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Spangler, Elizabeth

THE DIAGNOSIS AND EPIDEMIOLOGY OF PARATUBERCULOSIS IN DAIRY CATTLE IN CENTRAL OHIO

The Ohio State University

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THE DIAGNOSIS AND EPIDEMIOLOGY OF PARATUBERCULOSIS IN DAIRY CATTLE
IN CENTRAL OHIO

DISSERTATION

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

By
Elizabeth Spangler, A.B., M.S., D.V.M.

* * * * *

The Ohio State University
1987

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To my teachers
ACKNOWLEDGEMENTS

I owe a debt of gratitude to my advisor, Dr. Larry Heider, for his constant support throughout this project. He has helped me to set my professional goals, and allowed me the flexibility I needed to achieve them. His input into my career goes far beyond this project. Within the scope of the research project, he has alternately supported, challenged and facilitated from start to finish. Dr. Heider is, above all, a facilitator of the possible, and I have benefited from his enthusiasm and support.

It is difficult to put Dr. Steen Bech-Nielsen second in the list of acknowledgements. Dr. Bech-Nielsen has been the principal source of financial and laboratory support on this project. He has been very generous with project funds which facilitated the development of a paratuberculosis laboratory in the Department of Veterinary Preventive Medicine, and which enabled me to travel to NADC in Ames, Iowa, to Penn State University, to the University of Pennsylvania, and twice to Cornell University to exchange ideas with other paratuberculosis research workers. Steen saw to it that we attended meetings and that I received some professional exposure from the onset of the project.
I thank Dr. Dorn for his participation on this project and for his contribution to my graduate career at OSU. Dr. Dorn recognized my interests and ability when I was in practice, and is responsible for bringing me to Ohio from western Washington State. As department chair, he has given me a great deal of freedom to follow my professional interests outside of the dissertation while in graduate school. I learned from working with him that he is an idea man and a thorough but fair critic. His input has helped me to polish my work. Dr. Glen Hoffsis, the fourth member of my committee, has helped me put my research into a clinically relevant perspective. The questions he has raised have stimulated my thinking.

This project, a field study, could not have taken place without the dedication of the people in the field. They include: Dr. Douglas Yoder, Jim Yoder, Myron Yoder, Don King, Dr. Grant Johnson, John and Bonnie Ayars. These private veterinary practitioners and their clients gave generously of their time and made their records available to us. The Ohio Department of Agriculture, including but not limited to Dr. Jay Smith, Myrt Throckmorton, Dr. Sinnha and Brenda, supported us by supplying us with clinical material and laboratory results. Through their enthusiasm we were able to recruit many practitioners into contributing serum samples to the project. Thanks also are due to those veterinarians from all over the state of Ohio who took the time to send us specimens, either through ODA or on their own initiative. Many members of the Food Animal and Ambulatory sections of the Veterinary Teaching Hospital also submitted clinical material. These people included Drs. Paul Haas, Julie Hurley, Garnard Boner, Gary Bowman, John Andreas, Bill Shulaw, Bruce Hull, Mike Rings, Guy St. Jean and Peter...
Constable. DHI Inc. contributed time and financial assistance with record collection. Stuart Johnson, Ron Hovanec and Peter Goldsmith have been exemplary in their support of this project. Tim Friend at Spring Farm Packing Company was kind enough to allow me to follow cull cows to his plant.

I received daily help with sample collection, in the laboratory and at the computer from Tim Woodruff and Laura Huff. Without their help I would not be finished today. Kathy Medley and Dolores Fischer have been generous with both practical and moral support. Dr. Mel Moeschberger and Jim Ashton of the Biometrics Laboratory helped with the statistical analysis. Many of my colleagues in the department have come forth with favors, some extraordinary, at timely moments too numerous to elaborate. They include Drs. Ronald Warner, John Donahoe, Bill Shulaw and Will Hueston.

Finally, I would like to thank my husband, Robert David Macgregor, for his unending support in this and other life endeavors that started as independent projects and finished as a team effort. As always, he was there.
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CHAPTER 1
DIAGNOSIS AND CONTROL OF PARATUBERCULOSIS FROM AN EPIDEMIOLOGIC PERSPECTIVE: AN OVERVIEW

INTRODUCTION

Paratuberculosis is a difficult disease to study. Exposure to the agent, *Mycobacterium paratuberculosis*, occurs months to years before infection becomes apparent, most likely in the perinatal period. Little is known about the prepatent period. Infection does not generally become apparent until the animal reaches adulthood, and then, most infections are subclinical. Thus, the results of changes in disease management will not begin to produce measurable effects for a period of at least two years on the average farm. Furthermore, the use of inefficient diagnostic tests has lengthened this period by their failure to detect infected animals. Consequently, few herds become free of paratuberculosis once infection becomes established, in spite of conscientious efforts to eradicate it. Producers, practicing veterinarians and animal disease regulatory agencies alike are frustrated by this.

1personal communication, Dr. J. Graber, Ohio Department of Agriculture.
Just as we can set realistic goals in herd health programs for mastitis control, we should be concerned with setting goals for control of other endemic infectious diseases such as paratuberculosis. The availability of a sensitive and specific diagnostic test is of great value in achieving these goals.

Traditional control programs for paratuberculosis have relied upon fecal culture, a test which by definition gives no false positive results. Infections detected by cultural methods in culled dairy cattle have been shown to have detrimental effects upon reproductive performance and milk production. Fecal culture positive animals are also shedding organisms into the environment, thus perpetuating the opportunity for exposure of susceptible stock. The fecal-oral route is presently believed to be the major mode of transmission of paratuberculosis (Chiodini et al./1984), although there is much left to be learned about the significance of other routes of transmission. The disadvantages of fecal culture are its low sensitivity and a waiting period of 8 to 16 weeks between testing and test results.

A new diagnostic test for paratuberculosis should compare favorably with old methods not only in terms of sensitivity and specificity, but also in terms of its ability to detect "significant" infections. A significant infection is one which is likely to result in one or more of the following: clinical disease, reduced productivity, shedding of the organism, contamination of the environment, and increased risk of exposure of susceptible animals by any route of disease transmission. Insignificant
cases of paratuberculosis may never have much impact upon the host, or upon disease transmission on the endemically infected farm. To be successful in achieving herd health goals for paratuberculosis control, a new test should detect cases of the disease which are likely to escape from the reservoir. The test should ideally detect infections capable of producing a detrimental effect upon host function, particularly, in the case of food animals, upon production. It is most important that these cases of paratuberculosis be removed from known infected herds.

On the other hand, in eradication programs, in the sale and shipment of livestock, and in uninfected herds, all infections should be considered significant, because of the consequences of introducing paratuberculosis into uninfected herds, and because too little is known about the disease to predict which infections will progress. It is essential under these circumstances to have a test which is more sensitive and/or more rapid than current methods.

**BACKGROUND FOR THE RESEARCH**

The focus of this dissertation has been the investigation of a new diagnostic test for paratuberculosis, the enzyme-linked immunosorbent assay (ELISA) using an affinity purified antigen (Abbas *et al* 1983). The antigen was developed at the University of California at Davis and was provided to the Department of Veterinary Preventive Medicine at the Ohio State University by a commercial laboratory\(^2\) for field investigation. In our laboratory, we characterized the new antigen using polyacrylamide gel

\(^2\)California Animal Laboratory, Hemet, California
chromatography and immunoelectrophoresis (Bech-Nielsen et al. 1985). The antigen was field tested in over 2000 samples from calves, subclinically infected cattle, and clinically suspect cattle. The results of the field investigations are the subject of this dissertation.

THE RESEARCH OBJECTIVES

The primary objective of the field project is to evaluate the ELISA as a diagnostic tool for paratuberculosis and to determine how the use of ELISA would affect existing disease control programs for paratuberculosis. Four specific objectives were identified: 1) to describe the effect of calfhood vaccination for paratuberculosis upon the response of a vaccinated animal to an antibody test such as the ELISA, 2) to evaluate sensitivity and specificity of the ELISA among a population of clinically suspect animals, 3) to evaluate the ELISA in subclinically infected cattle in herds known to be infected with paratuberculosis, and 4) to compare milk production and mastitis prevalence among diseased and healthy cattle as classified by ELISA results.

The dissertation is organized around these four objectives. Each objective is the subject of a chapter that is designed to stand alone. They are preceded by two chapters on methodology. These chapters are included because the diagnosis of paratuberculosis raises some unique issues regarding the true infection status of tested animals. It is very difficult using traditional diagnostic methods to define with complete certainty the infection status of individual animals. Setting up the ELISA and establishing guidelines for transforming a continuous variable, the serum
ELISA response, into a dichotomous one, the positive or negative status of the animal as measured by the ELISA test, is the subject of one of the methodology chapters. The other is a brief discussion of the analytic methodology that was used in the absence of an accurate reference test to evaluate the infection status of the animal. The chapters were written to stand alone for publication; therefore, there may be some redundancy, particularly in the introductions and methods sections of some chapters.
REFERENCES


Chapter 2

VALIDATING NEW DIAGNOSTIC TESTS

INTRODUCTION

Three chapters of this dissertation are concerned with the validation of a new diagnostic test, the enzyme-linked immunosorbent assay (ELISA), for the diagnosis of paratuberculosis. In this chapter, an overview of the methods of validation used in the next three chapters is presented. The purpose in doing so is to establish a vocabulary for discussion and a framework that ties the three chapters together.

The goal of diagnostic testing is to establish, with known probability of error, the correct classification of a patient or subject with regard to the disease or condition of interest. To achieve this goal, the sensitivity and specificity of the test being used and the prevalence of the disease in the group being tested must be known. Methods for evaluating test sensitivity and specificity and estimating prevalence in animal populations have been reviewed (Martin 1977, Martin 1984). A discussion of the methods of test comparison used in this study follows.
DETERMINING SENSITIVITY AND SPECIFICITY

In the best of all possible worlds, the true disease status of a group of subjects is known. The sensitivity and specificity of a new diagnostic test is evaluated by running the test in this group and comparing the results to the "true state of nature" in these subjects (Table 1).

In actuality, the true disease status of the test subjects is often difficult, expensive or laborious to determine. For example, establishing the correct infection status of an animal with regard to paratuberculosis requires necropsy, histopathology and culture of several tissues (Summers 1981).

Most tests, including reference tests that are used to evaluate new diagnostic tests, measure a manifestation of disease rather than a disease process (Gerstman and Cappucci 1986). A textbook example is the use of blood glucose to diagnose diabetes mellitus. Feinstein (Feinstein 1974) calls this "surrogate testing". Other examples of surrogate testing include the use of somatic cells to detect intramammary infection and the use of antibodies to detect infectious diseases such as bovine virus diarrhea (BVD). In both infectious mastitis and BVD, disease can be present when the surrogate is not and disease can be absent when high levels of the surrogate are present.

When an imperfect reference test is used to approximate the true state of nature, the comparison results in relative sensitivity and relative specificity. Measures of relative sensitivity and specificity lead to biased estimates of the efficiency of a new test. If both sensitivity and
specificity of the reference test are low, then relative sensitivity and specificity are not very useful comparisons. If a test of low sensitivity is used as a reference, then relative specificity will be lower than the true specificity of the new test. Conversely, if a test of low specificity is used, the relative sensitivity will be a poor estimator of the true sensitivity. Microbial culture when used as a reference is a classic example of the latter error. For example, it is accepted that fecal culture as a diagnostic test for paratuberculosis is 100% specific, but may only be 50% sensitive. If fecal culture were used to evaluate the perfect diagnostic test for paratuberculosis, one with 100% sensitivity and specificity, the relative specificity of the perfect test would be only 66.7% (Table 2). Relative sensitivity, on the other hand, has some meaning. With paratuberculosis, measures of sensitivity relative to fecal culture have additional merit because of the importance of fecal shedding in the transmission of the disease (Chiodini et al. 1984). For this reason, sensitivity relative to fecal culture was used in this study.

In special cases where either the sensitivity or the specificity of the reference test is 100%, combined testing will result in improved estimates of the other parameter. If the specificity of a diagnostic test is assumed to be 100%, then the serial application of the reference test in a population may enhance its sensitivity. If testing errors are strictly random, then the sensitivity (SE) of the reference test increases as a function of the false negative rate (FN) and number of times that the test is applied:
If the errors are not strictly random, then the sensitivity of the reference test approaches but does not reach this level. However, the result will be an improvement on the use of a single test result to estimate the true disease state of an animal. This approach was used in Chapters 3 and 5, in which the definition of infection status was based on the results of three fecal culture tests.

If the sensitivity and specificity of the reference test are uncertain, then other measures of agreement are more appropriate than relative sensitivity and specificity (Fleiss 1981). The most frequently used measure of agreement is the proportion of specific agreement. The proportion of specific agreement, $p_{0}$, is measured by applying two tests to the same subjects, and calculating the proportion of the observations that fall in the concordant cells of a 2 x 2 table (Table 3). Some degree of agreement is expected by chance alone. The amount of agreement expected randomly, $p_{e}$, can be calculated from the marginals of the 2 x 2 table and compared to the observed agreement. The resulting statistic is called Kappa:

$$K = \frac{(p_{0} - p_{e})}{(1 - p_{e})}$$

Kappa is easy to interpret. When there is complete agreement, Kappa is equal to one. If agreement between two tests is less than the chance agreement, then Kappa is less than one. If observed is greater than the agreement expected by chance, then Kappa is greater than 1. A standard error for Kappa can be calculated and hypothesis tests performed. Kappa and
\( p_0 \) are reported in Chapter 5, where only one cross sectional measurement of infection status was made on each animal.

Maximum likelihood ratios (MLR) are the most sophisticated of the methods used to evaluate diagnostic tests in this study. When a maximum likelihood function is applied to several two by two tables of sample data, the MLR model finds the sensitivities and specificities which have the maximum probability of occurring (Madsen and Moeschberger 1980). The advantage of MLR is that a priori knowledge of the exact sensitivity and specificity of the reference test is not required. However, maximum likelihood ratio models must be used judiciously. They usually have rigorous assumptions and require large sample sizes. In the field study, the maximum likelihood ratio method of Hui and Walter (Hui and Walter 1980), which requires the application of a test to two groups with different disease prevalence, was used. In Chapter 5 the data distribution permitted the use of MLR methods.

GUIDELINES FOR AVOIDING BIAS AND MAKING VALID COMPARISONS BETWEEN DIAGNOSTIC TESTS

There are two major sources of bias in the evaluation of diagnostic tests. All methods of test comparison are biased when two tests that measure the same surrogate are used. The tests evaluated must be biologically independent, or else resulting estimates of sensitivity and specificity will be biased upward. Examples of biologically independent tests are tests which measure serum antibodies, microbial culture, and histopathology. This bias was avoided in this study by using fecal culture as a standard of comparison to serologic tests.
The sensitivity and specificity of tests used must also be constant over the groups if two or more groups are being compared. Differences in specificity are common in populations with different exposure history. For example, in a study of the ELISA for tuberculosis in human subjects using two control groups, a skin test negative control group and a skin test positive control group, the specificity of the ELISA was lower in the control group that was skin test positive (Benjamin and Daniel 1982). The sensitivity of a diagnostic test can also differ from one test group to another. Differences in test sensitivity occur when the prevalence of the test surrogate is different in two groups. For example, the prevalence of the antibody response to paratuberculosis is higher in clinical cases of the disease than in subclinical infections (de Lisle et al 1980). For that reason, evaluation of serologic tests must be performed in well-defined populations. The sample chosen should be representative of the population to which the test is applied, with respect to both cases of the disease and controls. Comparisons between samples should be made with caution, and only if statistical examination of the data fails to reject the hypothesis of no difference between groups in sensitivity and specificity respectively. In this study, bias was avoided by analyzing data from clinically infected cows separately from subclinically infected cows in endemically infected herds, and by combining data from different sources only after T-tests or chi square tests failed to find significant differences in sensitivity and/or specificity between the sources.
SUMMARY

Several methods can be used to validate a new diagnostic test. Methods discussed here are: a) comparison of the new test to the true state of nature, b) comparison to a reference test that approximates the true state of nature, c) an index of agreement between diagnostic tests, d) Kappa, a chance corrected measure of agreement between diagnostic tests and e) maximum likelihood ratios.

