# Evaluation of Conventional and Radiometric Fecal Culture and a Commercial DNA Probe for Diagnosis of *Mycobacterium paratuberculosis* Infections in Cattle

D.C. Sockett, D.J. Carr and M.T. Collins


## ABSTRACT

**Radiometric (RCM) and conventional fecal culture (HEY) and a commercial polymerase chain reaction/DNA probe were evaluated as diagnostic tests for subclinical paratuberculosis in dairy cattle using fecal specimens from a repository of paratuberculosis specimens. The case definition of subclinical bovine paratuberculosis was isolation of *Mycobacterium paratuberculosis*, by conventional or radiometric culture, from fecal samples or internal organs of dairy cattle without diarrhea or chronic weight loss. Animals designated as free of the disease originated exclusively from certified paratuberculosis-free herds in Wisconsin. Among 182 infected cattle, RCM and HEY fecal culture and the DNA probe had test sensitivities of 54.4%, 45.1% and 33.5%, respectively. Fecal samples from only 111 of the *M. paratuberculosis*-infected cows tested positive by at least one of the three tests and these cows were designated as fecal shedders; the remaining 71 were considered to have prepatent infections. Among the 111 *M. paratuberculosis* fecal shedders, RCM, HEY and the probe detected the organism in 89.2%, 73.8% and 55.0% of the fecal specimens, respectively. Herd prevalence significantly affected the sensitivity of all three diagnostic tests ($p < 0.05$) but only affected the fecal shedder detection efficiency of the DNA probe ($p < 0.01$). No positive DNA probe results were found on 100 randomly selected fecal samples from cows in four certified paratuberculosis-free herds, thus the DNA probe was 100% specific. Probe analyses could be performed in 24 h or less. Time to complete the culture-based tests was 12 wk for HEY and 7 wk for RCM. The estimated cost to perform each test was U.S. $12, $8 and $25 for HEY, RCM and DNA probe, respectively.**

## RÉSUMÉ

**Les cultures fécales conventionnelles (HEY) et radiométriques (RCM) ainsi qu'une sonde ADN générée par réaction d'amplification enzymatique (par PCR) disponible commercialement ont été évaluées comme outils de diagnostic pour la paratuberculose subclinique chez les bovins laitiers à partir d'échantillons fécaux provenant d'une banque de spécimens. Un cas de paratuberculose bovine subclinique était défini par l'isolement de *Mycobacterium paratuberculosis*, par la culture conventionnelle ou radiométrique faite à partir d'échantillons fécaux. Les animaux exempts de la maladie provenaient exclusivement de troupeaux certifiés exempts de paratuberculose provenant du Wisconsin. Sur 182 bovins infectés, les cultures fécales RCM et HEY, et la sonde ADN ont démontré respectivement une sensibilité de 54,4%, 45,1% et 33,5%. Seulement 111 vaches infectées avec *M. paratuberculosis* ont eu des échantillons fécaux positifs à au moins une des trois épreuves et ces vaches ont été désignées comme étant des porteurs fécaux; les 71 autres vaches ont été considérées avoir une infection prépatente. Parmi les 111 porteurs fécaux de *M. paratuberculosis*, RCM, HEY et la sonde ont détecté l'organisme dans respectivement 89,2%, 73,8% et 55,0% des échantillons fécaux. La prévalence dans le troupeau a affecté significativement ($p < 0,05$) la sensibilité des trois épreuves diagnostiques mais, pour l'efficacité de détection des porteurs fécaux, elle n'a affecté ($p < 0,01$) que la sonde ADN. Aucun résultat positif à l'épreuve de la sonde ADN n'a été trouvé parmi 100 échantillons fécaux sélectionnés au hasard dans quatre troupeaux certifiés exempts de paratuberculose, donc la sonde ADN a été jugée spécifique à 100%. Les analyses par la sonde ADN pouvaient être faites en 24 heures ou moins. Le temps requis pour compléter les épreuves de culture était de 12 semaines pour HEY et sept semaines pour RCM. L'estimation des coûts pour réaliser chaque épreuve était de 12$US, 8$US et 25$US pour HEY, RCM et la sonde ADN. (*Traduit par Dr Emile Bouchard*)**


## INTRODUCTION

For the past decade, researchers have been actively working to develop more accurate and faster diagnostic tests for Johne's disease (paratuberculosis), a gastrointestinal tract infection of ruminants caused by *Mycobacterium paratuberculosis*. Several new tests have been recently described for detection of the organism directly from fecal samples, with the most common being conventional and radiometric

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin 53706-1102 (Sockett, Collins) and Wisconsin Department of Agriculture, Trade and Consumer Protection, Animal Health Division, Madison, Wisconsin 53705 (Carr).

