageous.

Results of this study indicate that skin testing of  $\gamma$ -IFN positive individuals using a *M. paratuberculosis* cell-free sonicate yielded significantly increased amounts of  $\gamma$ -IFN produced from sensitized lymphocytes. Further research is warranted to determine if the addition of skin testing to the  $\gamma$ -IFN testing method could increase test sensitivity or durability

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