The true state of nature with respect to paratuberculosis is difficult to determine. The true disease negative animal is particularly elusive, because as a rule, diagnostic tests for paratuberculosis are not very sensitive, particularly in ruling out infection in apparently healthy animals (Riemann et al. 1984). This poses a problem in the validation of diagnostic tests for paratuberculosis. To overcome this problem, the last four of the five methods for test validation were employed in this study. The concept of relative sensitivity with respect to fecal culture is a useful comparison because of the high specificity of fecal culture, and because of the importance of the fecal shedder in the transmission of paratuberculosis. Relative sensitivity was calculated based on a single observation of test animals and, where the data existed, on observations repeated over time on the same animals. The result of repeated testing using fecal culture as a reference is increased sensitivity of the reference test. Where only a single observation was made on each animal, the proportion of specific agreement and Kappa, the agreement expected beyond random agreement, was calculated. Finally, where the data met the assumptions of the model, a
maximum likelihood ratio technique (Hui and Walter 1980) was used to estimate sensitivity and specificity. The guidelines presented for avoiding bias in comparing groups and combining data were followed. The study designs and methods used to validate the ELISA will be presented in the next three chapters.
Table 1

SENSITIVITY AND SPECIFICITY

<table>
<thead>
<tr>
<th>DISEASE +</th>
<th>DISEASE -</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEST +</td>
<td>A</td>
</tr>
<tr>
<td>TEST -</td>
<td>C</td>
</tr>
<tr>
<td>TOTAL</td>
<td>A+C</td>
</tr>
</tbody>
</table>

SENSITIVITY = A/(A+C)
SPECIFICITY = D/(B+D)

A = NUMBER OF TRUE TEST POSITIVES
B = NUMBER OF FALSE TEST POSITIVES
C = NUMBER OF FALSE TEST NEGATIVES
D = NUMBER OF TRUE TEST NEGATIVES
Table 2.

THE EFFECT OF A REFERENCE TEST OF LOW SENSITIVITY UPON THE ESTIMATE OF RELATIVE SPECIFICITY IN A BETTER TEST - AN EXAMPLE

A. SENSITIVITY AND SPECIFICITY OF THE TESTS RELATIVE TO THE TRUE STATE OF NATURE

<table>
<thead>
<tr>
<th></th>
<th>FECAL CULTURE (FC)</th>
<th>NEW TEST (NT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DISEASE +</td>
<td>FC + 50</td>
<td>NT + 100</td>
</tr>
<tr>
<td>DISEASE -</td>
<td>FC - 50</td>
<td>NT - 0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

SENSITIVITY = 50%
SPECIFICITY = 100%

B. SENSITIVITY AND SPECIFICITY OF THE NEW TEST (NT) RELATIVE TO FECAL CULTURE (FC)

<table>
<thead>
<tr>
<th></th>
<th>FC +</th>
<th>FC -</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT +</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>NT -</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>TOTAL</td>
<td>50</td>
<td>150</td>
</tr>
</tbody>
</table>

RELATIVE SENSITIVITY OF NEW TEST = 100%
RELATIVE SPECIFICITY OF NEW TEST = 66.7%
Table 3.
THE PROPORTION OF SPECIFIC AGREEMENT $p_0$ BETWEEN DIAGNOSTIC TESTS

<table>
<thead>
<tr>
<th>TEST 1</th>
<th>Positive</th>
<th>Negative</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>$a$</td>
<td>$b$</td>
<td>$p_2$</td>
</tr>
<tr>
<td>Negative</td>
<td>$c$</td>
<td>$d$</td>
<td>$q_2$</td>
</tr>
<tr>
<td>TOTAL</td>
<td>$p_1$</td>
<td>$q_1$</td>
<td>1</td>
</tr>
</tbody>
</table>

$p_0 = a + d$

$p_e = (p_1)(p_2) + (q_1)(q_2)$

$a = \text{proportion of positive agreement}$

$b = \text{proportion of test 2 positive/test 1 negative disagreement}$

$c = \text{proportion of test 2 negative/test 1 positive disagreement}$

$d = \text{proportion of negative agreement}$

$p_1 = \text{proportion of test 1 positives}$

$q_1 = \text{proportion of test 1 negatives}$

$p_2 = \text{proportion of test 2 positives}$

$q_2 = \text{proportion of test 2 negatives}$

$p_0 = \text{proportion of specific agreement observed}$

$p_e = \text{proportion of specific agreement expected by chance alone}$
REFERENCES


CHAPTER 3

DIAGNOSIS OF BOVINE PARATUBERCULOSIS USING THE ENZYME-LINKED IMMUNOSORBENT ASSAY OF ANTIBODY

INTRODUCTION

Several different serologic tests have been investigated for the screening and diagnosis of bovine paratuberculosis, including 1) the complement fixation (CF) test (de Lisle et al 1980a, 1980b, Goudswaard et al 1976, Ratnamohan et al 1986) 2) the agar gel immunodiffusion-precipitin (AGID) test (Sherman et al 1984, Goudswaard et al 1976), 3) fluorescent antibody (FA) test (Gilmour and Angus 1976, Abbas et al 1983a), 4) the enzyme-linked immunosorbent assay (ELISA) (Jorgensen and Jensen 1976, Abbas et al 1983b, Yokomizo et al 1983), 5) crossed immunoelectrophoresis (Brooks et al 1983), 6) radioimmunoassay (Worsae 1978), and 7) immunoperoxidase test (Nguyen and Buergelt 1983). The immunodiagnosis of paratuberculosis has been reviewed (Riemann and Abbas 1983, Chiodini et al 1984).

Serologic tests for paratuberculosis give many false positive and false negative reactions. One explanation for the number of false positives may be the use of crude antigen preparations, which cross react with related bacteria. Cross reactions are common in nature. Minden and coworkers
(Minden et al 1972) demonstrated antibodies to sonicated material from gram negative bacteria and nonpathogenic mycobacteria in the serum of healthy humans and rabbits. Inoculation of rabbits with homologous and heterologous bacteria increased the titers of these antibodies. Mycobacteria cross react with many gram positive bacteria such as corynebacteria and nocardia and nonpathogenic mycobacteria such as \textit{M. phlei} (Worthington 1967) that are commonly present in the farm environment. Mycobactin-independent mycobacteria have been isolated from fecal material of bulls that were positive for the CF test for paratuberculosis but had no visible lesions consistent with that diagnosis (Wilks et al 1981). \textit{M. paratuberculosis} can be characterized by at least 44 different protein antigens, possibly three of which are species specific (Gunnarson and Fodstad 1979). Research in immunodiagnosis of paratuberculosis has focused on improving the specificity of diagnostic antigens (Merkal 1961, de Lisle et al 1980a, Abbas et al 1983b).

The availability of newer more sensitive antibody assays coupled with new developments in antigenic specificity have stimulated interest in the serodiagnosis of mycobacterial diseases. The ELISA, developed by Engvall and Perlman (Engvall and Perlman 1972), is sensitive and specific for a number of diseases (Schuurs and Van Weemen 1977). Recently, it has been tried in mycobacterial diseases. It has been used in swine experimentally infected with \textit{M. avium} (Thoen et al 1978). Soluble surface antigens and sonicated whole cell extracts of \textit{M. bovis} (BCG strain) or \textit{M. tuberculosis} have been used in ELISA tests for tuberculosis in humans (Nassau et al
1976, Grange *et al.* 1980, Winters and Cox 1981, Zeiss *et al.* 1982). With one exception (Zeiss *et al.* 1982) there was considerable overlap in the range of responses in the tuberculous and control groups. Benjamin and Daniel (Benjamin and Daniel 1982), using an affinity purified antigen of *M. tuberculosis*, found significant differences in endpoint titer between patients with tuberculosis and controls. Specificity was 95.8% in a skin test negative control group and 79.4% in a skin test positive control group. Patients with nontuberculous mycobacterial infections had intermediate serum titers.

The ELISA has also been investigated for the diagnosis of bovine paratuberculosis. Jorgensen and Jensen used a heat extracted antigen of *M. paratuberculosis* to compare ELISA and CF titers in cows with weight loss and diarrhea (Jorgensen and Jensen 1978). The ELISA test was positive in all cases that were confirmed by bacteriologic culture. The specificity relative to the culture negative group was 60%, which was comparable to the CF test. Yokomizo and coworkers used an aluminum sulfate precipitated antigen of strain 18 *M. paratuberculosis* and an IgG1 specific conjugate in an ELISA test (Yokomizo *et al.* 1983). The ELISA was positive in 19 of 33 sera from fecal culture positive cows, and in 1 of 29 sera from fecal culture negative cows from herds with no history of paratuberculosis. Abbas and colleagues (Abbas *et al.* 1983b) reported on the use of an affinity purified protein extract of strain 18M as an ELISA antigen. When run at a single serum dilution, this assay discriminated between antisera to *M. paratuberculosis* and *Nocardia asteroides, M. fortuitum, M. phlei*, and *M. avium*. ELISA extinction values for *N. asteroides, M. fortuitum, and M. phlei*
antisera were the same as the negative control serum, or about 10% of the positive control. Extinction values for *M. avium* were about 25% of the positive control at the optimum working dilution of the test sera and antigen. When the crude protoplasmic extract was used in the ELISA, all sera reacted. Sensitivity and specificity of the ELISA using the affinity purified antigen relative to fecal culture were 80% and 89% respectively in 104 cows tested.

A practical ELISA test for the diagnosis of paratuberculosis infection in cattle must be easy to interpret, inexpensive to run, and the diagnostic accuracy must be significantly greater than chance. A continuous ELISA response must be transformed into a dichotomous, "yes or no" answer for the clinician. A problem with optimizing the diagnostic sensitivity and specificity of the ELISA at a single serum dilution has been the relatively broad spectrum of response in negative animals from known infected herds (Behymer 1984). The work of Abbas and coworkers (Abbas et al. 1983b) with an affinity purified antigen at a single working dilution has eliminated problems of crossreactivity with *nocardia* and some mycobacteria, and reduced crossreactivity to *M. avium*. When the affinity purified antigen is tested with serum from a cow infected with paratuberculosis, the number of immunoreactive protein bands is small relative to the bands identified with crude protoplasmic extracts of strain 18M or sonicated whole organisms of strain 19686 (Bech-Nielsen et al. 1985). A prospective evaluation of the test seems logical given these findings.
This report is an overview of the findings of a prospective study using the affinity purified protein antigen derived from strain 18M used in an ELISA test. The test was evaluated in three populations: 1) a dairy herd without previous history of paratuberculosis, 2) a group of sera from clinically suspect cattle that had been diagnosed positive for paratuberculosis by fecal culture, and 3) a group of three dairy herds with established paratuberculosis infection. The original testing was done prospectively, hence discrimination values between positive and negative were set on each ELISA plate at the time the plate was run. Estimates of sensitivity and specificity of the ELISA based on these discrimination values are reported elsewhere (Chapters 4 and 5). An advantage of the ELISA method used was that it allowed comparison between plates (de Savigny and Voller 1980), so the data could be pooled for retrospective analysis of sensitivity and specificity. In this report, the method of setting a positive/negative discrimination value is described first. The frequency distributions of the continuous ELISA response pooled over all plates in the separate populations tested are given. Finally, the data were examined retrospectively and the optimization of sensitivity and specificity based on a comparison of all tests run is discussed.

**METHODS**

**Study Sera**

Sera from four groups of cattle were studied: 1) a negative control herd, 2) fecal culture confirmed clinical cases of paratuberculosis, 3) fecal culture positive cows from endemic herds and 4) fecal culture negative cows from
endemic herds. The negative control herd was selected based on two criteria. First, the herd had no history of paratuberculosis over 15 years of veterinary service provided by the OSU Veterinary Hospital. Second, a herd fecal culture test at the time of serum collection was negative. The group of culture confirmed clinical cases was composed of cattle being cultured at the Ohio Department of Agriculture Laboratories or from cases presented to the Ohio State University Veterinary Hospital. Sera were submitted blindly for ELISA testing. Test results on sera from fecal culture positive cows were included in this report.

Three dairy herds with endemic paratuberculosis infection were sampled for fecal culture and ELISA testing. All animals in the herd were tested 3 times at 6 month intervals. Results of the first ELISA test are compared to three fecal culture tests in all animals six months old or older at the first test period. Cattle were classified as positive for paratuberculosis if they were fecal culture positive on either of the first two tests. Cattle were classified as negative for paratuberculosis if they were negative on all three fecal culture tests. These two groups are referred to as fecal culture positive cows from endemic herds and fecal culture negative cows from endemic herds.

Positive and negative reference sera were run on every ELISA plate. Ten positive reference sera came from the OSU Veterinary Hospital. One serum sample was selected to be used as the positive reference serum on all ELISA plates run. Negative reference sera were from two known paratuberculosis negative calves from Sweden (provided by Dr. Bech-Nielsen) and a
gnotobiotic calf. The discrimination value between positive and negative ELISA was defined using 10 positive reference sera from the OSU Veterinary Teaching Hospital and sera from the negative control herd.

**Fecal Culture**

Fecal samples were cultured according to the protocol used by the National Veterinary Services Laboratory (Mycobacteriology Unit, NVSL, 1985) with modifications in the decontamination procedure and in the media (Whipple and Merkal 1984). One gram of fecal material sample was suspended in 15 ml of 0.75% hexadecyl pyridinium chloride and shaken for 30 minutes. After settling for one to two hours, approximately 4 ml of the suspension was drawn up in a disposable transfer pipette that had been soaked for at least 30 minutes in 70% ethanol. The material in the transfer pipette was allowed to sediment overnight before being inoculated directly onto Herrold's egg yolk media. Amphotericin B was not included in the medium. A five tube system was used. Four out of the five tubes contained Mycobactin J. Two of the four tubes containing Mycobactin J also contained sodium pyruvate (0.4%). The control tube contained neither Mycobactin J nor pyruvate. Cultures were read at 1, 2, 3, 8, 12 and 16 weeks post-inoculation. All isolates were checked microscopically for acid fast organisms using the Ziehl-Neelsen technique (Smithwick 1976).
ELISA Serology

An indirect ELISA was used to detect antibodies to *M. paratuberculosis* using an affinity purified antigen derived from strain 18M (Abbas, et al. 1983b). Wells of an enzyme immunoassay microtiter plate\(^2\) were coated with a 1/64 dilution of *M. paratuberculosis* antigen\(^3\) in 50 μl of a carbonate buffer (0.015 M Na\(_2\)CO\(_3\), 0.035 M NaHCO\(_3\), 0.02% Na\(_3\)N, pH 9.6) and incubated overnight at 4° C. Plates were rinsed 3 times with a wash solution containing 0.15 M NaCl and 0.05% Tween-20. Test sera were diluted 1:50 in ELISA buffer (0.15 M NaCl, 0.05 M Tris, 0.1% BSA, 0.05% Tween-20) and 50 μl applied to each well. Plates were incubated for 60 minutes at 37° C, rinsed three times, and 50 μl of a 1:300 dilution of goat antibovine IgG (heavy and light chains) conjugated to horseradish peroxidase\(^4\) in ELISA buffer was added to each well. The plates were incubated for 1 hour and rinsed as before. One hundred microliters of substrate solution (0.06 mM 2,2'-azino-di-3-[ethyl-benzthiazoline sulfonic acid], 50 mM citric acid, 10 mM H\(_2\)O\(_2\), pH 4) was added to each well. Results were recorded as optical density at 405 nm\(^5\) when the positive reference serum reached an optical density of 0.8-1.0. Positive and negative reference sera were run on each plate. Each sample was run in duplicate wells on each plate, and repeated on at least two plates. Results were expressed as a percent of the positive reference serum (de Savigny and

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\(^2\)Linbro/TiterTek, Flow Laboratories, Mclean, VA 21202.