Reprint requests to Dr. M.T. Collins.

This study was supported in part by a grant from the United States Department of Agriculture grant 90-34116-5386.

Submitted October 21, 1991.



Can J Vet Res 1992; 56: 148–153

fecal culture and DNA probes (1-4). Procedures for measuring diagnostic test accuracy differ among published investigations making comparisons among tests difficult (1,3,5-7). One frequent problem has been that the *M. paratuberculosis* infection status of the animals tested has not been unequivocally resolved (8,9). In this report, we describe the evaluation of three organism detection-based diagnostic tests for Johne's disease using samples obtained from a repository for paratuberculosis specimens (10). The tests evaluated include conventional and radiometric fecal culture and a new commercially available DNA probe for *M. paratuberculosis*.

## MATERIALS AND METHODS

### SPECIMENS

The specimen collection used for this study is described in detail elsewhere (10). Briefly, serum and fecal specimens were collected concurrently from 855 dairy cattle (adult cows greater than two years of age) in 13 Wisconsin dairy herds. Nine of the herds, containing a total of 641 dairy cattle, were infected with *M. paratuberculosis* and had prevalences that ranged from 7% to over 60%. Four of the herds, containing a total of 214 cattle, were certified to be free of paratuberculosis by the state of Wisconsin Department of Agriculture, Trade and Consumer Protection based on a regular program of annual whole-herd testing for paratuberculosis by the conventional fecal culture method (11).

Fecal samples were collected per rectum with a disposable plastic obstetrical glove and approximately 50 g were placed in a plastic bag (Whirl-pack, Nasco Laboratories, Fort Atkinson, Wisconsin). Fecal samples were then placed in a styrofoam cooler, chilled with ice packs, taken to the laboratory and processed for isolation of *M. paratuberculosis* by conventional and radiometric methods after which they were frozen at −70°C.

Blood samples were collected at the same time as the fecal samples via the middle coccygeal vein using a 20 gauge, one inch needle and 10 mL Vacutainer tube (Corning Glass Works, Corning, New York). After clotting, and within 12 h, the samples were centrifuged and serum was harvested and stored at −70°C in a sterile cryogenic vial (Tekmar Co., Cincinnati, Ohio).

### CONVENTIONAL CULTURE

Conventioanl fecal culture on Herrold's egg yolk agar (HEY) was performed by the Wisconsin Central Animal Health Laboratory (WCAHL). Although a standard procedure for culture of *M. paratuberculosis* has been described (4), procedures vary somewhat between laboratories. In Wisconsin, the conventional method used 0.25% hexadecyl-cetylpyridinium chloride (HPC) as the decontaminant and two tubes of HEY with mycobactin and one tube without mycobactin as the culture medium. Cultures on HEY were incubated and observed for three months. In addition to the original fecal culture, the nine infected herds were cultured four more times by conventional methods at the WCAHL during the next two years. Isolation of a slow-growing, acid-fast organism with colonial morphology typical of *M. paratuberculosis* on HEY with mycobactin but not on HEY without mycobactin was considered a positive culture.

### RADIOMETRIC CULTURE

Adaptation of the BACTEC system for detection of *M. paratuberculosis* in bovine fecal specimens was recently described (1). It employs BACTEC 12B medium supplemened with 0.1 mL of a 40 μg/mL mycobactin J solution (Allied Laboratories, Glenwood Springs, Colorado), 1.0 mL of egg yolk suspension (Difco, Detroit, Michigan), and 0.1 mL of an antibiotic cocktail containing vancomycin, amphotericin B, and nalidixic acid; final concentrations in the radiometric broth are 10, 20 and 30 μg/mL, respectively. Fecal specimens or tissue homogenates were decontaminated in 1.0% hexadecylcetylpyridinium chloride at a ratio of 3 g per 30 mL. After 24 h, when large fecal or tissue debris had settled to the bottom of the tube, the top 10 mL was filter concentrated using a 10 mL syringe fitted with a 3 μm pore size filter in a Swinex filter holder (Millipore Corp., Bedford, Massachusetts). The entire filter was then placed into the vial containing the radiometric culture medium (RCM) and the vial was resealed. Vials of RCM were read weekly on a BACTEC 460. All positive vials (growth index >30) were subcultured on plate media with and without mycobactin and the identity of *M. paratuberculosis* isolates were confirmed by mycobactin dependency.

