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Evaluation of a rapid fecal PCR test for detection of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle

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ABSTRACT

A high throughput TaqMan® PCR assay for detection of bovine paratuberculosis was evaluated using fecal samples from 1808 dairy cattle in 7 naturally infected herds and 347 dairy cattle in 7 herds considered free of paratuberculosis. Fecal, blood, and milk samples were submitted to laboratories where the PCR-based assay, three different fecal culture procedures for *Mycobacterium paratuberculosis* (centrifugation, sedimentation and BACTEC-filter concentration method), two serologic ELISAs, and one milk ELISA were performed. Results from testing dairy cattle in herds free from *M. paratuberculosis* showed the PCR assay specificity was 99.7%. Twenty-three percent of the dairy cows fecal culture-positive by at least one of the 3 methods were test positive using the PCR assay. Using Bayesian non-gold standard analysis methods, the TaqMan® PCR assay had higher test specificity than serum ELISAs (99.3%; 95% confidence interval = 98.6-99.7%) and similar test sensitivity as serum ELISAs (29%; 95% confidence interval = 24-35%). Using classical methods, the estimate of relative sensitivity for the fecal PCR assay was 4% for light and moderate fecal shedders (compared to 12-13% for ELISAs) and 76% for heavy fecal shedders (compared to 67% for milk ELISAs). The PCR assay has higher sensitivity for detection of heavy fecal shedders than the evaluated milk ELISA, though lower sensitivity than serum or milk ELISAs for detection of light and moderate fecal shedders. This assay can be used as a quick test for detection of heavy fecal shedding cattle, those cattle with the highest risk of transmitting infection to susceptible cattle.

Keywords: *Mycobacterium paratuberculosis,* PCR, fecal culture, ELISA, cattle, Johne's disease, diagnosis, bovine.

Introduction

Johne's disease (JD), also called paratuberculosis, is one of the most economically important diseases in dairy cattle, costing over $250 per cow in inventory per year in highly infected herds (15). This disease causes enteritis, weight loss, reduced milk production, and premature culling in dairy cattle and other ruminant species. Transmission occurs primarily through fecal-oral routes and most herds are infected through introduction of subclinically infected cattle. Results from a 1996 USDA study showed an estimated 20-40% of dairy herds are infected with paratuberculosis, depending upon herd size using a herd testing method designed to detect herds with 10% seroprevalence with 90% confidence (20), with annual losses in U.S. dairy cattle herds exceeding $220 million (15). Due to ongoing expansion of dairy herds and widespread movement of cattle between herds, paratuberculosis transmission to uninfected herds is likely to continue. Additionally, concern has arisen that *M. avium* subsp. *paratuberculosis* (*M. paratuberculosis*) may be a cause of Crohn's disease in humans. The importance of controlling this disease has been recognized through a recent National Research Council report (1), yet will be difficult to achieve. Treatment is not cost-effective in infected cattle producing dairy products for human consumption, and vaccination is not widely used.

Diagnosis of *M. paratuberculosis* infection is challenging because of slow growth and the lack of diagnostic tests sensitive enough to detect most subclinically infected cattle, many of which intermittently shed the pathogen, thus serving as sources of infection to susceptible cattle. Detection of the pathogen itself is the most definitive method of diagnosis since the pathogen can often be detected during both subclinical and clinical stages of disease, but the typical method of pathogen detection (bacterial culture of feces) requires up to 16 weeks of incubation and is labor