\(^3\)California Animal Laboratory, Hemet, CA.

\(^4\)0.5 mg/ml, Kirkegaard and Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879.

\(^5\)Bio-Tek ELISA Reader, Bio-Tek Instruments, Burlington, VT 05401.
Voller 1980). The term ELISA response, when used in this report, refers to the average optical density of a sample expressed as a percent of the positive reference serum.

RESULTS

One hundred and two cows from the negative control herd were tested. The mean ELISA response expressed as a percent of the positive control serum in the negative control group was 26.3 (s.d. = 16). Ninety percent of the observations fell below 42.3 and 95% of the observations fell below 57.0. The distribution was skewed to the right (Fig. 1).

One hundred and ten sera from fecal culture confirmed clinical cases were tested. The mean ELISA response was 61.1 (s.d. = 34.5). The mean ELISA results for the negative control group and the culture confirmed clinical cases were significantly different (t = 9.55, p < 0.0001). The frequency distribution of confirmed clinical cases is shown in Figure 2, and compared to the negative control group in Figure 3.

Two hundred fifty two sera from cows in endemic herds were tested. The distribution of sera is shown in Figure 4. The overall mean ELISA result for all cows tested was 27.8 (s.d. = 19.4). The mean ELISA response of 161 animals that were fecal culture negative for paratuberculosis was 21.5 (s.d. = 15.3). The distribution of negative animals in endemically infected herds is shown in Figure 5. The mean ELISA response in 31 animals classified as positive to paratuberculosis was 49.4 (s.d. = 29.7). The mean ELISA response of fecal culture positive and fecal culture negative cows
from endemic herds was significantly different (t=5.02, p<0.0001). The frequency distribution of positive cattle from endemic herds is given in Figure 6, and compared to the distribution of negative cattle in endemic herds in Figure 7.

The sensitivity and specificity of the ELISA in the three populations tested are presented in Tables 4 and 5. Distributions of sera from the negative control herd and negative sera from herds with endemic paratuberculosis infection were very similar. The distribution of sera from culture confirmed clinical cases is narrower than the distribution of sera of culture positive animals from endemic herds. This results in lower test sensitivity observed at the same ELISA response level.

**DISCUSSION**

The discrimination value between positive and negative sera for the prospective evaluation of the ELISA was determined by the frequency distribution of the negative control sera (Table 4). An ELISA response of 50% or less of the positive control serum captured between 90 and 95% of the sera from the negative control herd. Since the ELISA frequency distribution of negative cows in other herds was not known, the discrimination value was based on the distribution of ELISA responses on each plate tested, expressed as a percent of the positive control serum. The cutoff between positive and negative was defined as two standard deviations above the mean of the population of values falling below 50% of the positive control serum on each plate. The positive reference sera consistently fell above the cutoff when this procedure was used.
ELISA results are expressed as a percent of the positive control value, this technique offers consistency of results between plates and does not depend upon rigorous quality control of substrate incubation time (de Savigny and Voller 1980). Thus results are comparable over many plates.

Results of this study show that the ELISA test has practical value for the identification of clinical and subclinical paratuberculosis in cattle. The assay is more sensitive in clinically suspect cattle. This is consistent with other studies of antibody response to paratuberculosis (de Lisle et al. 1980a, Riemann and Abbas 1983). Unique to this study is the similarity of distribution of negative sera from infected and uninfected herds (Table 4). There are at least two possible explanations for this. First, the negative control herd was not truly without paratuberculosis. One animal purchased 18 months before the initiation of the study developed clinical signs of paratuberculosis and was fecal culture positive a year after the herd fecal culture test. Since she was fecal culture negative at the time of the herd test, the risk of exposure of herdmates to paratuberculosis was probably minimal at that time. The second explanation is that the background level of crossreacting antibodies is about the same in both groups. Although this antigen is the result of several purification steps, it is still far from pure, and crossreacts to a degree with M avium (Abbas et al. 1983b), and probably other antigens. There is still a need for a more specific antigen for immunologic diagnosis for paratuberculosis.

Finally, the results indicate a broad range of responses in confirmed positive animals. There was considerable overlap with the distribution of
negative sera (Fig. 3 and Fig. 7), although the mean ELISA response was
different, both in the comparison of the negative control herd to confirmed
clinical cases and in the comparison of positive and negative cattle from
endemic herds. Some of the false negatives observed may be due to anergy
or to the spectral nature of the immune response (Bendixen 1978). The
efficiency of the CF and AGID tests is greater in infections that have
advanced to the clinical stage (Ratnamohan et al. 1986, Sherman et al. 1984),
possibly indicating some change in immune responsiveness with stage of
disease. Strain differences within the species \( M. \text{paratuberculosis} \) may
explain some missed infections with the ELISA. The laboratory attenuation
of strain 18M, the strain used in this assay, may have changed it
antigenically, and reduced its sensitivity. Purification may also have
reduced the sensitivity of the assay. Future investigations addressing these
questions will lead to improvements in the ELISA. The results of this study
indicate that it has practical value as a diagnostic test as it is used and
understood today.
Table 4.

Specificity of the ELISA in a Paratuberculosis Negative Herd (GROUP A) and in Fecal Culture Negative Cattle From Herds Endemically Infected with Paratuberculosis (GROUP B) at Different Cutoff Points of the ELISA Response.

<table>
<thead>
<tr>
<th>ELISA*</th>
<th>GROUP A (%)</th>
<th>GROUP B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>7.84</td>
<td>18.63</td>
</tr>
<tr>
<td>20</td>
<td>38.24</td>
<td>55.28</td>
</tr>
<tr>
<td>30</td>
<td>71.57</td>
<td>79.50</td>
</tr>
<tr>
<td>40</td>
<td>89.22</td>
<td>89.44</td>
</tr>
<tr>
<td>50</td>
<td>93.14</td>
<td>93.79</td>
</tr>
<tr>
<td>60</td>
<td>97.06</td>
<td>95.65</td>
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<tr>
<td>70</td>
<td>97.06</td>
<td>98.76</td>
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</tr>
<tr>
<td>90</td>
<td>99.02</td>
<td>100.00</td>
</tr>
<tr>
<td>100</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

n 102 161

1ELISA expressed as a percent of positive control serum
Table 5.

Sensitivity of the ELISA in Two Groups of Cattle, Confirmed Clinical Cases (GROUP C) and Subclinically Culture Positive Cows From Endemically Infected Herds (GROUP D), at Different Cutoff Points of the ELISA Response

<table>
<thead>
<tr>
<th>ELISA*</th>
<th>GROUP C</th>
<th>GROUP D</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>100.00</td>
<td>99.27</td>
</tr>
<tr>
<td>20</td>
<td>80.00</td>
<td>88.18</td>
</tr>
<tr>
<td>30</td>
<td>66.67</td>
<td>81.82</td>
</tr>
<tr>
<td>40</td>
<td>53.33</td>
<td>72.73</td>
</tr>
<tr>
<td>50</td>
<td>43.33</td>
<td>57.27</td>
</tr>
<tr>
<td>60</td>
<td>33.33</td>
<td>46.36</td>
</tr>
<tr>
<td>70</td>
<td>26.67</td>
<td>37.27</td>
</tr>
<tr>
<td>80</td>
<td>23.33</td>
<td>30.00</td>
</tr>
<tr>
<td>90</td>
<td>13.33</td>
<td>20.00</td>
</tr>
<tr>
<td>100</td>
<td>6.67</td>
<td>13.64</td>
</tr>
<tr>
<td>110</td>
<td>0.0</td>
<td>7.27</td>
</tr>
<tr>
<td>120</td>
<td>0.0</td>
<td>6.36</td>
</tr>
<tr>
<td>130</td>
<td>0.0</td>
<td>3.64</td>
</tr>
<tr>
<td>140</td>
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<tr>
<td>150</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

n 102 31

*Elisa expressed as a percent of positive control serum
Figure 1.

DISTRIBUTION OF ELISA OPTICAL DENSITY EXPRESSED AS A PERCENT OF POSITIVE REFERENCE SERUM OPTICAL DENSITY (% POS) OF SERA FROM 102 COWS IN A PARATUBERCULOSIS NEGATIVE HERD.
Figure 2.

DISTRIBUTION OF ELISA OPTICAL DENSITY EXPRESSED AS A PERCENT OF POSITIVE REFERENCE SERUM OPTICAL DENSITY (% POS) OF SERA FROM 110 FECAL CULTURE CONFIRMED CLINICAL CASES OF PARATUBERCULOSIS.
Figure 3.

COMPARATIVE DISTRIBUTION OF SERUM ELISA OPTICAL DENSITY EXPRESSED AS A PERCENT OF POSITIVE REFERENCE SERUM OPTICAL DENSITY (% POS) IN 102 COWS IN A PARATUBERCULOSIS NEGATIVE HERD (NEG CONTROLS) AND FECAL CULTURE CONFIRMED CLINICAL CASES (FC+ CLINICALS).
Figure 4.

DISTRIBUTION OF ELISA OPTICAL DENSITY EXPRESSED AS A PERCENT OF POSITIVE REFERENCE SERUM OPTICAL DENSITY (% POS) OF SERA FROM 252 COWS IN 3 ENDEMIC HERDS.
Figure 5.

DISTRIBUTION OF ELISA OPTICAL DENSITY EXPRESSED AS A PERCENT OF POSITIVE REFERENCE SERUM OPTICAL DENSITY (% POS) OF SERA FROM 161 COWS NEGATIVE TO SERIAL FECAL CULTURE IN 3 ENDEMIC HERDS.
DISTRIBUTION OF ELISA OPTICAL DENSITY EXPRESSED AS A PERCENT OF POSITIVE REFERENCE SERUM OPTICAL DENSITY (% POS) OF SERA FROM 31 FECAL CULTURE POSITIVE COWS IN 3 ENDEMIC HERDS.
Figure 7.

COMPARATIVE DISTRIBUTION OF SERUM ELISA OPTICAL DENSITY EXPRESSED AS A PERCENT OF POSITIVE REFERENCE SERUM OPTICAL DENSITY (% POS) IN 31 FECAL CULTURE POSITIVE COWS (FC POS) AND 161 FECAL CULTURE NEGATIVE COWS (FC NEG) IN 3 ENDEMIC HERDS.
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Chapter 4
USE OF SEROLOGIC TESTS TO DIAGNOSE PARATUBERCULOSIS INFECTION IN CLINICALLY SUSPECT CATTLE

INTRODUCTION

Reasons for performing diagnostic tests for paratuberculosis are: a) to establish estimates of prevalence of infection; b) to identify diseased animals for removal in the implementation of control programs on farms; c) to identify the "index case" on farms where paratuberculosis has never been diagnosed previously; d) to certify animals free of disease for sale, purchase or export and e) to establish herd negative status which may be required for sale or export of animals from the herd. Tests with high specificity are required in culling programs where low prevalence exists. The economic loss of culling healthy animals based on false positive diagnostic test results may be greater than the cost of lost production without a culling program. Tests with high specificity are also useful in confirming clinical diagnoses. On the other hand, tests of high sensitivity are required to be confident that an animal is free of disease. The consequences of using a test of moderate or low sensitivity in control or eradication programs go far beyond the loss of an individual animal and could involve damaged reputation or professional liability for such "errors" in diagnosis. In many instances, one test is used to perform all the functions
listed above. Its use is met with variable levels of satisfaction by veterinarians, regulatory officials and producers, depending upon the need or reason for testing.

Fecal culture has been accepted as the most suitable test for detecting infection in apparently healthy cattle in known infected herds (Merkal 1973, Chiodini et al. 1984, Thoen and Muscoplat 1979). A recent survey showed that 24 states have laboratory facilities for the culture of *Mycobacterium paratuberculosis* (Anonymous 1986). The optimum test for other purposes has not been determined. Fecal culture lacks sensitivity and culture results cannot be determined for 8-16 weeks. These two characteristics of fecal culture are serious deficiencies. Serologic tests offer a practical alternative to fecal culture for use by state diagnostic laboratories and other institutions involved in testing large numbers of animals for paratuberculosis. One benefit of a serologic test is more rapid availability of results. Another potential benefit of serologic tests is a decrease in the proportion of false negative results and, hence, more reliable disease screening, as in certifying animals free of disease for sale, import or export.

Many serologic tests have been evaluated for the diagnosis of paratuberculosis. The efficiency of these tests is variable because of both false positive and false negative reactions. This is evident from Table 6, which summarizes findings on the sensitivity and specificity of four immunoserologic tests: the complement fixation (CF) test, the fluorescent

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1Dr. Jay Graber, Ohio Department of Agriculture, personal communication
antibody (FA) test, the agar gel immunodiffusion (AGID) test, and the enzyme-linked immunosorbent assay (ELISA) for paratuberculosis. Some of the variability observed is due to the nature of the different techniques. For example, within the same laboratory, using the same sera and the same test antigen, the ELISA appears to be more sensitive than the AGID (Yokomizo et al/1983) or the CF test (Jorgensen and Jensen 1978). The AGID test is the least sensitive assay, but the most specific in those studies evaluating two or more assays (Buergelt et al/1977, Yokomizo et al/1983).

There is also considerable variation in sensitivity and specificity within the same test (Table 6). For example, the sensitivity of the CF test ranges from 19% (Buergelt et al 1977) to 83% (Jorgensen and Jensen 1978). The sensitivity reported for the AGID test ranges from 21% (Buergelt et al/1977) to 97% (Sherman et al/1984). Different conditions under which the tests are compared are capable of producing systematic biases, the impacts of which are difficult to assess. Some of the sources of bias are: the population from which the sera were collected to evaluate the test, the antigen preparation used, and the standard reference test to the state of health or disease used.

The population of animals chosen to evaluate the test affects the sensitivity and specificity observed when the relationship between the attribute of disease being measured and the disease is not stable from one population to the next (Martin 1977). In serologic tests, the attribute being measured is serum antibody. The immune response to infection with \textit{M. paratuberculosis} is spectral in nature, with a progression from cell-
mediated to humoral immunity (Bendixen 1978). A measurable antibody response begins relatively late in infection. The antibody response as measured by CF titers has been correlated with the degree of fecal shedding in naturally exposed cattle (de Lisle et al. 1980a). Cattle showing clinical signs are more likely to be shedding organisms in the feces (Merkal et al. 1968b, Hole 1958). Therefore, a serologic test may appear to be more sensitive when evaluated in a population of animals showing clinical signs or when fecal culture is chosen as the reference test. No study has used two different populations or different reference tests in the same population, but the trend is evident in different studies using the same test. The highest sensitivities for the CF test (Jorgensen and Jensen 1978, Ratnamohan et al. 1986) the AGID test (Sherman et al. 1984) and the ELISA (Jorgensen and Jensen 1978), were evaluated in clinically suspect cattle (Table 6).