### DNA PROBE ASSAY

The DNA probe assays were performed with the commercial kits (U.S. Vet. Licence no. 313) according to the directions of the manufacturer, IDEXX Laboratories (Portland, Maine). In the probe kit, the polymerase chain reaction (PCR) is used to improve the sensitivity of the test (12). Oligonucleotide primers, derived from the insertion element IS900 and specific for *M. paratuberculosis*, are used to amplify a 229 base pair product which is detected by a hybridization probe internal to the PCR primers (3). Fecal samples were frozen prior to probe analyses and had undergone a maximum of two previous thawings. A 1.0 g sample was used for each test. Positive and negative control fecal samples as well as positive (amplified product) control samples were run with each assay. Samples were usually processed in batches of 50.

### SEROLOGY

In an effort to identify animals with the full spectrum of *M. paratuberculosis* infection severity, serum samples, collected at the same time as the fecal samples, were tested by a commercial enzyme-linked immunosorbent assay (ELISA; Allied Laboratories, Glenwood Springs, Colorado) originally described by Yokomizo (13,14). If ELISA positive results were found, but the *M. paratuberculosis* infection could not be confirmed by fecal culture, ileum and regional lymph node biopsies were obtained for histopathology and culture, either by surgery or at the abattoir.

### SURGICAL BIOPSY PROCEDURE

The biopsy procedure collected a full thickness (1 by 2 cm) piece of terminal ileum, taken 10 to 20 cm proximal to the ileocecal junction, and an ileocecal lymph node through a right flank laparotomy incision. Sections stained with both hematoxylin-eosin and acid-fast stains were prepared from half of each tissue, and the remainder was

homogenized and cultured for *M. paratuberculosis* by the radiometric technique (1). Tissues collected at slaughter were the same size and from the same anatomical location and they were processed and cultured in the same way.

### GOLD STANDARD FOR TEST SPECIFICITY ANALYSIS

Isolation of *M. paratuberculosis* from fecal samples is generally considered definitive for diagnosis of paratuberculosis, hence, by definition, the culture-based procedures are 100% specific. For DNA probe specificity analysis, only animals from herds certified to be free of paratuberculosis were used as the negative control population. Herds in Wisconsin certified to be free of paratuberculosis are those in which all animals ≥ 20 months of age have been tested and found to be negative for paratuberculosis by the conventional fecal culture method performed on at least three consecutive samples taken not less than 10 months but not more than 14 months apart. Herd certification is renewed annually and all four herds tested have passed at least one annual recertification (11). One hundred animals were randomly selected from the paratuberculosis-free herds for probe specificity analysis.

### GOLD STANDARD FOR TEST SENSITIVITY ANALYSIS

The diagnostic difficulty in paratuberculosis is detection of *M. paratuberculosis* infection in clinically normal animals. Animals with signs of paratuberculosis, chronic diarrhea and weight loss were excluded from analysis. The case definition for subclinical paratuberculosis was isolation of *M. paratuberculosis* from any fecal sample or tissue sample collected. Thus, using this microbiological case definition, only infected animals were used for diagnostic test sensitivity analysis. Of the 641 animals tested in the nine paratuberculosis infected herds, 182 were confirmed to be infected. Herd prevalence for *M. paratuberculosis* infection ranged from as low as 7% in herd A to greater than 60% in herd I (Table I).

### *M. PARATUBERCULOSIS* DETECTION EFFICIENCY

Among the 182 *M. paratuberculosis*-infected cattle, 111 (61.0%) were positive on at least one of the tests. These were designated *M. paratuberculosis* fecal shedders. The ability of the three tests to detect fecal shedders was compared on this subset of 111 animals.

### STATISTICAL METHODS

Test sensitivity and specificity estimates with 95% confidence limits were calculated by standard methods (15). Diagnostic test sensitivity determinations for the three tests were compared by using McNemar's test (16). In addition, the effect of prevalence on diagnostic test sensitivity was evaluated by comparison of test results in herds with high or moderate to low paratuberculosis prevalence by the binomial distribution method (17).

## RESULTS

### TEST SPECIFICITY

Since isolation of *M. paratuberculosis* from feces or internal organs is considered definitive for diagnosis of paratuberculosis, RCM and HEY techniques are 100% specific (5). All 100 samples randomly selected from cattle in the four certified-free herds were negative when tested by the DNA probe, giving the probe a diagnostic test specificity of 100%.