intensive. Contamination of cultures is an added problem due to the frequent inability of current decontamination protocols to inactivate all non-mycobacterial microflora in feces, resulting in contamination of some cultures during the lengthy incubation period. These issues have led to widespread use of diagnostic methods to detect host immune response to *M. paratuberculosis*. Available serologic assays, however, are problematic as well, both in detecting infected cattle and misclassifying uninfected cattle (23). Because no rapid diagnostic test to detect most subclinically infected cattle prior to fecal shedding is currently available, test-and-cull strategies for control of JD are not by themselves cost-effective (12), and cattle producers must implement lengthy (up to 10 years) herd control programs. Currently needed are high volume diagnostic tests suitable for detection of this pathogen in dairy and beef herds to facilitate efficient operation of state and national control programs, including animal movement controls. As new diagnostic tests and testing methods become available, rational design of herd "certification" programs for non-infected herds and paratuberculosis control programs for *M. paratuberculosis*-infected herds require precise, objective estimation of the sensitivity and specificity of available diagnostic tests to define which tests are most cost-effective for use in these programs (5, 6).

Cattle shed *M. paratuberculosis* in feces at varying levels, depending upon the stage of disease of individual animals (14). Quantification of fecal shedding is routinely performed by certain laboratories, especially those using Herrold's egg yolk (HEY) media, and are commonly categorized into levels (light, moderate, and heavy fecal shedding) corresponding to the number of colonies observed per tube. This quantification provides an estimate of the risk of transmission from cattle at various stages of clinical disease through fecal-oral routes. In addition, this information helps to estimate the risk of transmission through other routes (milk,

colostrum, and placenta), because cattle at later stages of infection are more likely to be infectious to susceptible cattle than cattle at earlier stages. Rapid identification of these high risk cattle enable management of cattle by risk category to reduce transmission, thereby contributing to overall JD control.

High throughput PCR tests have the potential to provide rapid (less than 1 week) detection of *M. paratuberculosis* at a cost comparable to or less than that of conventional culture. Estimation of the sensitivity and specificity of these assays is necessary, however, before implemented routinely for JD diagnosis. The objective of this study was to evaluate the validity of a TaqMan® based PCR assay as compared to that of other available assays for detection of *M. paratuberculosis* in infected and uninfected dairy cattle.

Materials and Methods:

Development of PCR diagnostic assay. A previously identified target gene (MAV2, an insertion sequence from *M. paratuberculosis* (17) was systematically analyzed for optimal oligonucleotide primer and TaqMan® probe sequences by computational methods using ABI Primer Express software (Foster City, CA) and tested for uniqueness by BLAST (Basic local alignment search tool) analysis against all identified genes deposited in GenBank (24). *M. paratuberculosis* and other control microorganisms were identified and cultured at the Minnesota Veterinary Diagnostic Laboratory (MVDL) for use as positive and negative controls. DNA were extracted from cultured *M. paratuberculosis* and negative control microorganisms (*M. avium* subsp *avium*, *M. terrae* complex, MAIS, and *M. avium* complex). The optimal conditions for amplification and detection of of the *M. paratuberculosis* ISMAV2 gene were refined. The primer pair ISMav2

Forward (5'-GATGAGTGGGTCGAGGACTACAA; 40 mM)/ISMav2 Reverse (5'-CCGTTGAGCCGGTGTGAT; 40 mM) was used to amplify the target gene from 5 µl template DNA in the presence of a TaqMan fluorescence probe (6FAM-CCAAGCCCTAAAGAT-MGB; 5mM; ABI, Foster City, CA). The MGB (minor groove binder) probe was utilized to increase the Tm of the probe, which allows clearer differentiation between positive and negative diagnostic samples. The cycling conditions for amplification and detection were: 1 cycle for 15 min at 95°C, 50 cycles at 94°C for 15 secs followed by 1 min at 57°C, and then held at 4°C. Extraction of 1 gram of fecal material was completed using the QIAamp DNA Stool Mini Kit (Qiagen) and supplemented with bacterial lysis (FastPrep® System, QBiogene, MP Biomedicals). The PCR kit used was the Taqman Universal PCR Master Mix (Applied Biosystems). *M. paratuberculosis* negative fecal matter spiked with selected agents known to be present in uninfected herds and with *M. paratuberculosis* was used to confirm specificity. The MAV2 TaqMan® PCR test was then repeated on spiked feces in tandem with the existing experimental Johne's IS900 TaqMan® PCR test based on the detection of a known insertion sequence (IS*900*).