The antigen preparation used may also affect test results, particularly the specificity. The variation in specificity observed with serologic tests may be explained by the use of heterogenous antigen preparations containing a number of crossreacting antigens. Mycobacterial antigens in general are complex and crossreact with many other organisms (Daniel and Janicki 1978). Crude preparations of M. paratuberculosis cross react with M. avium, M. phlei and other saprophytic mycobacteria, and with gram positive bacteria including corynebacteria and nocardia (Yokomizo et al. 1983). The use of purer reagents should improve specificity. The use of an affinity purified antigen in the ELISA for paratuberculosis improved the specificity
of the assay from 59% (with the crude antigen) to 89% (with the affinity purified antigen) in one study (Abbas et al/1983b).

The reference test used to estimate the true disease status of individual animals may also affect the apparent sensitivity and specificity of a diagnostic test. Use of a standard of comparison that is not an accurate reflection of the disease status of the target population results in information about relative sensitivity and specificity at best (Fleiss 1981). At worst, the new test being compared may be discarded on the merits of an inferior test. Use of a standard with low sensitivity leads to underestimation of the specificity of the new test (Greenberg and Jekel 1969). This may be a problem with evaluation of diagnostic tests for paratuberculosis because fecal culture is frequently used as the reference test (see Table 6). Fecal culture misclassifies as many as 50% of known infected animals (Hutchinson et al/1986, Buergelt et al/1977, Merkal 1970). Use of fecal culture as a standard may also affect sensitivity. Merkal and others (Merkal 1984, Julian 1975) have postulated that the onset of a detectable humoral immune response is coincident with the onset of fecal shedding. Therefore, tests evaluated on fecal culture alone may have high relative sensitivity but may in fact fail to detect a high proportion of infections that can be detected by other methods. The most reliable reference test for paratuberculosis is necropsy with histopathology and culture of gut tissues and associated lymph nodes. Necropsy with impression smears of the same tissues is a less sensitive measure of infection status than histopathology (Summers 1981), although it is occasionally used (Larsen et al/1963, Ratnamohan et al/1986). The
difference in reference tests used may explain why the sensitivity of the AGID test using necropsy and histopathology results as a reference test (Buergelt et al/1977) was 21%, while the sensitivity of the AGID relative to fecal culture alone was 45% (Yokomizo et al/1983).

Necropsy with complete histopathology and tissue culture may be a more reliable "gold standard" than a single fecal culture, but it is laborious and expensive. With fecal culture the disease status of negative cattle is in doubt. It is particularly impractical to slaughter and necropsy the nonreactors in a clinical trial. When evaluating efficiency of tests for paratuberculosis, other measures of test validation must be sought. The proportion of observed agreement between tests is one such measure (Table 7). The Kappa statistic is useful to see if the observed agreement between two diagnostic tests is in excess of the agreement expected by chance alone (Fleiss 1981). An advantage of Kappa is that it can be interpreted even in the absence of reliable estimates of sensitivity and specificity. If the observed level of agreement is significantly greater than chance, then the tests probably measure the same thing, and further validation of either or both of the tests is justified. If agreement is not in excess of chance, then reasons for this can be sought in a systematic manner.

Maximum likelihood ratio estimates for sensitivity and specificity have been proposed for evaluating tests in the event that there is no definitive way to establish the true health status of the animal (Quade et al/ 1980, Hui and Walter 1980). These work well as long as the assumptions of the model are met, but if biologically related tests are used (such as two tests that
measure serum antibody), then maximum likelihood estimates are biased
(Dohoo 1981). Other methods for measuring agreement between tests have
been reviewed elsewhere (Martin 1977, Martin 1984).

Two serologic test have recently been introduced for testing cattle for
paratuberculosis. The agar gel immunodiffusion test (AGID), using a crude
protein extract of *M. paratuberculosis* strain 18M, was developed in goats
(Sherman and Gezon 1980). The test was evaluated in a sample of 51 cattle
with chronic weight loss and diarrhea. The positive predictive value was
96.9%, but an unbiased estimate of sensitivity was not determined because
AGID positive cattle were followed more aggressively than AGID negative
cattle (Sherman et al 1984). Relative to fecal culture, the AGID test
sensitivity was 97% (27/28) and specificity was 81% (17/21). This test
looked very promising in this group of clinically suspect animals, and should
be evaluated prospectively in a larger sample.

The ELISA using an affinity purified protein antigen of strain 18M (Abbas et
a/1983a) has not been evaluated in a group of clinically suspect cattle, but
results in a mixed group of cattle from endemic herds look promising. The
potential of either of these tests for augmenting or replacing fecal culture
in the diagnosis of paratuberculosis needs to be investigated further. The
purpose of this study is to compare the two serologic tests, the AGID and
the ELISA, in the diagnosis of paratuberculosis in a sample of sera from
clinically suspect cattle. Sensitivity and specificity of each test relative
to fecal culture, the proportion of specific agreement, and Kappa, the
proportion of agreement beyond the agreement expected by chance, are determined for the two tests.

MATERIALS AND METHODS

Sample Selection

A total of 162 samples was cultured and tested serologically using AGID and ELISA for paratuberculosis. Sera came from two sources. Fifty-nine samples were submitted by clinicians at the Ohio State University (OSU) Veterinary Hospital requesting serology for paratuberculosis on clinically suspect cattle. Fecal samples were submitted with serum. The remaining 103 samples were obtained from private practitioners submitting fecal samples for culture to the Ohio Department of Agriculture Diagnostic Laboratory at Reynoldsburg (ODA). If fecal culture results were positive for *M. paratuberculosis*, a letter requesting serum samples was mailed out with the laboratory results to the practitioner who submitted the positive fecal. After practitioners became aware of the serologic testing program, serum samples were received simultaneously with fecal submissions.

Fecal Culture

Samples submitted to the Ohio Department of Agriculture Laboratory were cultured according to the protocol used by the National Veterinary Services Laboratory (Mycobacteriology Unit, NVSL 1985). A four tube system consisting of three tubes of Herrold's egg yolk medium enriched with Mycobactin J² for the isolation of *M. paratuberculosis* and one control tube

²Allied Labs, Inc., Ames IA.
of Herrold's without Mycobactin J was used. Samples submitted through the Ohio State University (OSU) Veterinary Hospital were cultured at OSU with a modified technique using 0.75% hexadecyl pyridinium chloride in the decontamination step (Whipple and Merkal 1983, Merkal 1984). Amphotericin B was not included in the medium. A five tube system was used. Four out of the five tubes contained Mycobactin J. Two of five tubes contained media to which sodium pyruvate (0.4%) had been added, and two tubes with Mycobactin J plus the control tube contained no pyruvate. Cultures in both labs were read at 1, 2, 3, 8, 12 and 16 weeks post-inoculation. All isolates were checked microscopically for acid fast organisms using the Ziehl-Neelsen technique (Smithwick 1976).

**ELISA Serology**

An indirect ELISA was used to detect antibodies to *M. paratuberculosis* using a partially purified antigen derived from strain 18M (Abbas et al. 1983b). Wells of an enzyme immunoassay microtiter plate were coated with a 1/64 dilution of *M. paratuberculosis* antigen in 50 μl of a carbonate buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, 0.02% NaN₃, pH 9.6) and incubated overnight at 4° C. Plates were rinsed 3 times with a wash solution containing 0.15 M NaCl and 0.05% Tween-20. Test sera were diluted 1:50 in ELISA buffer (0.15 M NaCl, 0.05 M Tris, 0.1% BSA, 0.05% Tween-20) and 50 μl applied to each well. Plates were incubated for 60 minutes at 37° C, rinsed three times, and 50 μl of a 1:300 dilution of goat

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3Linbro/Titerlek, Flow Laboratories, Mclean, VA 21202
4California Animal Laboratory, Hemet, CA
antibovine IgG (heavy and light chains) conjugated to horseradish peroxidase\textsuperscript{5} in ELISA buffer was added to each well. The plates were incubated for 1 hour and rinsed as before. One hundred microliters of substrate solution (0.06 mM 2,2'-azino-di-3-[ethyl-benzthiazoline sulfonic acid], 50 mM citric acid, 10 mM H\textsubscript{2}O\textsubscript{2}, pH 4) was added to each well. Results were recorded as optical density at 405 nm\textsuperscript{6} when the positive reference serum reached an optical density of 0.8-1.0. Positive and negative reference sera were run on each plate. Results were expressed as a percent of the positive control. This method of expressing results offers the advantage of being easy to quantify and understand, and diminishes the need for rigorous quality control of substrate incubation time since, within limits, ratios remain constant throughout substrate incubation time. In addition, sera need not be run at more than one dilution to achieve interpretable results (de Savigny and Voller 1980).

Samples were run in duplicate wells on each plate, and each sample was run on at least two plates. Thus each sample was run in at least four wells and on at least two plates. Samples were run in the order in which they were logged into the laboratory. The discrimination value between positive and negative was set on each plate by comparing the sample with the distribution of other samples on the same plate. Other samples consisted of sera submitted in a herd testing program being conducted in known infected

\textsuperscript{5} 0.5 mg/ml, Kirkegaard and Perry Laboratories, Inc., 2 Cessna Court, Gaithersburg, MD 20879.
\textsuperscript{6} Bio-Tek ELISA Reader, Bio-Tek Instruments, Burlington, VT 05401.
herds. Behymer (Behymer 1984) found that when a partially purified antigen (Abbas et al. 1983b) is used for ELISA testing in herds with a history of exposure to paratuberculosis, a frequency distribution of samples on the plate falls into two groups representing infected and uninfected cows. Frequency plots of known infected herds in our research demonstrated two overlapping groups of sera, with a division at about 50% of the positive control. The distribution of the low group was used to set the discrimination level between positive and negative sera. The population of low values was defined as any well reading less than 50% of the positive control serum. The cutoff value for distinguishing positive and negative results on an individual plate was set at two standard deviations above the mean of the population of low values on that plate.

**AGID Serology**

Agarose gels were prepared according to the method of Sherman (Sherman et al. 1984), using a crude protoplasmic extract of strain 18 \textit{M. paratuberculosis}7. Results were read at 24 and 48 hours after incubation at room temperature. Results were recorded as positive if there was at least one clearly defined line of precipitation at either reading. A negative result was recorded if no lines of precipitation were observed.

**Data Analysis**

Sensitivity and specificity percentiles of the AGID and ELISA results relative to fecal culture were calculated on Ohio Department of Agriculture.

7Allied Labs, Inc., Ames, IA
(ODA) and OSU Veterinary Hospital samples separately and combined. Relative sensitivity of AGID and ELISA tests performed on ODA samples and on OSU samples were compared using Chi Square. Kappa was calculated on the index of agreement for the pooled data between fecal culture and ELISA, between fecal culture and AGID, and between ELISA and AGID (Fleiss 1981). The index of agreement used was the overall proportion of agreement, \( p_o = a + d \), or the sum of the proportions of the sample that fall in the concordant cells of a two by two table (Table 7). If all the results agree under the two ratings being compared, then \( p_o \) assumes a maximum value of unity. Kappa is defined as the ratio of the excess observed agreement beyond chance to the maximum possible agreement beyond chance alone:

\[
\text{Kappa} = \frac{(p_o - p_e)}{(1 - p_e)}.
\]

RESULTS

There were 108 fecal culture positive samples (66.7%) among the 162 samples tested. Sixty-one of 103 samples (59.2%) from ODA were fecal culture positive and 47 out of 59 submissions (79.7%) from the OSU Veterinary Hospital were positive to fecal culture (Tables 8, 9 and 10).

Over the 18 month sample collection period, the Ohio Department of Agriculture cultured 1182 diagnostic submissions for paratuberculosis. \( M. \) paratuberculosis was isolated from 32.5% of these samples. Serum samples were available for 103 fecal samples (8.7%) of the total samples submitted for culture for \( M. \) paratuberculosis. Serum was received with
15.8% (61/381) of the fecal samples that were culture positive for *M. paratuberculosis* and 5.2% (42/801) of the samples that were culture negative.

Sensitivity of the ELISA relative to fecal culture (relative sensitivity) was 79% in the ODA submissions, 87% among the submissions from the OSU Veterinary Hospital, and 82% overall. Specificities relative to fecal culture (relative specificity) in the two groups was 69% and 58% respectively. Differences in sensitivity between the two groups were not statistically significant (Chi Square = 0.813, p = 0.367). When the two groups were combined, the pooled relative sensitivity was 82% and the pooled relative specificity was 66.7%.

Sensitivity of the AGID test relative to fecal culture was 50.8% in the ODA submissions, and 55.3% among the submissions from the OSU Veterinary Hospital. Relative specificities were 92.9% and 91.7% respectively. Differences in sensitivity between the group of samples from ODA and the group from OSU were not statistically significant (Chi Square = 0.073, p = 0.787). Relative sensitivity of the AGID test in the pooled sample was 52.8%. Relative specificity was 92.6% in the pooled sample.

Kappa, the measure of agreement beyond chance between two raters, was calculated for fecal culture vs. ELISA, fecal culture vs AGID, and ELISA vs. AGID over all samples combined. When fecal culture and ELISA results were compared, the proportion \( p_0 \) of samples that was classified the same way by both tests was 0.7716, the proportion \( p_0 \) expected by chance alone was 0.5535, and Kappa was 0.4885, or significantly greater than the agreement
expected by chance alone (p<.001). When fecal culture and AGID were compared, $p_0$ was 0.6605, $p_e$ was 0.4589, and Kappa was 0.3726. Kappa was significantly greater than the agreement expected by chance alone (p<.001). Kappa for ELISA vs AGID was 0.3365, based on a $p_0$ of 0.6420 and a $p_e$ of 0.4604. The observed agreement was also significantly greater than expected (p<.001).

**DISCUSSION**

In this study samples were collected from two different sources and combined for analysis. The primary justification for doing so is that samples from the OSU Veterinary Hospital, while representing a specialty practice, would have been submitted to the Ohio Department of Agriculture Diagnostic Laboratory had an in-house culture service not been available. Culture techniques in the two laboratories were different and could have contributed to differences in relative sensitivity and specificity observed, although the differences between the two laboratories were not statistically significant.

Two thirds of the ODA sample was comprised of cases that remained on farms for up to 16 weeks after paratuberculosis was initially included in the differential diagnosis. The ODA sample may therefore overrepresent mild and subclinical cases in contrast to advanced clinical cases. The former may also be harder to classify correctly by serologic tests. There may be better agreement between serologic test results and fecal culture in more advanced cases of paratuberculosis. Finally, it should be remembered that in the present study, differences in sensitivity of the order of
magnitude observed between ODA and OSU samples could have been explained by chance alone.

In this study, there was no readily available reference of comparison to the true state of nature with regard to infection with *M. paratuberculosis*. Estimates of sensitivity and specificity of diagnostic tests for paratuberculosis relative to fecal culture are of interest because animals that are fecal culture positive are also shedding organisms into the environment. The most important route of transmission of paratuberculosis is reported to be horizontal through the contact of young animals with contaminated feed and water (Julian 1975). A rapid serologic test that correlates well with the shedding state may be an advantage in a culling program. However, this does not replace the need for a test which can accurately identify all animals infected with paratuberculosis, regardless of fecal culture status. Further investigations should examine the disease status of fecal culture negative cows more thoroughly either directly through necropsy or indirectly using maximum likelihood ratio estimates.