### TEST SENSITIVITY

Test results for each of the nine *M. paratuberculosis*-infected herds are summarized in Table I. Among the 182 *M. paratuberculosis*-infected animals, 82 were HEY fecal culture positive, 99 were RCM fecal culture positive and 61 were DNA probe positive, giving test sensitivities of 45.1% ± 7.2%, 54.4% ± 7.3% and 33.5% ± 6.9%, respectively (Table II). McNemar's test indicated there were significant differences in sensitivity among all three tests (Table III). The effect of herd prevalence on diagnostic test sensitivity was examined by dividing the herds into two groups; those with a high (>48%) prevalence of infection (herds G,H,I), and those with a low to moderate (<27%) prevalence of infection (herds A,B,C,D,E,F). The average infection prevalence in the high prevalence group was 53.9% and in the moderate prevalence group it was 16.3%. Comparison of the three tests on these two separate populations revealed a significant effect of pre-

**TABLE I. Distribution of test positive animals for each infected herd**

| Farm | No. cows | No. infected cows | Prev % | No. any test[a] + | No. HEY + | No. RCM + | No. probe + | All tests + | Only HEY + | Only RCM + | Only probe + |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 84 | 6 | 7.1 | 2 | 0 | 2 | 0 | 0 | 0 | 2 | 0 |
| B | 45 | 4 | 8.9 | 2 | 0 | 2 | 0 | 0 | 0 | 2 | 0 |
| C | 116 | 19 | 16.4 | 11 | 9 | 10 | 5 | 5 | 1 | 2 | 0 |
| D | 68 | 11 | 16.2 | 3 | 3 | 3 | 2 | 2 | 0 | 0 | 0 |
| E | 61 | 15 | 24.6 | 5 | 3 | 5 | 1 | 1 | 0 | 2 | 0 |
| F | 61 | 16 | 26.2 | 10 | 6 | 9 | 4 | 4 | 1 | 2 | 0 |
| G | 104 | 50 | 48.1 | 28 | 20 | 25 | 16 | 14 | 1 | 6 | 2 |
| H | 66 | 39 | 59.1 | 31 | 25 | 25 | 16 | 13 | 6 | 3 | 0 |
| I | 36 | 22 | 61.1 | 19 | 16 | 18 | 17 | 16 | 0 | 2 | 1 |
| Total | 641 | 182 | 28.1 | 111 | 82 | 99 | 61 | 55 | 9 | 21 | 3 |

[a]Animals positive by HEY, RCM or probe were defined as fecal shedders
Prev = Prevalence of Johne's disease
RCM = Radiometric culture of fecal specimens
HEY = Conventional culture of fecal specimens on Herrold's egg yolk agar
Probe = Commercial DNA probe for *M. paratuberculosis* detection

**TABLE II. Sensitivity of RCM and HEY fecal culture and a DNA probe for diagnosis of *M. paratuberculosis* infections in dairy cattle**

| Test | Moderate prevalence herds | High prevalence herds | All nine herds |
|---|---|---|---|
| RCM | 43.7% ± 11.6% | 61.3% ± 9.1% | 54.4% ± 7.3% |
| HEY | 29.6% ± 10.7% | 55.0% ± 9.3% | 45.1% ± 7.2% |
| Probe | 16.9% ± 8.8% | 44.1% ± 10.1% | 33.5% ± 6.9% |

**TABLE III. Comparison of test sensitivities using McNemar's test**

| Test comparison | Moderate prevalence herds | | High prevalence herds | | All nine herds | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | p | $\chi^2$ | p | $\chi^2$ | p |
| HEY vs RCM | 5.79 | < 0.016 | 1.7 | < 0.19 | 7.3 | < 0.007 |
| HEY vs probe | 4.92 | < 0.027 | 5.0 | < 0.025 | 10.8 | < 0.001 |
| RCM vs probe | 17.05 | < 0.0001 | 12.96 | < 0.0003 | 31.11 | < 0.0001 |

$\chi^2$ = Chi-square distribution, df = 1
HEY = Conventional culture of the fecal specimens on Herrold's egg yolk agar
RCM = Radiometric culture of the repository fecal specimens
Probe = Commercial DNA probe testing of the repository fecal specimens

valence on test sensitivity for all three diagnostic tests (HEY, z = 3.53, $p < 0.001$, RCM, z = 2.35, $p < 0.05$, Probe, z = 4.20, $p < 0.0001$). The diagnostic test sensitivities of HEY and RCM fecal culture and the DNA probe were higher in the high prevalence group than in the moderate prevalence group (Table II). McNemar's test indicated there was a significant difference in sensitivity between the DNA probe and either culture-based test in the two groups of animals. There was no significant difference in the sensitivities of HEY and RCM fecal culture in the high prevalence group ($p < 0.19$), but there was a significant difference in sensitivities of HEY and RCM in the moderate prevalence group ($p < 0.016$) (Table III).