Two types of controls were used for the fecal PCR assay. Extraction controls were tested once per extraction. For negative extraction controls, DNA was extracted from fecal samples from cattle from known uninfected dairy herds (Level 4 of the Voluntary Johne's Disease Test Negative Program for Cattle) and tested using the MAV2 TaqMan® PCR assay. Positive extraction controls were created by extracting DNA from fecal samples from known uninfected cattle that was spiked with *M. paratuberculosis* and tested using the MAV2 TaqMan® PCR assay. In the MAV2 TaqMan® PCR assay, positive template controls consisted of dilutions of *M. paratuberculosis* DNA (previously extracted), with 2 positive template controls per plate.

Eight no template controls (NTC: sterile water) were tested per test plate. To determine the threshold above which diagnostic samples were considered positive, the high and low NTC values were first deleted and the mean of the remaining 6 values was determined for use in analyses described below.

The reactions in the plates were pre-read at 518 nm (6-FAM). After the reactions were analyzed by PCR as outlined above, the reactions were post-read at the same wavelength. ABI 7000 System Detector Software normalized the reporter dye (6-FAM) and calculated Rn, the fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. The degree of fluorescence due to probe hydrolysis (interpreted as the degree of amplification) was determined by calculating the delta Rn, the difference between the Rn value after the PCR reaction took place and the Rn value before the PCR reaction for each sample. The "cutpoint" was calculated from the following equation: 6 x [standard deviation of the nontemplate controls (NTC) x *t*] + average NTC value. The value *t* (5.894) was provided by ABI. The Johne's ISMAV2 TaqMan® PCR assay detection limit was 7.5 x 10^3 CFU per ml of isolated *M. paratuberculosis*, estimated at 7.5 x 10^3 CFU per g of feces.

Sample collection for validation of PCR assay. The sensitivity and specificity of the PCR test for detection of *M. paratuberculosis* were estimated using fecal samples collected from dairy cattle in US dairy herds, with sampling methods previously described by the study investigators (7). To estimate test specificity, we used fecal samples from dairy herds known to be free of paratuberculosis. Study herds included 7 dairy herds at Level 4 of the Voluntary Johne's Disease Test Negative Program for Cattle (VJDTNP; n = 347 cows). Fecal samples from each cow were

tested by 3 bacterial culture methods to provide further evidence of lack of infection. To estimate test sensitivity, we collected fecal samples from 1808 cattle in 7 known infected dairy herds. Criteria for herd selection included *M. paratuberculosis* seroprevalence of >10% and no regular testing and culling for Johne's disease, to provide a natural spectrum of infected cattle in a study population not artificially influenced by prior culling of test-positive cattle.

From each cow that had calved at least once within the study herds, fecal, blood, and milk samples were collected simultaneously, labeled, and transported with refrigeration for processing. Fecal samples were submitted fresh, within 48 hours of collection, to 3 different laboratories for laboratory testing, including the Minnesota Veterinary Diagnostic Laboratory (MVDL), the University of Wisconsin-School of Veterinary Medicine (UW-SVM), and the University of Pennsylvania (UP). The MVDL performed conventional bacterial culture using the Herrold's egg yolk agar (HEY)-sedimentation method (SED, 21) and performed the fecal PCR assays. The UW-SVM cultured fecal samples by the BACTEC-filter concentration method (BACTEC, 4) and the UP performed *M. paratuberculosis* culture using the HEY-centrifugation technique (CENT, 9, 22, 23). Blood samples were centrifuged and sera were harvested to perform ELISA assays at the University of Wisconsin Johne's Testing Center using two different kits for serum antibody detection (ELISA A=IDEXX Laboratories, Inc., Westbrook, ME and ELISA B=CSL/Biocor, Omaha, NE). Milk samples were refrigerated and sent to a testing laboratory to perform a milk ELISA test (ELISA E, Antel Biosystems, Inc., Lansing, MI). All antibody assays were performed by each laboratory according to manufacturers' instructions and interpreted as prescribed.