In this study, sensitivity and specificity relative to fecal culture were 82.4% and 66.7% for the ELISA. For the AGID test, relative sensitivity was 52.8% and relative specificity was 92.6% overall.

Specificity relative to fecal culture is not a very valuable measure because of misclassification errors. Kappa, a descriptive statistic on the index of agreement between ELISA and fecal culture, was 0.4885, and was significantly greater than the agreement expected by chance. Kappa in
excess of 0.4 indicates a substantial degree of reliability between two independent raters, and suggests that the ELISA could be used independently of fecal culture to measure the same thing. Further work is needed to define the ELISA relative to infection status, but work reported here and elsewhere (Abbas et al/1983b) supports the use of ELISA as a practical and rapid screening test for paratuberculosis.

The AGID test has been evaluated previously in a small sample of cattle with chronic weight loss and/or chronic diarrhea (Sherman et al/1984). Sensitivity relative to fecal culture was 97% in 28 fecal culture positive animals. This is significantly higher than the pooled sensitivity of 52.8% reported in this study (Chi Square = 16.8, p < .01). Earlier workers using different techniques have reported lower sensitivity and specificity for gel precipitation tests (Buergelt et al/1977, Goudswaard et al/1976, Merkal et al/1968b; see Table 6). In experimentally infected sheep, measurable precipitating antibody response increased in intensity and number of precipitin bands with increasing severity of infection as documented by post mortem lesions (Merkal et al/1968a). Since the same technique was used in this and Sherman's study, the differences are more likely to be due to differences in severity of the clinical disease in the two samples than to differences in laboratory methods.

Kappa was somewhat lower for the agreement between AGID and fecal culture than between fecal culture and ELISA. This is a reflection of the lower sensitivity of the AGID test. The AGID test has a higher relative specificity than the ELISA. Thus, there is a correlation between the absence
of a measurable precipitin response and the absence of detectable shedding. Because it is conservative, the AGID test offers a reasonably specific and rapid test for ruling in a diagnosis of paratuberculosis. A diagnosis of paratuberculosis cannot be ruled out on the basis of a negative AGID test alone, because of its low sensitivity.

Kappa was used in combination with measures of relative sensitivity and specificity to evaluate use of serologic tests in a clinical diagnostic setting. In three comparisons, ELISA vs. fecal culture, AGID vs. fecal culture and ELISA vs. AGID, the agreement between tests was significantly beyond the agreement expected by chance alone. Agreement between ELISA and fecal culture was greater than the agreement between AGID and fecal culture or between AGID and ELISA. Differences in agreement are probably explained by differences in relative sensitivity between the two tests. Either of the two serologic tests could be used alone or in conjunction with fecal culture to diagnose paratuberculosis. The ELISA is appropriate where sensitivity and negative predictive value are important, such as in screening for paratuberculosis or in certifying animals for sale or movement. The AGID test is appropriate where specificity and positive predictive value are important, as in establishing a rapid clinical diagnosis in a valuable animal. The use of fecal culture as an adjunct test would enhance accuracy in both situations. Crossreactivity to \textit{M. avium} can be diminished by careful adjustment of the assay (Abbas \textit{et al} 1983b), but the antigen and serum concentrations that optimize the assay have very narrow tolerances. The ELISA specificity may be enhanced by running an antigen control well for \textit{M. avium} with each serum tested. The results could be expressed as a ratio
and a infection status determined from a grid, just as is done in comparative cervical intradermal testing for tuberculosis in cattle.
Table 6

A REVIEW OF IMMUNODIAGNOSTIC METHODS EVALUATED FOR USE IN THE DIAGNOSIS OF PARATUBERCULOSIS

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>POP'N</th>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
<th>ANTIGEN</th>
<th>STANDARD</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF</td>
<td>C, SC</td>
<td>59%(23/39)</td>
<td>35%(42/65)</td>
<td>MP Str 18</td>
<td>N, S</td>
<td>Larsen <em>et al</em> 1963</td>
</tr>
<tr>
<td>CF</td>
<td>C</td>
<td>46%(12/26)</td>
<td>76%(70/92)</td>
<td>MP</td>
<td>N, HP, C</td>
<td>Gilmour and Angus 1976</td>
</tr>
<tr>
<td>CF</td>
<td>C, SC</td>
<td>19% (8/42)</td>
<td>88% (15/17)</td>
<td>MP</td>
<td>N, HP, C</td>
<td>Buergelt <em>et al</em> 1977</td>
</tr>
<tr>
<td>CF</td>
<td>C</td>
<td>83% (25/30)</td>
<td>90% (9/10)</td>
<td>MP</td>
<td>FC</td>
<td>Jorgensen and Jensen 1978</td>
</tr>
<tr>
<td>CF</td>
<td>C, SC</td>
<td>67% (20/30)</td>
<td>99% (420/423)</td>
<td>MP CHO Ag</td>
<td>FC</td>
<td>de Lisle <em>et al</em> 1980b</td>
</tr>
<tr>
<td>CF</td>
<td>C, SC</td>
<td>57% (20/35)</td>
<td>57% (25/44)</td>
<td>MP Promise</td>
<td>FC (2x)</td>
<td>Abbas <em>et al</em> 1983a</td>
</tr>
<tr>
<td>CF</td>
<td>C</td>
<td>74% (128/172)</td>
<td>92% (84/91)</td>
<td>M AVIUM</td>
<td>N, S</td>
<td>Ratnamohan <em>et al</em> 1986</td>
</tr>
<tr>
<td>FA</td>
<td>C</td>
<td>62% (16/26)</td>
<td>92% (85/92)</td>
<td>MP</td>
<td>N, HP, C</td>
<td>Gilmour and Angus 1976</td>
</tr>
<tr>
<td>FA</td>
<td>C, SC</td>
<td>62% (22/35)</td>
<td>50% (22/44)</td>
<td>MP Promise</td>
<td>FC (2x)</td>
<td>Abbas <em>et al</em> 1983a</td>
</tr>
<tr>
<td>AGID</td>
<td>S, SC</td>
<td>21% (9/42)</td>
<td>100% (17/17)</td>
<td>MP</td>
<td>N, HP, C</td>
<td>Buergelt <em>et al</em> 1977</td>
</tr>
<tr>
<td>AGID</td>
<td>C, SC</td>
<td>45% (15/33)</td>
<td>100% (29/29)</td>
<td>MP Str 18</td>
<td>FC</td>
<td>Yokomizo <em>et al</em> 1983</td>
</tr>
<tr>
<td>AGID</td>
<td>C</td>
<td>97% (27/28)</td>
<td>81% (17/21)</td>
<td>MP Str 18</td>
<td>FC</td>
<td>Sherman <em>et al</em> 1984</td>
</tr>
</tbody>
</table>

1 CF=complement fixation, FA=fluorescent antibody, AGID=agar gel immunodiffusion, ELISA=enzyme-linked immunosorbent assay.
2 POP'N=population sampled, C=clinically suspect cattle, SC=subclinically infected cattle.
3 MP=M. paratuberculosis, M AVIUM=M. avium. Strains (Str 18, Promise) when reported follow species names. CHO Ag=carbohydrate antigen, crude=crude extract, ap=affinity purified extract.
4 STANDARD(reference test used. N=necropsy, HP=histopathology, C=tissue culture, FC=fecal culture, S=impression smears.)
Table 6, continued

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>POP'N</th>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
<th>ANTIGEN</th>
<th>STANDARD</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA C</td>
<td>100%(30/30)</td>
<td>60%(6/10)</td>
<td>MP</td>
<td>FC</td>
<td>Jorgensen and Jensen 1978</td>
<td></td>
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<tr>
<td>ELISA C, SC</td>
<td>56%(19/33)</td>
<td>97%(28/29)</td>
<td>MP 18M</td>
<td>FC</td>
<td>Yokomizo et al/1983</td>
<td></td>
</tr>
<tr>
<td>ELISA C, SC</td>
<td>62%(37/60)</td>
<td>59%(26/44)</td>
<td>MP 18M crude FC(2x)</td>
<td>Abbass et al/1983b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA C, SC</td>
<td>83%(50/60)</td>
<td>89%(39/44)</td>
<td>MP 18M ap</td>
<td>FC(2x)</td>
<td>Abbass et al/1983b</td>
<td></td>
</tr>
</tbody>
</table>

1^CF=complement fixation, FA=fluorescent antibody, AGID=agar gel immunodiffusion, ELISA=enzyme-linked immunosorbent assay.
2^POP'N=population sampled, C=clinically suspect cattle, SC=subclinically infected cattle.
3^MP=M paratuberculosis, M AVIUM=M avium. Strains (Str 18, Promise) when reported follow species names. CHO Ag=carbohydrate antigen, crude=crude extract, ap=affinity purified extract.
4^STANDARD=reference test used. N=necropsy, HP=histopathology, C=tissue culture, FC=fecal culture, S=impression smears.
Table 7.

Format for Measuring Observed Agreement Between Two Tests

<table>
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<tr>
<th></th>
<th>TEST 1</th>
<th></th>
<th>TEST 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>TOTAL</td>
</tr>
<tr>
<td>Positive</td>
<td>a</td>
<td>b</td>
<td>p₁</td>
</tr>
<tr>
<td>Negative</td>
<td>c</td>
<td>d</td>
<td>q₁</td>
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<tr>
<td>TOTAL</td>
<td>p₂</td>
<td>q₂</td>
<td>1</td>
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</table>

Table 8.

Results of Tests Performed on Submissions to the Ohio Department of Agriculture

<table>
<thead>
<tr>
<th>n</th>
<th>Fecal Culture</th>
<th>ELISA</th>
<th>AGID</th>
</tr>
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<tr>
<td>28</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>+</td>
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<tr>
<td>1</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

| Total Positive/ | 61 | 61 | 34 |
| Total Tested    | 103| 103| 103 |
Table 9.

Results of Tests Performed on OSU Veterinary Hospital Submissions

<table>
<thead>
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Table 10.

Laboratory Results for All Diagnostic Submissions

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</tr>
<tr>
<td>35</td>
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<td>-</td>
</tr>
</tbody>
</table>

Total Positive 108 107 61
Total Tested 162 162 162
REFERENCES


CHAPTER 5

USE OF SEROLOGIC TESTS FOR THE DIAGNOSIS OF PARATUBERCULOSIS IN
SUBCLINICALLY INFECTED CATTLE FROM KNOWN INFECTED HERDS

INTRODUCTION

Traditional approaches to the control of paratuberculosis in known infected herds include a) the culling of subclinically infected animals, b) the prevention of exposure of susceptible stock by environmental management and, sometimes, c) calfhood vaccination. Subclinically infected animals in known infected herds are usually identified by the repeated application of a single diagnostic method, fecal culture. The primary advantage of using fecal culture to screen for subclinical infection is the virtual absence of false positive results. While the sensitivity increases each time the test is used on the same group of animals, there is no corresponding decrease in specificity, because the false positive rate is zero. Another advantage of fecal culture is that it selectively eliminates animals that are contributing to the spread of infection through environmental contamination. However, the relatively low sensitivity of fecal culture (Buergelt et al 1977, Merkal 1970) and long waiting period for results dictate that infected animals are left in the herd longer than is desirable, thus increasing the risk of disease transmission. There are also significant production losses associated with
subclinically infected cattle as detected by fecal culture (Buergelt and Duncan 1978, Merkal et al/1975).

**SEROLOGIC TESTS FOR PARATUBERCULOSIS**

Many different types of immunoserologic tests for paratuberculosis have been developed. A tangible benefit of serologic testing is a shortened turnaround time for laboratory results compared to fecal culture. A good serologic test would also be more sensitive. Some tests have shown promise as confirmatory tests for paratuberculosis in cattle with weight loss and diarrhea, but have low sensitivity even in clinical suspects and are too unreliable to use for subclinical infections (Sherman et al/1984, Ratnamohan et al/1986). While these tests confirm the presence of Johne’s disease when positive, a negative test result is not very useful. A potential benefit of serologic tests is that animals may be detected before they become active fecal shedders. If paratuberculosis can be detected earlier, the potential for transmission of infection within the herd and for negative impacts of the disease upon production will be reduced.

The primary disadvantage of serologic testing in the past has been the high crossreactivity of mycobacterial antigens with other mycobacteria and with corynebacteria, nocardia and other gram positive bacteria (Minden et al/1972). The resulting low specificity has severely limited the value of serologic tests in control programs for paratuberculosis within infected herds. New antigens and new technology offer the promise of serologic tests with higher diagnostic efficiency. Recently, an enzyme-linked
immunosorbent assay (ELISA) for human tuberculosis using an affinity purified protein antigen has been investigated (Benjamin and Daniel 1982). Endpoint titers of the test were significantly different for tuberculous patients and control groups. Infections with nontuberculous mycobacteria gave endpoint titers that were intermediate between patients with tuberculosis and control groups. An affinity purified protein antigen of *Mycobacterium paratuberculosis* has recently been developed and used in an ELISA for cattle (Abbas et al 1983). The test discriminates between animals that are positive to fecal culture and animals that are fecal culture negative, and gives a reduced response with sera from animals experimentally infected with *M. avium*. This antigen contains fewer immunoreactive proteins as identified by immunotransfer blotting than do crude antigens (Bech-Nielsen et al 1985). In contrast to other ELISA tests for paratuberculosis (Yokomizo et al 1983, Jorgensen and Jensen 1978), results can be interpreted at a single serum dilution.

**TEST VALIDATION: THE "GOLD STANDARD"**

When evaluating a new diagnostic test, the ideal is to compare the new test to the true state of nature. Often, however, the true state of nature is difficult or expensive to determine, and old diagnostic tests are used as a "gold standard" of comparison (Fletcher et al 1982). If the reference test lacks precision, the relative sensitivity and specificity of the new test thus derived may not be a good estimation of the ability of the test to discriminate between diseased and healthy animals. In particular, when tests with low sensitivity are used as a reference standard, the specificity
of the new test appears lower than it really is (Greenberg and Jeckel 1969, Gart and Buck 1966). This is a major problem with relying on a single fecal culture to evaluate serologic tests for paratuberculosis. One solution to the problem is to retest the same population one or more times. If the testing errors are strictly random and the specificity is assumed to be 100%, then the sensitivity (SE) of the reference test improves as a function of the false negative rate (FN) and the number of times (n) that the test is applied:

\[
SE = 1 - (FN)^n
\]

If the errors are not strictly random, then sensitivity of the reference test approaches but does not reach this level, but the result will be an improvement on the use of a single reference test result to estimate the true disease status of the animal. With most diagnostic tests, the errors are a mixture of random and nonrandom events. In any case, the results of repeated testing in the same population will yield better estimates of disease status within individuals than a single test result.

Recently, maximum likelihood ratio estimates have been developed to evaluate sensitivity and specificity of diagnostic tests (Quade et al. 1980, Hui and Walter 1980). In simple terms, in the comparison of two diagnostic tests with unknown sensitivity and specificity, a maximum likelihood function is applied to the sample data, usually several two by two tables derived either from repeated applications of the tests to the same subjects (Quade et al. 1980) or the result of using two tests in more than one sample (Hui and Walter 1980). The maximum likelihood function finds the sensitivities and specificities which have the maximum probability of
occurring, given the observed data (Feinberg 1980). Both serial testing and
maximum likelihood ratio methods are applicable to evaluation of diagnostic
tests for paratuberculosis.