### *M. PARATUBERCULOSIS* DETECTION EFFICIENCY

Fecal shedding was defined as a positive test on the fecal sample by any one of the three test methods. Of the 182 infected cattle, 111 (61.0%) were fecal shedders; 55 (30.2%) were positive on all three tests, 23 (12.7%) were positive on two of the three tests, and 33 (18.1%) were positive on only a single test. Among 111 animals found to be shedding *M. paratuberculosis*, the rate of detection of the organism was 73.8% ± 8.2%, 89.2% ± 5.8% and 55.0% ± 9.3% for HEY, RCM and DNA probe, respectively. These rates were all significantly different from each other ($p < 0.007$) (Table IV). When the herds were divided into high and moderate prevalence groups as previously described, the fecal shedder detection rates for HEY, RCM and DNA probe were 78.2% ± 9.2%, 87.2% ± 7.5% and 62.8% ± 10.8% respectively in the high prevalence group, and 63.6% ± 16.7%, 93.9% ± 8.3% and 36.4% ± 16.7% respectively in the moderate prevalence group. Herd prevalence only caused a significant change in the *M. paratuberculosis* detection ability of the DNA probe test (z = 2.65, $p < 0.01$). Except for comparison of HEY to RCM in high prevalence herds, there was a significant difference in the *M. paratuberculosis* detection ability of the three tests in the high and moderate infection prevalence populations of animals as well as in all nine herds as a whole. McNemar's test analysis on the rates of detection of animals shedding *M. paratuberculosis* in feces was identical to that for test sensitivities shown in Table III.

**TABLE IV. Comparison of the ability of HEY and RCM fecal culture and a DNA probe to detect the *M. paratuberculosis* in bovine feces**

| Test | Moderate prevalence herds | High prevalence herds | All nine herds |
|---|---|---|---|
| RCM | 93.9 ± 8.3% | 87.2 ± 7.5% | 89.2 ± 5.8% |
| HEY | 63.6 ± 16.7% | 78.2 ± 9.2% | 73.8 ± 8.2% |
| Probe | 36.4 ± 16.7% | 62.8 ± 10.8% | 55.0 ± 9.3% |

RCM = Radiometric culture
HEY = Culture on Herrold's egg yolk agar
Probe = Commercial DNA probe assay

## DISCUSSION

When evaluating diagnostic accuracy it is important to include patients with the entire spectrum of disease as it is normally found in the population being tested (18). For subclinical Johne's disease this should include unequivocally disease-free populations of animals and infected populations of animals composed of both those shedding *M. paratuberculosis* in their feces and those not shedding. The distribution of infection severity among animals that contributed fecal and serum samples for the repository for paratuberculosis specimens meets this criterion and is probably representative of the proportion of animals at each stage of *M. paratuberculosis* infection normally found in infected herds prior to implementation of paratuberculosis control procedures. Including only fecal culture positive animals in sensitivity analysis, and using fecal culture negative animals for specificity analysis will not give a proper estimate of test accuracy, particularly for those tests not based on detection of the organism. This is most important for specificity analysis since one cannot be certain that a fecal culture negative animal is disease-free especially if it resides in a known infected herd, due to the long prepatent period of the disease. To illustrate this point, if sensitivity analysis was done using only HEY fecal culture positive cows, and specificity analysis was done on animals in the same herd but having a minimum of three negative HEY fecal cultures spaced over a 24 month period, the sensitivity and specificity of the commercial DNA probe would have been 43.0% and 98.8%, respectively. In reality, the DNA probe had a sen-

sitivity and specificity of 33.5% and 100%, respectively.

There was a significant difference in the sensitivity of the three diagnostic tests evaluated, with RCM fecal culture being the most sensitive and the commercial DNA probe being the least sensitive. Since all three tests evaluated are based on organism detection, differences in test sensitivity can be explained by differences in the specimen processing techniques and sample size. The radiometric technique which uses filter concentrated fecal samples can detect as few as ten viable organisms per gram of feces (1,19), conventional fecal culture using the sedimentation technique has a detection limit of $10^3$ viable organisms per g of feces (4), and the DNA probe test requires at least $10^2$ organisms per g of feces for a positive test, when the procedure is performed according to the manufacturer's recommendations and assuming the PCR reaction is 100% efficient (3). On only three fecal samples was the DNA probe positive and both fecal culture techniques negative. This occurrence probably reflects a nonuniform distribution of *M. paratuberculosis* in bovine fecal samples of infected cows.