Classical analysis. PCR assay specificity was defined as the percentage of samples yielding a negative PCR assay result among the *M. paratuberculosis*-free fecal samples from noninfected herds. PCR assay relative sensitivity was estimated (with 95% confidence intervals) within the infected cattle population by comparing fecal PCR results to the *M. paratuberculosis* status of each sample defined by all three culture methods collectively. Differences in relative specificity and sensitivity between PCR and ELISA assays were evaluated using McNemar's tests of association. Because of the variability in fecal culture results among laboratories and culture methods, we defined a fecal sample as positive for *M. paratuberculosis* if at least one of three laboratories performing a fecal culture procedure designated the sample as positive.

Relative sensitivity of the PCR assay was also estimated by *M. paratuberculosis* fecal shedding level by comparing the mean *M. paratuberculosis* culture scores on each fecal sample to PCR assay results. Each laboratory using solid media ranked the number of *M. paratuberculosis* recovered as 1+ (less than 10 colonies per tube), 2+ (mean of 10-49 colonies per tube), 3+ (mean of 50-99 colonies per tube), or 4+ (>100 colonies per tube), considering all culture tubes inoculated. The laboratory using liquid media also developed a comparable ranking system (1+, 2+, 3+, 4+) for test results by categorization of time to detection. A composite *M. paratuberculosis* score based on reports from the three independent laboratories was calculated from the arithmetic mean of scores. PCR test comparisons were made to results from fecal culture and serum and milk serologic assays. Standard errors for confidence interval estimation were calculated using the formula SE = pq/n. Assay differences in relative sensitivity between fecal shedding levels were evaluated using McNemar's test of association, after collapsing data

into categories based on fecal culture shedding levels described above (no shedding, light to moderate shedding (1+ to 2+), and heavy shedding (3+ to 4+)).

Bayesian analysis. True sensitivity and specificity of the PCR assay, centrifugation fecal culture (CENT), serum ELISA assay A, and serum ELISA assay B were estimated using a Bayesian method. The Bayesian method can be used to estimate the accuracy of diagnostic tests in an absence of a gold standard. The method takes into account for uncertainty in sensitivity and specificity of the tests estimated and allows prior information or expert knowledge to be incorporated into the analysis. Moreover, the parameter estimate based on Bayesian analysis has a probability distribution which allows direct probability interpretation (13). The analysis using Bayesian method involves 3 main components**:** likelihood function $L(y|\theta)$, prior distribution $g(\theta)$, and posterior distribution $f(\theta|y)$. These 3 components are combined through Bayes' rule: $f(\theta|y) \alpha L(y|\theta) \times g(\theta)$, where α denotes proportionality (3). In the present study, the likelihood function was derived from the test results from the 7 infected dairy herds; the prior distributions presented prior knowledge on the test sensitivity and specificity of each testing method; and the posterior distributions combined all information about the parameters of interest (sensitivity and specificity) from the likelihood function and the prior distributions. Then, the estimation of the parameters of interest from the posterior distributions was carried out using the Gibbs sampler, an iterative algorithm that constructs a Markov chain and permits empirical estimation of posterior distributions (3).

In the present study, we used a Bayesian model for estimation of sensitivity and specificity of 4 tests in multiple populations, which was modified from a model for estimation of test validity of

2 correlated tests in multiple populations as described elsewhere (2). Our model accounted for the possible effect of conditional dependence between 2 tests that measured similar biological process by including sensitivity covariance (Covse) and specificity covariance (Covsp) for bacterial culture and fecal PCR assay and for the 2 serum assays in the model. The magnitude of Covse and Covsp are affected by the magnitude of test sensitivities and test specificities, respectively, and their limits were defined previously (10).