OBJECTIVES OF THE FIELD STUDY

The ELISA using an affinity purified protein antigen (Abbas et al 1983)
shows promise as a rapid, sensitive and specific assay for paratuberculosis.
Its effectiveness in the diagnosis of subclinical infection awaits a
prospective evaluation of the test in a controlled field study, with followup
of the paratuberculosis infection status of fecal culture negative animals.
This test could replace or augment fecal culture if field studies confirm
that it is specific for *M. paratuberculosis*, if its sensitivity is greater than
fecal culture in subclinically infected animals, or if it identifies infected
animals for removal before fecal culture.

The purpose of this study was to evaluate the ELISA using an affinity
purified protein antigen (Abbas et al 1983) in the diagnosis of
paratuberculous cattle in dairy herds where *M. paratuberculosis* infection
is present. The agar gel immunodiffusion test (AGID) (Sherman et al 1984)
was applied to the same animals for comparison. The sensitivity and
specificity of a single serologic test was compared to single and serial
fecal culture as a reference test. The use of the ELISA as a serially applied
test in control programs was also evaluated. Maximum likelihood ratio
estimates of sensitivity and specificity were derived using fecal culture as
a standard of comparison (Hui and Walter 1980). Finally, the temporal
relationship between a positive fecal culture response and a positive ELISA or AGID test was investigated.

MATERIALS AND METHODS

Sample Selection and Study Design

A convenience sample of three dairy herds located in the same veterinary practice was selected on the basis of similarity of size, calf rearing practices and owner cooperation. Every animal in these herds was tested at six month intervals. Two herds were tested 4 times over an 18 month time period and one herd was tested 3 times over a 12 month time period. All animals older than 6 months when the study began were included in the analysis. Samples for fecal culture and serology were collected. Blood and fecal samples were processed the same day they were collected. Fecal culture was initiated on collection day and serum was separated, frozen and held at -70° C for later testing. Fecal culture results were reported to the private practitioner and to owners for their use. The reporting of culture results was used as an incentive for participation in the testing program. Sixteen animals that left the farms during the study were followed to the packing plant, where samples were taken of feces, terminal ileum and ileocecal lymph node.

Tissue and Fecal Culture

Fecal samples were cultured according to the protocol used by the National Veterinary Services Laboratory (Mycobacteriology Unit, NVSL, 1985) with modifications in the decontamination procedure and in the media (Whipple
and Merkal 1984, Merkal 1984). One gram of fecal material was suspended in 15 ml of 0.75% hexadecyl pyridinium chloride (HPC) and shaken for 30 minutes. After settling for one to two hours, approximately 4 ml of the suspension was drawn up in a disposable transfer pipette that had been soaked for at least 30 minutes in 70% ethanol. The material in the transfer pipette was allowed to sediment overnight before being inoculated directly onto Herrold’s egg yolk media (HEM). Amphotericin B was not included in the medium. A five tube system was used. Four out of the five tubes contained Mycobactin J. Two of the four tubes containing Mycobactin J also contained sodium pyruvate (0.4%). The control tube contained neither Mycobactin J nor pyruvate. Tissue cultures were set up according to the same protocols. A 1 g sample was dissected and placed in a sterile 15 ml tissue grinder with 4 ml of 0.75% HPC. Tissues were ground and then the suspension was drawn up in a transfer pipette, allowed to settle overnight and inoculated onto five tubes of HEM. All cultures were read at 1, 2, 3, 8, 12 and 16 weeks post-inoculation. All isolates were checked microscopically for acid fast organisms using the Ziehl-Neelsen technique (Smithwick 1976).

ELISA Serology

An indirect ELISA was used to detect antibodies to *M. paratuberculosis* using an affinity purified antigen derived from strain 18M (Abbas et al. 1983). Wells of an enzyme immunoassay microtiter plate were coated

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1Allied Labs, Ames, IA
2Linbro/TiterTek, Flow Laboratories, Mclean, VA 21202
with a 1/64 dilution of *M. paratuberculosis* antigen\(^3\) in 50 μl of a carbonate buffer (0.015 M Na\(_2\)CO\(_3\), 0.035 M NaHCO\(_3\), 0.02% NaN\(_3\), pH 9.6) and incubated overnight at 4° C. Plates were rinsed 3 times with a wash solution containing 0.15 M NaCl and 0.05% Tween-20. Test sera were diluted 1:50 in ELISA buffer (0.15 M NaCl, 0.05 M Tris, 0.1% BSA, 0.05% Tween-20) and 50 μl applied to each well. Plates were incubated for 60 minutes at 37° C, rinsed three times, and 50 μl of a 1:300 dilution of goat antibovine IgG (heavy and light chains) conjugated to horseradish peroxidase\(^4\) in ELISA buffer was added to each well. The plates were incubated for 1 hour and rinsed as before. One hundred microliters of substrate solution (0.06 mM 2,2'-azino-di-3-[ethyl-benzthiazoline sulfonic acid], 50 mM citric acid, 10 mM H\(_2\)O\(_2\), pH 4) was added to each well. Results were recorded as optical density at 405 nm\(^5\) when the positive reference serum reached an optical density of 0.8-1.0. Positive and negative reference sera were run on each plate. Results were expressed as a percent of the positive reference serum optical density (de Savigny and Vollr 1980).

Samples were run in duplicate wells on each plate, and each sample was run on at least two plates. Thus, each sample was run in at least four wells and on at least two plates. Samples were run in the order in which they were received in the laboratory. The discrimination value between positive and negative was set on each plate by comparing the sample with the

\(^3\)California Animal Laboratory, Hemet, CA
\(^4\)0.05 mg/ml, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD 20879
\(^5\)Bio-Tek Elisa Reader, Bio-Tek Instruments, Burlington, VT 05401
distribution of other samples on the same plate. Behymer (1984) found that when an affinity purified antigen (Abbas et al/ 1983) is used for ELISA testing in herds with a history of exposure to paratuberculosis, a frequency distribution of samples on the plate falls into two groups representing infected and uninfected cows. In this study, the population of low values was defined as any well reading less than 50% of the positive control serum. The distribution of the low group was used to set the discrimination level between positive and negative sera. The cutoff value to distinguish between positive and negative results on an individual plate was set at two standard deviations above the mean of the population of low values on that plate.

**AGID Serology**

Agarose gels were prepared according to the method of Sherman (Sherman et al/ 1984), using a crude protoplasmic extract of *M. paratuberculosis* strain 18M. Results were read at 24 and 48 hours after incubation at room temperature. Results were recorded as positive if there was at least one clearly defined line of precipitation at either reading. A negative result was recorded if no lines of precipitation were observed.

**Data Analysis**

Sensitivity and specificity of ELISA and AGID tests relative to fecal culture were calculated for first ELISA and AGID tests. Sensitivity and specificity of three ELISA and AGID tests in series relative to three serial fecal culture tests were also calculated. The maximum likelihood procedure of Hui and

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6Allied Labs, Inc., Ames, IA
Walter (1980) was used to generate estimates of sensitivity and specificity and their standard errors for the ELISA tests as a single test and as a serial test. With serial testing, an animal was defined as positive for paratuberculosis if any of three fecal culture tests were positive, or if post mortem or slaughter inspection revealed evidence of infection with *M. paratuberculosis*. An animal was negative if all three fecal culture tests were negative, or if the animal had been necropsied and/or sampled at slaughter and not found to be positive by culture of terminal ileum, ileocecal lymph node or feces taken at the time of slaughter. A positive serial ELISA or AGID result was recorded for any animal that tested positive on any of the first three test dates. A negative serial ELISA or AGID result was recorded if the animal was serologically negative on all of the dates it was tested. The sign test (Hollander and Wolfe 1973) was used to determine if the ELISA and AGID tests became positive before or after the fecal culture became positive.

**RESULTS**

Of 224 cows and heifers tested by both fecal culture and ELISA on the first herd test, 14 were fecal culture positive and 210 were fecal culture negative. Relative sensitivity for the ELISA test was 71% and specificity was 83% (Table 11, Table 13). Point estimates of relative sensitivity and specificity for serial test results were 73% and 61% for the ELISA relative to fecal culture (Table 12, Table 13). Maximum likelihood ratio (MLR) estimates of sensitivity were 91% for the ELISA based on first test results only and 73% for the test applied as a serial test, relative to serial fecal
culture. MLR estimates of specificity were 82% and 66% for single and serial ELISA tests respectively (Table 13). Estimates of relative sensitivity of ELISA and their standard errors, and MLR estimators and their standard errors are given in Table 13.

AGID sensitivity relative to fecal culture was 38% for first AGID and 33% for serial AGID (Tables 14, 15 and 16). There were no AGID positive animals that were not fecal culture positive in either single or serial tests.

The sign test was performed on 33 fecal culture positive animals with both ELISA and AGID results available (Table 17). The ELISA test detected 9 infected animals in an earlier test period than fecal culture and fecal culture identified 11 animals in an earlier test period than the ELISA. There were no significant differences between the ELISA and fecal culture in the test period in which infection was first identified (p=0.38). In the absence of evidence of earlier detection by fecal culture, ELISA results of samples collected during the same test period are still available 8-16 weeks sooner than fecal culture results. The AGID test detected only one animal earlier than the fecal culture test. Twenty-five out of thirty-three fecal culture positive animals were detected at least six months later or not at all by the AGID test. The fecal culture test identified subclinical infections earlier than the AGID test (p<0.001).

DISCUSSION

Results indicate that the AGID test is much less sensitive than fecal culture or ELISA. The only AGID positive animals were also shedding detectable
levels of *M. paratuberculosis* in the feces at the time of testing. Both sign test results and estimates of relative sensitivity and specificity indicate that the AGID is not the test of choice for screening for paratuberculosis. However, the AGID test had a very high positive predictive value in this population of subclinically infected cattle, just as it did in a population of clinically affected cattle (Sherman et al. 1984). The AGID is best used as a confirmatory test, with a positive result being much more useful than a negative result. In as much as clinically infected cattle are more likely to be shedding mycobacteria (Duncan et al. 1978), the AGID test will have higher sensitivity in a group of clinically affected cattle. Advantages of the AGID are that it is easy to set up, run and interpret, and could be distributed in kit form. Its use will result in more rapid elimination of paratuberculosis suspect cattle from farms, if fecal culture is the only other test available. Reliance solely upon the AGID test for diagnosis of paratuberculosis is not recommended, on the basis of the low sensitivity estimates obtained.

The ELISA, on the other hand, shows promise as a substitute for fecal culture as a screening test for disease. It is both more sensitive and results are available much faster than with fecal culture. It could not be demonstrated that it identifies infected animals in an earlier test period than fecal culture, but laboratory results from the same test period will be available much sooner than fecal culture results. Serial application of the ELISA resulted in no gains in sensitivity and loss of specificity. When diagnostic tests are applied in series with a positive result on one test being taken as positive, the sensitivity of a test is expected to increase.
The study design used biased these results toward lower, rather than higher, relative sensitivity of the ELISA in this serial testing situation. If an animal was negative by fecal culture but remained in the herd for less than three tests, the observation was discarded because of the relative uncertainty of the disease status of the animal being tested, regardless of the ELISA test results. If an animal tested positive to fecal culture, it was likely that the animal was removed from the herd before the next test, but the observation was included in the analysis. In such cases the ELISA test had smaller chance of becoming positive than if the animal had been observed for three tests. Roughly one third of observed fecal culture positive animals were ELISA negative at the time of the positive fecal culture. It is not known how many of these would have seroconverted had the animals not been removed. This bias would still be present in the MLR estimate of serial ELISA sensitivity. The reason for evaluating the ELISA test in this manner is to compare the ELISA test in a simulated control program. Point estimates for sensitivity of the ELISA test are still higher than accepted estimates of sensitivity of fecal culture (Buergelt et al 1977). As expected in serial testing, the specificity of the test dropped. While confidence limits are wide, MLR estimators of sensitivity and specificity support the estimates of relative sensitivity and specificity of the ELISA test. The ELISA using a partially purified protein antigen offers a test of higher sensitivity than fecal culture or AGID in detecting paratuberculosis among subclinically infected cattle, and does well in terms of specificity, in an environment where use of an indirect test might be expected to yield a high percentage of false positive results.
Table 11
FREQUENCY DISTRIBUTION OF FIRST ELISA RESULTS (E1) BY FIRST FECAL CULTURE RESULTS (F1)

<table>
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<tr>
<th>F1</th>
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<th>-</th>
<th>TOTAL</th>
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</thead>
<tbody>
<tr>
<td>E1+</td>
<td>10</td>
<td>36</td>
<td>46</td>
</tr>
<tr>
<td>E1-</td>
<td>4</td>
<td>174</td>
<td>178</td>
</tr>
<tr>
<td>TOTAL</td>
<td>14</td>
<td>210</td>
<td>224</td>
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</table>

Table 12
FREQUENCY DISTRIBUTION OF SERIAL ELISA RESULTS (E3) BY SERIAL FECAL CULTURE (F3)

<table>
<thead>
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<th>+</th>
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<tbody>
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<td>E3+</td>
<td>22</td>
<td>58</td>
<td>80</td>
</tr>
<tr>
<td>E3-</td>
<td>8</td>
<td>92</td>
<td>100</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30</td>
<td>150</td>
<td>180</td>
</tr>
</tbody>
</table>
**Table 13**

**ESTIMATES OF SENSITIVITY AND SPECIFICITY OF ELISA TESTS FOR PARATUBERCULOSIS BY FOUR METHODS:**
- **FIRST ELISA BY FIRST FECAL CULTURE (E1 x F1),**
- **SERIAL ELISA BY SERIAL FECAL CULTURE (E3 x F3),**
- **MAXIMUM LIKELIHOOD RATIO OF FIRST ELISA BY FIRST FECAL CULTURE (MLR E1 x F1), AND**
- **MAXIMUM LIKELIHOOD RATIO ESTIMATE OF SERIAL ELISA BY SERIAL FECAL CULTURE (MLR E3 x F3)**

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<th>METHOD</th>
<th>SENSITIVITY</th>
<th>+/- 2 x (S.E.)</th>
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</thead>
<tbody>
<tr>
<td>E1 x F1</td>
<td>0.71</td>
<td>(0.47, 0.96)</td>
</tr>
<tr>
<td>E3 x F3</td>
<td>0.73</td>
<td>(0.57, 0.96)</td>
</tr>
<tr>
<td>MLR E1 x F1</td>
<td>0.91</td>
<td>(0.28, 1.0)</td>
</tr>
<tr>
<td>MLR E3 x F3</td>
<td>0.73</td>
<td>(0.45, 1.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SPECIFICITY</th>
<th>+/- 2 x (S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 x F1</td>
<td>0.83</td>
</tr>
<tr>
<td>E3 x F3</td>
<td>0.61</td>
</tr>
<tr>
<td>MLR E1 x F1</td>
<td>0.82</td>
</tr>
<tr>
<td>MLR E3 x F3</td>
<td>0.66</td>
</tr>
</tbody>
</table>
Table 14

FREQUENCY DISTRIBUTION OF FIRST AGID (A1) BY FIRST FECAL CULTURE (F1)

<table>
<thead>
<tr>
<th>F1</th>
<th>+</th>
<th>-</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>A1</td>
<td>8</td>
<td>209</td>
<td>217</td>
</tr>
<tr>
<td>TOTAL</td>
<td>13</td>
<td>209</td>
<td>212</td>
</tr>
</tbody>
</table>

Table 15

FREQUENCY DISTRIBUTION OF SERIAL AGID (A3) BY SERIAL FECAL CULTURE (F3)

<table>
<thead>
<tr>
<th>F3</th>
<th>+</th>
<th>-</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>A3</td>
<td>20</td>
<td>150</td>
<td>170</td>
</tr>
<tr>
<td>TOTAL</td>
<td>30</td>
<td>150</td>
<td>180</td>
</tr>
</tbody>
</table>
### Table 16