The costs of the three tests are approximately $12.00 for conventional fecal culture on HEY, $8.00 for radiometric fecal culture and $25.00 for the commercial DNA probe which includes the cost of the kits, labor and all the expendables required to run the test. The advantage of the DNA probe is speed. The commercial test can be performed in 24 h (3) compared with conventional fecal culture which takes at least 12 weeks (4,5) and the radiometric technique which takes seven weeks to declare specimens negative (1).

Most investigators believe that cattle primarily become infected with *M. paratuberculosis* by ingesting the organism as neonates, although *in utero* transmission of the bacterium has been shown to occur (20). Animals actively shedding the organism in their feces are more infectious and thus tests based on organism detection in feces are highly desirable. In this report, RCM fecal culture detected the highest number of fecal shedders (89.2%) and the commercial DNA probe detected the least (55.0%). In addition, the probe assay was the only test in which the *M. paratuberculosis* detection ability was significantly affected by herd prevalence of infection, although a similar trend was found with conventional culture. The detection ability of the DNA probe increased as the infection prevalence in the herd increased suggesting that heavily infected herds contain a larger percentage of animals shedding large numbers of *M. paratuberculosis*, and low prevalence herds contain a higher proportion of light or nonshedders.

It is currently accepted that the sensitivity and specificity are fixed intrinsic characteristics of a diagnostic test (21-25). This assumption may only be correct when the characteristics of the populations being tested are not substantially different. Human populations are relatively homogeneous by comparison with domesticated animals, e.g. in Wisconsin alone, there are approximately 34,000 dairy herds (26). The prevalence of paratuberculosis among herds has been shown to vary widely, as seen in the nine selected for this study. In this report, there was a significantly lower test sensitivity for all three diagnostic tests in the group of herds with moderate *M. paratuberculosis* infection prevalence as compared to the high prevalence herds. This observation has been reported for the commercial agar gel immunodiffusion (AGID) test for Johne's disease as well; the AGID test had a much higher sensitivity in populations containing clinically ill animals than in populations where the animals were not clinically ill (9,27,28). This observation is most likely due to the biology of Johne's disease.

Johne's disease is a slow, progressive infection of the terminal ileum of cattle and other ruminants. Animals are infected usually at the time of birth but do not begin to shed the organism in detectable amounts in their feces until several years later (5). There are several factors that influence the time it takes for an animal to begin shedding the organism, but two of the most important factors are thought to be the infectious dose ingested by the neonate, and how soon after birth infection occurs (5). It is well established that animals develop increasing resistance to infection as they mature (29,30). In heavily infected herds, there are larger numbers of viable *M. paratuberculosis* in the environment, and calves are more likely to be exposed to *M. paratuberculosis* soon after birth. It is conceivable that in high prevalence herds virtually every calf born on a farm becomes infected. This correlates well with field observations that as Johne's disease becomes more severe in dairy herds, animals begin fecal shedding and develop Johne's disease at a younger age (5). Infection pressure therefore could explain why the sensitivity of the three diagnostic tests evaluated increased with increasing herd prevalence. Test positive adult animals in high prevalence herds may have more advanced cases of *M. paratuberculosis* infection and thus be more readily diagnosed.

The observed decrease in sensitivity for the three tests as Johne's disease herd prevalence declined has important implications for disease eradication efforts in infected herds. Diagnostic tests for Johne's disease are used to select animals for culling. Obviously, if the sensitivity of a test decreases as the number of infected animals in the herd decreases, it might become very difficult to completely eradicate paratuberculosis from infected herds using only test and cull methods. This is consistent with the experience of many veterinarians and livestock producers involved in paratuberculosis control or eradication programs (5). Also, the effect of prevalence on test sensitivity will affect the ability of the test to estimate the true disease prevalence from the apparent prevalence (number of test positive animals) (31). Without adjusting for changing test sensitivity, in low *M. paratuberculosis* infection prevalence herds, the tests would underestimate the true prevalence of Johne's disease, and in high prevalence herds the tests would overestimate the true prevalence. This observation would also affect the predictive value model used to calculate the probability a given test result for an individual animal is correct (21,23,24). Since all three tests are 100% specific, prevalence would obviously not affect the positive predictive value of these three tests. Prevalence would, however, affect the predictive value of negative test results (NPV); in low prevalence herds the NPV would be calculated to be higher than it really was, and in high prevalence herds the NPV will be calculated to be lower than it really was.