The prior information about test sensitivities and specificities was provided by consensus of 5 experts selected by USDA-APHIS for development of optimal testing strategies for control of Johne's disease in cattle (Michael Collins, Ian Gardner, Franklyn Garry, Allen Roussel, and Scott Wells). The experts provided the most likely value, and either the lowest or the highest possible value for all parameters in the model with 95% confidence. Uncertainty about the prior information was represented by use of beta (α, β) distributions where the values of α and β determine the shape of the distribution. In this study, the prior beta distributions were assessed by use of software called Betabuster (version 1; downloadable at http://www.epi.ucdavis.edu/diagnostic tests). Elicitations of prior information for all parameters in the models and the corresponding beta distributions are presented in Table 4.

All Bayesian analyses were performed using WinBUGS (1996–2001, version 1.4, Imperial College & MRC, UK, available at www.mrc-bsu.cam.ac.uk/bugs). Model convergence was assessed through visual examination of trace plots, assessment of Monte Carlo error, and by running multiple chains from dispersed starting values (11). We generated 2 parallel runs of 50,000 iterations of each model, and the first 10,000 were discarded as the burn-in period.

Posterior inferences were based on summaries of the final 40,000 iterations, and presented as a median, and a 95% probability interval (2.5 and 97.5 percentiles) of each parameter estimate. In addition to the estimation of test sensitivities and specificities, the probability that the differences in test sensitivity or specificity between the PCR assay and other tests (CENT, ELISA A, and ELISA B) were assessed using the step function in WinBUGS. If the probability was < 0.05, we can conclude that the sensitivity (or specificity) of the PCR assay was significantly lower than that of the compared test. On the other hand, if the probability was > 0.95, we can conclude that the sensitivity (or specificity) of the PCR assay was significantly higher than that of the compared test. If the value falls between 0.05 and 0.95, no significant difference between the test sensitivities (or specificities) can be concluded.

Results

Evaluation of extraction control data showed all 33 positive extraction controls were positive at 1-7 SD above the mean negative template control value. All negative extraction controls were negative using a cutpoint of 6 SD above the mean negative template control value. At 1 SD above the mean negative template control value on each plate, 36% (12/33) of plates were positive, 12% (4/33) were positive at 2 SD, 6% (2/33) were positive at 3 SD, and 3% (1/33) were positive at 4 and 5 SD.

Using samples from noninfected herds, PCR test specificity varied by cutpoint used. At a cutpoint of 1 SD above the mean negative template control, the test specificity was 48.4% (95% CI = 43.2 – 53.7%). At cutpoints of 2, 3, 4, 5, and 6 SD above the mean negative template control, the test specificity was 80.4% (95% CI = 76.2 – 84.6%), 94.0% (95% CI = 91.4 –

96.5%), 98.3% (95% CI = 96.9 – 99.6%), 99.1% (95% CI = 98.2 – 100%), and 99.7% (95% CI = 99.1 – 100%). While recognizing that a high cutpoint leads to high test specificity at the expense of sensitivity, our objective was a highly specific test, and the 6 SD cutpoint was used to categorize test results in further data analyses. In comparison to antibody detection tests (Table 1), the fecal PCR assay specificity was higher (99.7%) than that of one of the currently available serum ELISA assays (A) and similar to those of the other serum ELISA assay (B) and the milk ELISA assay (E).

In the infected dairy herds, 25% of cows were culture-positive by at least one of 3 culture-based tests but only 5% of cows were culture-positive using all 3 culture methods (Table 2). Approximately 10% of cows in these herds were test-positive using 2 of the culture methods, while 18% were test-positive using the most sensitive method (centrifugation). Study cows reflected cows at varying stages of infection with *M. paratuberculosis*, including 5% heavy fecal shedders. Over 300 BACTEC culture tests were invalid due to bacterial or fungal overgrowth, limiting the test comparisons for this method to 1481 observations. In addition, approximately 100 test results were missing from each of the serum ELISA test results and over 200 test results were not available for the milk ELISA test since these cows were not lactating at the time of collection.