**SENSITIVITY AND SPECIFICITY OF FIRST AGID BY FIRST FECAL CULTURE (A1 x F1) AND SERIAL AGID BY SERIAL FECAL CULTURE (A3 x F3)**

<table>
<thead>
<tr>
<th>METHOD</th>
<th>SENSITIVITY</th>
<th>+/- 2 x (S. E.)</th>
<th>SPECIFICITY</th>
<th>+/- 2 x (S. E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 x F1</td>
<td>0.38</td>
<td>(0.12, 0.66)</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>A3 x F3</td>
<td>0.33</td>
<td>(0.16, 0.51)</td>
<td>1.0</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 17

**TIME OF ONSET OF SEROLOGIC RESPONSE (ELISA AND AGID) RELATIVE TO DETECTABLE FECAL SHEDDING OF MYCOBACTERIUM PARATUBERCULOSIS**

<table>
<thead>
<tr>
<th>TEST</th>
<th>SEROLOGY FIRST</th>
<th>SAME</th>
<th>CULTURE FIRST</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA(^1)</td>
<td>9</td>
<td>13</td>
<td>11</td>
<td>33</td>
</tr>
<tr>
<td>AGID(^2)</td>
<td>1</td>
<td>7</td>
<td>25</td>
<td>33</td>
</tr>
</tbody>
</table>

\(^1\)p=0.32  
\(^2\)p<0.001
REFERENCES


INTRODUCTION

The impact of paratuberculosis on production and disease has been the subject of much speculation and little research. A study on the impact of paratuberculosis on cull cows (Merkal et al./1975) found that mastitis and infertility were given as reasons for culling more frequently in cows with paratuberculosis than in uninfected cows culled from the same herd. In a study of transformation of lymphocytes from blood and milk Elsken and Nonnecke (Elsken and Nonnecke 1986) found that milk lymphocytes from cows infected with paratuberculosis were less responsive to a purified protein derivative of *Mycobacterium paratuberculosis* than milk lymphocytes from paratuberculosis negative herdmates. From this, they hypothesize that udder defense mechanisms may be diminished in cows with paratuberculosis. Thus, there is circumstantial evidence for an association between paratuberculosis and mastitis. Buergelt and coworkers (Buergelt et al./1978) found significant differences in 305 day mature equivalent milk production (305 day ME) between infected cull cows with clinical signs of
paratuberculosis and uninfected cull cows from the same herd. No difference between 305 day ME in subclinical and uninfected cull cows was demonstrated.

There is a need for more information on the relationship between paratuberculosis infection, the risk of other diseases and production. Proof of causality requires the analysis of complex interrelationships between production and disease. The determination of causality goes beyond the finding of a statistical association. However, investigation of the association between paratuberculosis, mastitis and production will be an important step in understanding these biological relationships and, ultimately, in controlling these diseases and their associated production losses. No concurrent comparisons between herdmates have been made of production or disease relationships with respect to paratuberculosis. The objective of this study is to measure associations between milk production, mastitis and subclinical paratuberculosis in a cohort of herdmates.

METHODS

Study Design

Laboratory and production data were collected from two central Ohio dairy herds. All animals in both herds were tested for paratuberculosis three times at six month intervals by fecal culture and ELISA serology. Fecal culture results were reported to herd owners. Production data and somatic
cell counts (SCC) were collected using DHI records. All dairy cows with lactation records in progress on the date of the first herd test for paratuberculosis or beginning a lactation between the first and the second herd test for paratuberculosis were eligible to enter the study. Lactation records from six months before the first herd test for paratuberculosis to six months after the last herd test for paratuberculosis were used.

**Laboratory Data**

Fecal culture and ELISA were used for diagnosis of paratuberculosis. Fecal culture was performed according to the protocol used by the National Veterinary Services Laboratory (Mycobacteriology Unit, NVSL 1985) with modifications in the decontamination procedure and in the media (Whipple and Merkal 1984). An animal was classified as fecal culture positive for *M. paratuberculosis* if isolates were acid fast and mycobactin dependent. The fecal culture method is described in detail in Chapters 3, 4 and 5 of this dissertation.

An indirect ELISA with an affinity purified antigen of strain 18 *M. paratuberculosis* (Abbas *et al.* 1983) was also used to diagnose paratuberculosis. This method is described in greater detail in Chapter 3.

Cows were classified by laboratory data into test positive and test negative groups for paratuberculosis five different ways, based on the results of serial and single tests (Table 18).

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1Dairy Herd Improvement, Inc., Powell, Ohio 43065
The results of serial fecal culture (FC) were compared in the following manner. A positive cow is defined as one having a positive FC test in any of the herd surveys. A negative cow was negative to all three FC tests for paratuberculosis. The small number of FC positive animals at each test precluded comparisons of ME and SCC based on a single test result.

Cows were classified by serial ELISA testing, referred to simply as ELISA in this report, in a similar manner. Cows were also classified by individual ELISA results. These are referred to as E1, E2, and E3.

**Milk Production**

Three hundred five day mature equivalent milk production (ME) was obtained from Dairy Herd Improvement (DHI) records. For each cow, the lactation studied was the lactation in progress at the time of the first herd test for paratuberculosis. If cows were dry on that date, or if days in milk was greater than 305, then the lactation which began after the first herd test was used. Records of cows in milk less than 150 days during the study period were not eligible because of incompleteness.

**Milk Somatic Cell Counts**

Somatic cell count in milk were used as a predictor of mastitis infection status (Meek *et al.* 1980). Ohio DHI reports somatic cell counts as the linear score\(^2\) as a whole number ranging from 0 to 9. The mean linear score somatic cell count was calculated for each lactation from monthly DHI records.

\[2\text{Linear Score Somatic Cell Count} = \left(\log_{e}(\text{SCC}/0.6931)\right)-2.6439, \text{where SCC is the raw somatic cell count.}\]
reports of linear score somatic cell counts for the lactation in progress. Lactations were included in the analysis if days in milk was 305 days or less on the first herd test for paratuberculosis, and if 4 or more monthly linear scores were reported. The mean linear score somatic cell count (SCC) was used as a measure of mastitis because milk production loss is linear with SCC, and the average of four or more monthly scores is an accurate predictor of the presence of pathogens in milk (Shook 1982).

**Covariates and Statistical Analysis**

In addition to ME and SCC, days in milk (DIM) on the first herd test day for paratuberculosis and lactation number were recorded. Lactation number was reduced to a categorical variable, LAC, with three levels: first lactation, second lactation, third or later lactation. Herd of origin (HERD) was also considered in the models tested. Milk production is correlated with days in milk and lactation number. Since ME is corrected for DIM and lactation number (Bath et al. 1978), DIM and LAC were not included as covariates in the statistical analysis of ME. Somatic cell counts in milk have been correlated with stage of lactation, lactation number and milk production (Dohoo and Meek 1982, Leslie et al. 1983, Kirk 1984). SCC as it is defined in this study is the lactation average cell count. Thus all stages of lactation are represented in this summary statistic and DIM was not included in the statistical analysis of SCC. DIM was used only as a guideline for entry of records into the analysis.

The individual cow 305 day milk production (ME) and somatic cell count expressed as a mean linear score for the lactation (SCC) were analyzed by
least squares analysis of variance (Winer 1971) using SAS GLM\(^3\) to test for the association between the dependent variable (ME or SCC) and the laboratory diagnosis of paratuberculosis (LAB DX). The design was a 2x2 factorial with independent variables LAB DX and HERD. When ME was used as the response variable, SCC was included as a covariate. When SCC was the response variable, ME and LAC were included as covariates. First order interactions between HERD and LAB DX were also tested. The models used are shown in Table 19.

RESULTS

Descriptive statistics for ME, SCC and lactation number are given in Table 20. No significant differences between herds were observed (p>.05 in all cases). Mean ME was 16,211 lbs, mean SCC was 3.66 and mean lactation number was 3.331.

The analysis of variance for the association between ME and FC was significant (p=.01; r\(^2=.189\); Table 21). Least squares means for 15 FC positive and 60 FC negative cows were 14,149 lbs. and 17,424 lbs. respectively. First order interactions between HERD and FC were not significant. No significant association was found between ME and serial ELISA, E1, E2 or E3. However the simple regression of SCC on ME was significant (p=.02, r\(^2=.087\)). Since ME was not normally distributed, alternate analyses were considered. The data set was truncated and the analyses repeated. Elimination of the top and bottom fifth and tenth percentiles had no effect upon the results. Analysis of the data based on

\(^3\)SAS Institute, Inc., SAS Circle, Cary, N. C. 27511
ranks using the Wilcoxon rank sum test (Hollander and Wolfe 1973) for the effect of LAB DX on ME was not significant. A nonparametric analysis of covariance (Hollander and Wolfe 1973) blocking on HERD and using SCC as a covariate with LAB DX as the treatment and ME as the response was not significant.

No association was found between SCC and FC or between SCC and any comparison based on ELISA. The regression of ME and LAC on SCC was highly significant, however (p=.001, r²=.198). The distribution of SCC did not violate assumptions of normality.

DISCUSSION

The results indicate that there is a significant relationship between paratuberculosis and milk production, corrected for the differences in mastitis prevalence between paratuberculosis infected and uninfected herdmates. The fact that FC or any other measure of paratuberculosis infection was not significantly associated with SCC in the models tested can be interpreted three ways. It may mean that the association between paratuberculosis and mastitis does not exist, that it is a weak relationship that indirect measures of mastitis and paratuberculosis could not detect, or that there are other predictors of mastitis that need to be considered in the model. The use of indirect measures of infection such as SCC and ELISA, with attendant errors of disease classification, may have clouded the relationship between mastitis and paratuberculosis. The classification errors associated with the ELISA test may also explain why there was no
association between paratuberculosis infection and ME when ELISA was used as a classification variable for paratuberculosis.

Because FC positive cows were culled, the number of complete lactation records in this group was smaller than in the FC negative group. In the data analysis, the effect of estimating ME and SCC in more records in this group is not known. Schaeffer and Burnside (Schaeffer and Burnside 1976) have shown that lactation curves can be predicted after 150 DIM. Shook (Shook 1982) reported that the lactation average linear score somatic cell count based on 4 or more tests was a more accurate predictor of presence of pathogens in milk than measurements of a single linear score during a lactation. Thus, limiting the analysis to lactations greater than 150 days and with 4 or more observations on somatic cell count should reduce the effect of this bias.

Results of this study support the hypothesis that paratuberculosis infection has a negative impact upon milk production. The difference in milk production observed between fecal culture positive and fecal culture negative herdmates is over 3,000 lbs per lactation. A difference of this magnitude, multiplied by the number of cows that were fecal culture positive, represents 45,000 lbs. of milk lost over the two year period, taking into account ME only during the lactation that infection was detected. The concurrent design of this study provides a more direct estimate of the impact of paratuberculosis upon production in infected and uninfected herdmates than estimates from a previously reported study on cull cows (Buergelt et al/1978). The estimate thus derived represents
production loss at the time that disease is detected, and is an estimate of the loss that could be avoided if regular testing for paratuberculosis and culling of infected animals was part of the herd health program on these farms.
Table 18.

CLASSIFICATION OF COWS BASED ON RESULTS OF DIAGNOSTIC TESTS FOR PARATUBERCULOSIS

<table>
<thead>
<tr>
<th>NAME</th>
<th>DEFINITION</th>
</tr>
</thead>
</table>
| FC   | + = positive to any fecal culture test  
- = negative to three fecal culture tests |
| ELISA | + = positive to any ELISA test  
- = negative to three ELISA tests |
| E1   | + = positive to first ELISA test  
- = negative to three ELISA tests |
| E2   | + = positive to second ELISA test  
- = negative to three ELISA tests |
| E3   | + = positive to third ELISA test  
- = negative to three ELISA tests |
Table 19.

STATISTICAL MODELS USED TO TEST FOR ASSOCIATIONS BETWEEN ME, SCC AND PARATUBERCULOSIS INFECTION STATUS

I. ME AND PARATUBERCULOSIS

\[ ME_{i j k} = \mu + LAB \, DX_i + HERD_j + SCC_k + e_{ijk} \]

II. SCC AND PARATUBERCULOSIS

\[ SCC_{ijkl} = \mu + LAB \, DX_i + HERD_j + LAC_k + ME_l + e_{ijk} \]

(First order interactions between HERD and LAB DX were also tested in models I and II)

ME = 305 day mature equivalent milk production
\( \mu \) = Herd average ME (analysis I) or herd average SCC (analysis II)
LAB DX = FC, ELISA, E1, E2, and E3 (See Table 18)
HERD = Herd of origin
SCC = Somatic cell count expressed as the individual cow average linear score
\( e \) = error
### Table 20.

**MATURE EQUIVALENT MILK PRODUCTION (ME), MILK SOMATIC CELL COUNTS (SCC) AND LACTATION NUMBER (LAC) OF COWS IN HERDS WITH SUBCLINICAL PARATUBERCULOSIS INFECTION**

<table>
<thead>
<tr>
<th></th>
<th>HERD 1</th>
<th>n</th>
<th>mean</th>
<th>sd</th>
<th>HERD 2</th>
<th>n</th>
<th>mean</th>
<th>sd</th>
<th>TOTAL</th>
<th>n</th>
<th>mean</th>
<th>sd</th>
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<tbody>
<tr>
<td>ME</td>
<td></td>
<td>39</td>
<td>16,197</td>
<td>3240</td>
<td></td>
<td>45</td>
<td>16,224</td>
<td>4133</td>
<td></td>
<td>84</td>
<td>16211</td>
<td>3723</td>
</tr>
<tr>
<td>SCC</td>
<td></td>
<td>41</td>
<td>3.61</td>
<td>1.30</td>
<td></td>
<td>39</td>
<td>3.71</td>
<td>1.56</td>
<td></td>
<td>80</td>
<td>3.66</td>
<td>1.43</td>
</tr>
<tr>
<td>Lac. *</td>
<td></td>
<td>41</td>
<td>3.31</td>
<td>2.07</td>
<td></td>
<td>45</td>
<td>3.49</td>
<td>2.34</td>
<td></td>
<td>86</td>
<td>3.41</td>
<td>2.20</td>
</tr>
</tbody>
</table>

**Table 21.**

**ANALYSIS OF VARIANCE TABLE: EFFECT OF FECAL CULTURE STATUS (FC), HERD OF ORIGIN (HERD), AND SOMATIC CELL COUNT (SCC) ON 305 DAY MATURE EQUIVALENT MILK PRODUCTION**

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>D. F.</th>
<th>F VALUE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>1</td>
<td>10.01</td>
<td>.0026</td>
</tr>
<tr>
<td>HERD</td>
<td>1</td>
<td>.26</td>
<td>.6123</td>
</tr>
<tr>
<td>SCC</td>
<td>1</td>
<td>1.79</td>
<td>.1870</td>
</tr>
</tbody>
</table>
REFERENCES


Meek, A. H., Barnum, D. A., and Newbold, F. H. S. Use of total and differential somatic cell counts to differentiate potentially infected from potentially non-infected quarters and cows between herds of various levels of infection. J. Food Protection 1980; 43: 10-14.