All three of the diagnostic tests evaluated were 100% specific but were significantly different in both fecal *M. paratuberculosis* detection efficiency and sensitivity for diagnosis of infection. The sensitivity of diagnostic tests for Johne's disease, based on detection of *M. paratuberculosis* in fecal samples, is adversely affected by the long prepatent period of the infection. Such tests have the advantage, however, of detecting the more infectious animals in a population and are thus useful tools in disease control efforts. Differences in test sensitivity between RCM, HEY and the probe were most likely due to differences in specimen processing procedures. Fecal culture by the radiometric technique was the most sensitive and least expensive of the tests evaluated. The DNA probe for *M. paratuberculosis* has the same problems of low sensitivity and high cost as other nucleic acid probes used in clinical microbiology (32). This will likely restrict the use of the probe in veterinary diagnostic laboratories to special circumstances.

## ACKNOWLEDGMENTS

We thank K.B. Kenefick, T.A. Conrad and B.-X. Ji for their help with the radiometric fecal culture and DNA probe evaluation. We also thank the Wisconsin Central Animal Health Laboratory for performing the conventional fecal cultures, in particular T.J. Larsen, V.B. Rice, R.L. Lindauer and S. Lesar.

## REFERENCES

1. **COLLINS MT, KENEFICK KB, SOCKETT DC, LAMBRECHT RS, McDONALD J, JORGENSEN JB.** Enhanced radiometric detection of *Mycobacterium paratuberculosis* using filter concentrated fecal specimens. J Clin Microbiol 1990; 28: 2514–2519.
2. **KIM YG, BECH-NIELSEN S, GORDON J, SELMONS RD, SPANGLER E.** Comparison of two methods for isolation of *Mycobacterium paratuberculosis* from bovine fecal samples. Am J Vet Res 1989; 50: 1110–1113.
3. **VARY PH, ANDERSEN PR, GREEN E, HERMON-TAYLOR J, McFADDEN JJ.** Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. J Clin Microbiol 1990; 28: 933–937.
4. **WHIPPLE DL, MERKAL RS.** Procedures for the field and laboratory processing of fecal specimens for the isolation of *Mycobacterium paratuberculosis*. In: Proc 28th Annu Meet Am Assoc Vet Lab Diagnost 1985: 239–245.
5. **CHIODINI RJ, VAN KRUININGEN HJ, MERKAL RS.** Ruminant paratuberculosis (Johne's disease): The current status and future prospects. Cornell Vet 1984; 74: 217–262.
6. **COLGROVE CS, THOEN CO, BLACKBURN BO, MURPHY CD.** Paratuberculosis in cattle: A comparison of three serologic tests with results of fecal culture. Vet Microbiol 1989; 19: 183–187.
7. **JONES RL.** Review of recent research studies in the United States related to Johne's disease with emphasis on diagnosis and control of the disease. In: Milner AR, Wood PR, eds. Johne's Disease. Current Trends in Research, Diagnosis and Management. 1st ed. Melbourne: CSIRO, 1989: 1–8.
8. **HURLEY SS, SPLITTER GA, WELCH RA.** Development of a diagnostic test for Johne's disease using a DNA hybridization probe. J Clin Microbiol 1989; 27: 1582–1587.
9. **SHERMAN DM, GAY JM, BOULEY DS, NELSON GH.** Comparison of the complement fixation and agar gel immunodiffusion tests for the diagnosis of subclinical bovine paratuberculosis. Am J Vet Res 1990; 51: 461–465.
10. **SOCKETT DC, CARR DJ, RICHARDS WD, COLLINS MT.** A repository of specimens for comparison of diagnostic testing procedures for bovine paratuberculosis. J Vet Diagnost Invest 1992; (in press).
11. **COLLINS MT, McLAUGHLIN AR.** Experience in Wisconsin in control and accreditation of Johne's disease infected herds. In: Milner AR, Wood PR, eds. Johne's Disease. Current Trends in Research, Diagnosis and Management. 1st ed. Melbourne: CSIRO, 1989: 67–73.