Classical analysis results. Overall, 23% of cows detected as positive using at least one of the 3 culture methods were test-positive using fecal PCR assay (Table 3). This relative sensitivity was slightly lower than that for the serum ELISAs (28%) though not significantly different than that for the milk ELISA (26%). A comparison of results among tests by fecal shedding level

indicated that the relative sensitivity of the fecal PCR assay as defined using the composite fecal culture level of shedding was much lower in light and moderate fecal shedders (4%) than that of the serum and milk ELISAs (12-13%). On the other hand, the fecal PCR assay had a relative sensitivity for heavy fecal shedders of 76%, which was higher than that for the milk ELISA (67%) though not statistically different from that of serum ELISAs.

Bayesian analysis results. For each parameter estimate, the Monte Carlo error was small, autocorrelation values indicated that iterates were not overly correlated with subsequent values, and visual examination of the trace plots indicated convergence of the models. Bayesian analysis indicated conditional independence between PCR and CENT (Covse median of -0.02 with 95% PI between -0.06 and 0.001; Covsp median of -0.0003 with 95% PI between -0.00006 and 0.002). The Covse between ELISA A and ELISA B was small and positively correlated (Covse median of 0.15 with 95% PI between 0.13 and 0.17), whereas the Covsp between the tests was clustered around zero (Covsp median of 0.0004 with 95% PI between -0.0009 and 0.005).

From Bayesian analyses, the estimated sensitivity of the fecal PCR assay (29%) was much lower than the estimated sensitivity of bacterial culture using centrifugation (CENT, 75%) and similar to that of ELISA A (26%) and ELISA B (27%). The probability that the sensitivity of the fecal PCR assay was greater than sensitivity of CENT, ELISA A, and ELISA B was 0, 0.74, and 0.49, respectively, which indicated that PCR sensitivity was significantly lower than CENT sensitivity, but not significantly different from the sensitivity of ELISA A and ELISA B. The estimated specificity of the fecal PCR assay was 99.3%, compared to estimated specificities of CENT

(99.8%), ELISA A (94.9%), and ELISA B (98.0%). The probability that the specificity of the fecal PCR assay was greater than the specificity of CENT, ELISA A, and ELISA B was 0.04, 1.0, and 0.99, respectively, which indicated that the specificity of PCR was significantly lower than specificity of CENT, and significantly higher than the specificity of either ELISA A or ELISA B.

Discussion

This study presents the most thorough field validation of a fecal PCR assay for detection of *M. paratuberculosis* to date. The study design included large samples of well-characterized naturally infected and noninfected cattle characterized using multiple tests, including 3 different culture methods used to characterize fecal shedding levels of cattle. Our specificity estimates derived from sampling known (and confirmed) paratuberculosis noninfected herds.

One of our objectives was to estimate the sensitivity of the fecal PCR assay for detection of *M. paratuberculosis*. Our estimate from classical methods indicated an overall relative sensitivity of 23%, which was slightly lower than that of serum ELISAs. Due to lack of a perfect gold standard test to identify all infected cattle, we also estimated the sensitivity of the fecal PCR test using a Bayesian approach. From Bayesian analyses, the true sensitivity estimate of the PCR assay was 29% and not significantly different from that of serum ELISAs.

The sensitivity of all assays evaluated in this study for detection of *M. paratuberculosis* infected cattle is dependent on stage of infection. Cattle that shed large numbers of *M. paratuberculosis* in feces are more likely to have positive test results. Our results agree with another study using

serum ELISA B (Sweeney et al., 1995) that showed an overall ELISA sensitivity of 45% ± 5% and a specificity of 99% ± 1%. The sensitivity of the test in that study was highest for cows with clinical paratuberculosis (87% ± 8%) and was lowest for subclinical, light fecal shedding cattle (15% ± 7%). A study evaluating serum ELISA A with the same sera (8) similarly estimated the sensitivity varying from 15% in light shedders to 88% in cattle with clinical signs. The specificity of this test was 97% overall across several groups of presumed uninfected cattle.