Chapter 7

SEROLOGIC RESPONSES OF CALVES TO VACCINATION FOR

MYCOBACTERIUM PARATUBERCULOSIS

INTRODUCTION

Calfhood vaccination is used as a part of the control effort on farms infected with paratuberculosis. The value of vaccination and its contribution to control is not clear. It does seem to be effective in reducing the number of clinical cases and animals shedding organisms in feces (Larsen et al. 1978). It seems likely that vaccination is of benefit by increasing host resistance and by decreasing environmental exposure to Mycobacterium paratuberculosis. Maximum benefits are derived when vaccination is practiced in conjunction with other control practices (Wilesmith 1982).

The key to effective control of paratuberculosis is the rapid and accurate detection of infected cows. Cows shedding Mycobacterium paratuberculosis organisms in high numbers can be detected by fecal culture. However, incubation of cultures takes 8-16 weeks and only heavily infected animals are detected by this method. Animals that are capable of contaminating the herd environment and thus spreading infection may remain in the herd for long periods of time. Efforts are being made to develop tests that can detect infected cows before they have become fecal shedders. Serologic
tests may be used to monitor infection with *M. paratuberculosis*; and results are available more rapidly than results of fecal culture. If vaccination interferes with identification of infection by serologic methods, then the contribution of vaccination to control programs also incorporating test and removal methods needs to be evaluated. The purpose of the present study is to monitor the serologic responses of vaccinates and nonvaccinates within the same herd, using an indirect enzyme-linked immunosorbent assay (ELISA) for *M. paratuberculosis* antibody.

**MATERIALS AND METHODS**

**Study Herd**

This investigation was undertaken in a 75 cow registered Guernsey herd selected on the basis of owner cooperation and the recent initiation of a vaccination program for *M. paratuberculosis*. This is an open herd that is comingle with a commercial Holstein herd. The Guernsey herd was tested for paratuberculosis by an indirect ELISA test using a partially purified protein antigen (Abbas *et al* 1983) and fecal culture once at the initiation of the study and again eleven months later. All lactating and nonlactating Guernseys that had calved at least once were tested. The Holstein herd was not tested. A separate calving area was provided for Guernseys. Guernsey calves were separated from their dams at birth and raised on another farm. They were kept in calf hutch until weaning and then placed in dry lot in groups of six to ten heifers by age, through the first year of life.
Experimental Design

Five control calves (numbered C1 through C5) and 15 vaccinates (numbered V1 through V15) were bled at monthly to bimonthly intervals from the first through the twelfth month of life. Fecal samples were collected from the calves at the last test date and cultured. Treatment calves were vaccinated between 5 and 35 days of life by a private veterinary practitioner contracted by the herd owner. The vaccine used was a commercial product of killed *M. paratuberculosis* strain 18\(^1\). Initial blood samples were drawn at the time of vaccination by the veterinarian administering the vaccine and mailed to the laboratory, or prior to vaccination by one of the research team. Control calves entered the study on the first farm visit made by one of the team members after it was determined that they were not candidates for vaccination. Calves became ineligible for vaccination either by surpassing 35 days of age without receiving the vaccine (three heifer calves) or were not candidates for vaccination for other reasons (two bull calves).

Fecal Culture

Isolation of *M. paratuberculosis* from feces was done on Herrold's Egg Yolk medium supplemented with Mycobactin J (Merkal 1973). Approximately 1 g of fecal material was placed in a 50 ml centrifuge tube containing 0.7% hexadecyl pyridinium chloride and shaken for 30 minutes. After settling for two hours 3 ml were drawn up in a disposable transfer pipette disinfected

\(^1\)Fromm Laboratories, Inc., Grafton, Wisconsin.
In 70% ethanol. The sample was allowed to settle overnight before inoculating 0.2 ml onto slants of Herrold's egg yolk medium. A five tube system consisting of two tubes of Herrold's supplemented with sodium pyruvate (4.1 g/l) and mycobactin, two tubes supplemented with mycobactin only, and one tube with neither mycobactin nor pyruvate added was used. Samples were incubated for 16 weeks at 37° C. Acid fast isolates were identified as *M. paratuberculosis* by mycobactin dependency.

**Enzyme-Linked Immunosorbent Assay**

An indirect ELISA was used to detect antibodies to *M. paratuberculosis* using an affinity purified antigen derived from a Ribi press extract of strain 18 (Abbas *et al* 1983). Wells of a microtiter enzyme immunoassay plate were coated with 50 μl of a 1/64 dilution of the antigen in carbonate buffer and incubated overnight at 4° C. Plates were rinsed 3 times with a wash solution containing 0.15 M NaCl and 0.05% Tween-20. Test sera were diluted 1:50 in ELISA buffer (0.15 M NaCl, 0.05 M TRIS, 0.1% BSA and 0.05% Tween-20) and 50 μl applied to each well. Plates were incubated for 60 minutes at 37° C, and rinsed three times. Horseradish peroxidase-conjugated goat antibovine immunoglobulin G (heavy and light chains) was diluted 1:300 in ELISA buffer and 50 μl was added to each well. The plates were incubated for 1 hour and rinsed as before. One hundred microliters of substrate containing 10 mM H₂O₂, 0.06 mM 2,2'-azino-di-3-

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2Linbro/Titertek, Flow Laboratories, Mclean, VA 21202.
3Antigen obtained from California Animal Laboratory, Hemet, CA.
40.05 mg/ml, Kirkegaard and Perry Laboratories, Gaithersburg, MD 20879.
[ethyl-benzthiazoline-6-sulfonic acid] in 50mM sodium citrate (pH 4.0) was added to each well. Plates were read when positive control sera reached an optical density of 1.0. Results of samples from adult animals were expressed as a percent of positive control and the discrimination value between positive and negative determined by comparing the distribution obtained to that of a known negative population as previously described (de Savigny and Voller 1980; for details see Chapter 3). Results of calf sera were expressed as raw optical density.

All calf sera were run in triplicate. Serum samples from different test days from the same calf were run on the same plate. Control samples were run on all plates. Results are expressed as the mean of the raw optical density readings for the three replicates.

RESULTS

The adult Guernsey herd was tested by fecal culture and ELISA twice eleven months apart. On the first test, two of 75 milking and dry dairy animals were fecal culture positive. These were purchased animals that had been in the herd less than a year; they were removed from the herd immediately. There were no fecal culture positive animals out of 77 animals cultured on the second test. The prevalence of ELISA positives was 12% on the first test and 24% on the second test. Among the dams of study calves, one was fecal culture positive on the first test (Table 22). ELISA responses of study dams are given in Table 22. Five of the study dams were removed from the herd between the first and second herd screening tests. Genetic dams of nonvaccinates were negative to all tests. However, the five control calves
were all embryo transfers from two dams, and the surrogate dams were not available for testing.

Control calves entered the study at 46, 52, 59, 60 and 120 days of age (mean 67 days, median 59 days). Vaccinates were 5 to 40 days of age at vaccination. Mean age at vaccination was 23 days, and the median age was 22 days. Control calves were older because of their different method for entry into the study.

The length of the followup period ranged from 290 to 448 days of life in the control group (mean 412 days, median 437 days). Length of followup for vaccinated calves varied from 286 to 482 days of life. The mean length of followup of vaccinated calves was 390 days of age or 367 days post vaccination (median 373 days of life or 347 days post vaccination).

The threshold value for a positive ELISA response to vaccination was set at an optical density of 0.250, or twice the mean of the highest ELISA responses of the negative control calves. If the ELISA response of a very young calf with a low exposure potential to paratuberculosis represents the background noise in the assay, then this threshold represents a signal to noise ratio of two. Thirteen of fifteen calves responded to vaccination by this criterion. The maximum optical density reached by the responder calves ranged from four to twelve times the cutoff level (Table 22). The maximum optical density readings of the calves that failed to respond to vaccination were 0.062 and 0.137, which was below the cutoff level of 0.250 (Table 22). No consistent patterns were observed between culture
status of dam, ELISA status of dam, vaccination date and vaccination response of the calf as measured by the indirect ELISA (Table 22).

Control calves had low optical density readings throughout the study period (Figure 8). Calf V1, the calf that was born to a fecal culture positive dam, had a serum ELISA reading over 0.400 at the initial sample (Figure 9). Her ELISA response curve from the second sampling period on resembled those of other calves (Figures 9 and 10). The two calves that failed to respond to vaccination also stayed low throughout the sampling period, with the exception of V15, which had an ELISA titer of 0.363 twelve days before vaccination (Figure 11). Her dam was ELISA positive. Other calves out of ELISA positive dams had low titers initially (Figures 10, 12-15). Calves that responded to vaccination did so at the earliest between 65 and 106 days post vaccination and at the latest sometime after the 181st day following vaccination. The average calf developed a measurable ELISA response between the 118th day and the 181st day following vaccination. The highest response measured occurred on the last test day in nine out of thirteen calves responding to vaccination, up to 454 days after vaccination.

**DISCUSSION**

This study was conducted in a herd with a low prevalence of Johne's disease, as reflected in the fecal culture results. The source of the ELISA titers in cows is unknown; however, cows could be infected but not shedding, exposed but not infected, or the ELISA result could be a false positive. The potential for exposure exists in this herd, but the degree of exposure is unknown. It seems likely that it could be moderate given the
open status of the herd, the history of infection in Guernsey cows and the comingling of cows with a commercial Holstein herd that is used for leasing.

ELISA responses of control calves stayed low during the study period while 13 of 15 vaccinates developed a positive ELISA, indicating that the response observed was probably a result of vaccination. The relationship between protective immunity and the presence of a serologic response as detected by the ELISA was not studied.

Reasons for failure of two calves (V13, V15) to develop an ELISA response to vaccination are speculative and include 1) prenatal exposure and immunologic tolerance, 2) interference from colostral immunoglobulins and c) defects in the vaccine or the vaccination technique. Failure due to the vaccine or the vaccination technique is unlikely in this study because other calves vaccinated the same day developed a positive ELISA response (Table 22). One of the calves (V15) showed serologic evidence of pre-vaccine exposure to *M. paratuberculosis*, while the other calf (V13) did not (Figure 11). Both calves had dams that were ELISA positive on both tests (Table 22); it seems plausible that either tolerance or passively acquired immunity could have interfered with vaccination response in these calves. Another calf (V11) with a dam that was ELISA positive on both tests developed a strong ELISA response following vaccination (Figure 12). One calf (V1) had both a fecal culture positive dam and a positive ELISA prior to vaccination and developed a strong response following vaccination (Figure 9). Circumstantial evidence indicates that exposure history may play a role in
serologic responses of calves to vaccination, although it does not appear to be an accurate predictor of serologic response.

The ELISA response of individual vaccinated calves was extremely variable in time of onset and peak response. All calves that responded did so by six months after vaccination and they all remained positive at the end of the study period, which was, on the average, a year following vaccination.

Lymphocyte stimulation indices of vaccinated calves are reportedly low (median stimulation index < 2) in the first 20 weeks of life in a vaccinated exposed herd, but high in yearlings in a vaccinated unexposed herd (Hintz 1981). Complement fixation titers have been measured at six and eighteen months post vaccination in calves inoculated with a disrupted cell vaccine (Larsen et al/1969). Fewer calves were CF positive 18 months following vaccination than at six months. In that study, intradermal test responses declined over the same time period.

It is clear from the literature that both cell-mediated and humoral immunity as measured by lymphocyte stimulation and complement fixation tests remains high for at least a year after paratuberculosis vaccination. In this study, ELISA response remained high for the length of followup, up to 482 days post vaccination (mean followup = 367 days post vaccination). During this period the ELISA response of control calves stayed low. A knowledge of the duration and stability of the ELISA response to vaccination becomes increasingly important if we want to rely on rapid serologic methods for diagnosis of paratuberculosis in vaccinated herds. This study demonstrates that vaccination interferes with ELISA testing through the
fifteenth month of life. If this response persists into the second year of life, serologic methods will be of little value in diagnosing infection in vaccinated herds.
Table 22

VACCINATED CALVES: ELISA RESPONSE, VACCINATION DATE, MAXIMUM ELISA OPTICAL DENSITY (MAX ELISA) AND RESULTS OF SCREENING TESTS FOR PARATUBERCULOSIS IN DAMS

<table>
<thead>
<tr>
<th>CALF</th>
<th>ELISA</th>
<th>VACC. DATE</th>
<th>MAX ELISA</th>
<th>FC 1(^2)</th>
<th>ELISA 1</th>
<th>FC 2</th>
<th>ELISA 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>+</td>
<td>12/18/84</td>
<td>1.012</td>
<td>+</td>
<td>MISSING</td>
<td>SOLD</td>
<td>SOLD</td>
</tr>
<tr>
<td>V2</td>
<td>+</td>
<td>12/18/84</td>
<td>1.435</td>
<td>-</td>
<td>MISSING</td>
<td>SOLD</td>
<td>SOLD</td>
</tr>
<tr>
<td>V3</td>
<td>+</td>
<td>12/18/84</td>
<td>0.478</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V4</td>
<td>+</td>
<td>12/18/84</td>
<td>0.874</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V5</td>
<td>+</td>
<td>2/14/85</td>
<td>1.528</td>
<td>-</td>
<td>+</td>
<td>SOLD</td>
<td>SOLD</td>
</tr>
<tr>
<td>V6</td>
<td>+</td>
<td>2/14/85</td>
<td>1.699</td>
<td>-</td>
<td>MISSING</td>
<td>SOLD</td>
<td>SOLD</td>
</tr>
<tr>
<td>V7</td>
<td>+</td>
<td>3/30/85</td>
<td>1.285</td>
<td>-</td>
<td>+</td>
<td>SOLD</td>
<td>SOLD</td>
</tr>
<tr>
<td>V8</td>
<td>+</td>
<td>3/30/85</td>
<td>0.727</td>
<td>-</td>
<td>MISSING</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>V9</td>
<td>+</td>
<td>4/4/85</td>
<td>1.008</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V10</td>
<td>+</td>
<td>4/4/85</td>
<td>1.403</td>
<td>-</td>
<td>MISSING</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>V11</td>
<td>+</td>
<td>4/4/85</td>
<td>1.008</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V12</td>
<td>+</td>
<td>4/4/85</td>
<td>1.286</td>
<td>-</td>
<td>MISSING</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>V13</td>
<td>-</td>
<td>4/4/85</td>
<td>0.062</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V14</td>
<td>+</td>
<td>5/16/85</td>
<td>1.072</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>V15</td>
<td>-</td>
<td>5/16/85</td>
<td>0.137</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^1\)Test dates for dams: FC1 on 4/4/85; ELISA 1 on 2/19/85; FC 2 and ELISA 2 on 1/16/86

\(^2\)FC = Fecal Culture
Figure 8

ELISA RESPONSE (OPTICAL DENSITY) OF NONVACCINATES
Figure 9

ELISA RESPONSE (OPTICAL DENSITY) OF A CALF (V1)
BORN TO A FECAL CULTURE POSITIVE DAM
Figure 10

ELISA RESPONSE (OPTICAL DENSITY) OF CALVES VACCINATED ON DECEMBER 18, 1984
Figure 11

ELISA RESPONSE (OPTICAL DENSITY) OF VACCINATED CALVES THAT FAILED TO DEVELOP A MEASURABLE ANTIBODY RESPONSE TO VACCINATION
Figure 12

ELISA RESPONSE (OPTICAL DENSITY) OF CALVES VACCINATED ON FEBRUARY 14, 1985
Figure 13

ELISA RESPONSE (OPTICAL DENSITY) OF CALVES VACCINATED ON MARCH 30, 1985
Figure 14

ELISA RESPONSE (OPTICAL DENSITY) OF CALVES VACCINATED ON APRIL 4, 1985
Figure 15

ELISA RESPONSE (OPTICAL DENSITY) OF CALVES VACCINATED ON MAY 16, 1985
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