12. **SAIKI RK, GELFAND DH, STOFFEL S, SCHARF SJ, HIGUCHI R, HORN GT, MULLIS KB, ERLICH HA.** Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988; 239: 487–491.
13. **YOKOMIZO Y, NISHIMORI K, KISHIMA M, YUGI H.** Evaluation of an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis. A proposal of replacing the complement fixation test with the ELISA as the official diagnostic test for paratuberculosis in Japan. In: Merkal RS, Thorel MF, eds. Proceedings of the Second International Colloquium on Paratuberculosis. 1st ed. Maisons-Alfort: Laboratoire Central de Recherches Vétérinaires, 1988: 206–214.
14. **YOKOMIZO Y, YUGI H, MERKAL RS.** A method of avoiding false-positive reactions in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis. Jpn J Vet Sci 1985; 47: 111–119.
15. **SACKETT DL, HAYNES RB, TUGWELL P.** The interpretation of diagnostic data. In: Clinical Epidemiology. A Basic Science for Clinical Medicine. 1st ed. Boston: Little, Brown and Company, 1985: 59–138.
16. **REMINGTON RD, SCHORK MA.** Chi-square tests for frequency data. In: Statistics with Applications to the Biological and Health Sciences. 1st ed. Englewood Cliffs, New Jersey: Prentice-Hall, Inc., 1970: 229–252.
17. **REMINGTON RD, SCHORK MA.** Hypothesis testing. In: Statistics with Applications to the Biological and Health Sciences. 1st ed. Englewood Cliffs, New Jersey: Prentice-Hall, Inc., 1970: 192–228.
18. **RANSOHOFF DF, FEINSTEIN AR.** Problems of spectrum and bias in evaluating the efficacy of diagnostic tests. New Engl J Med 1978; 299: 926–930.
19. **LAMBERT JR, BORROMEO M.** Possible association between *Mycobacterium paratuberculosis* infection and Crohn's disease in humans. In: Milner AR, Wood PR, eds. Johne's Disease. Current Trends in Research, Diagnosis and Management. 1st ed. Melbourne: CSIRO, 1989: 116–120.
20. **SEITZ SE, HEIDER LE, HUESTON WD, BECH-NIELSEN S, RINGS M, SPANGLER L.** Bovine fetal infection with *Mycobacterium paratuberculosis*. J Am Vet Med Assoc 1989; 194: 1423–1426.
21. **DIERKSHEIDE WC.** Medical decisions: Interpreting clinical tests. ASM News 1987; 53: 677–680.
22. **MARTIN SW, MEEK AH, WILLEBERG P.** Measurement of disease frequency and production. In: Veterinary Epidemiology — Principles and Methods. 1st ed. Ames, Iowa: Iowa State University Press, 1987: 48–76.
23. **THORNER RM, REMEIN QR.** Principles and procedures in the evaluation of screening for disease. Public Health Monograph No. 67. Washington, DC: US Government Printing Office, 1961: 1–24.
24. **VECCHIO TJ.** Predictive value of a single diagnostic test in unselected populations. New Engl J Med 1966; 274: 1171–1173.
25. **ROGAN WJ, GLADEN B.** Estimating prevalence from the results of a screening test. Am J Epidemiol 1978; 107: 71–76.
26. **WISCONSIN AGRICULTURAL STATISTICS SERVICE.** Wisconsin 1990 Agricultural Statistics. Madison: Wisconsin Dept. Agriculture, Trade and Consumer Protection, 1990: 1–94.
27. **SHERMAN DM, BRAY B, GAY JM, BATES F.** Evaluation of the agar gel immunodiffusion test for the diagnosis of subclinical paratuberculosis. Am J Vet Res 1989; 50: 525–530.
28. **SHERMAN DM, MARKHAM RJF, BATES F.** Agar gel immunodiffusion test for diagnosis of clinical paratuberculosis in cattle. J Am Vet Med Assoc 1984; 185: 179–182.
29. **HAGAN WA.** Age as a factor in susceptibility to Johne's disease. Cornell Vet 1938; 28: 34–40.
30. **LARSEN AB, MERKAL RS, CUTLIP RC.** Age of cattle as related to resistance to infection with *Mycobacterium paratuberculosis*. Am J Vet Res 1975; 36: 255–257.
31. **MARCHEVSKY N.** Errors in prevalence estimates in population studies: A practical method for calculating real prevalence. Zoonosis 1974; 16: 85–109.
32. **ENGLEBERG NC.** Nucleic acid probe tests for clinical diagnosis — where do we stand? ASM News 1991; 57: 183–186.