A further goal of this study was to estimate the sensitivity of the fecal PCR assay to detect cattle shedding *M. paratuberculosis* in feces (relative sensitivity), especially in heavy fecal shedding cattle. Identification of heavy fecal shedding cattle is important because these cattle are the most infectious in terms of risk of transmission to susceptible cattle. While fecal shedding is the primary route of transmission of infection, cows shedding high numbers of *M. paratuberculosis* in feces are more likely than light fecal shedders to transmit infection to calves transplacentally (19) and through milk and colostrum (18). Cattle in this study were present in the study herds at varying stages of infection, including heavy fecal shedders (Table 2). For this study, we used the mean bacterial culture score across the 3 culture methods to create *M. paratuberculosis* concentration categories for test evaluations, since different bacterial culture methods have different sensitivities.

Based on results from this study, this fecal TaqMan® PCR assay can be used as a quick test for detection of subclinically infected heavy fecal shedding cattle (76% relative sensitivity), those cattle at highest risk of transmitting infection to susceptible cattle. It is not an effective assay for detection of other subclinically infected cattle shedding fewer *M. paratuberculosis* (4% relative

sensitivity). While lack of sensitivity in light to moderate shedders is a limitation to this PCR assay, detection of heavy fecal shedders is more critical to herd control. A proportion of light fecal shedders may be passing *M. paratuberculosis* bacteria directly through the gastrointestinal tract after oral ingestion (e.g., passive fecal shedders). Identification of light fecal shedders, especially in heavily infected high prevalence herds, can be problematic to herd managers, as actively infected cattle and passive shedders cannot currently be differentiated. The fecal PCR assay is more sensitive for detection of high risk cattle (heavy fecal shedders) than the milk ELISA tests and at least as sensitive for detection of these cattle as serum ELISAs while less sensitive for light fecal shedders.

Veterinary practitioners must always interpret test results for paratuberculosis in light of the estimated within-herd prevalence of *M. paratuberculosis* infection as well as the performance characteristics of the diagnostic test. Predictive values of positive and negative tests provide interpretive context and vary depending upon within-herd prevalence of infection as well as sensitivity and specificity of diagnostic tests used. Cost-benefit is another important aspect of diagnostic test utility and must be considered before incorporation in disease control programs. Currently, the cost per test of the fecal PCR assay is approximately 80% of the cost of traditional Herrold's egg yolk fecal culture and over 3 times that of serum ELISAs. At the decision-level, the critical issues to consider are tradeoffs in validity and cost among available tests. The fecal PCR assay is a faster and cheaper replacement for fecal culture and, though less sensitive, is good at detection of heavy fecal shedders. In comparison to ELISAs, the fecal PCR assay has higher sensitivity than milk ELISA for detection of heavy fecal shedders (and lower sensitivity to detect light fecal shedders) at a much higher cost.

In summary, these results demonstrate that the sensitivity of a novel high-throughput molecular diagnostic test for paratuberculosis is less than that of fecal culture and similar overall sensitivity to ELISAs, while less sensitive than ELISAs for detection of light to moderate fecal shedders. It is particularly effective at rapid detection of heavy fecal shedding cattle. Rapid identification of cattle actively shedding high numbers of *M. paratuberculosis* in feces can allow removal from the herd or segregation of the highest risk cattle from susceptible cattle thereby avoiding further environmental contamination.

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Table 1. Specificity of tests for detection of *M. paratuberculosis*-infected cattle in uninfected herds (n=7)

Test	Test negative / Total cows tested	Specificity *	95% confidence interval
Fecal PCR	346 / 347	99.7%[a]	99.1 – 100 %
Serum ELISA A	342 / 359	95.3%[b]	84.0 - 100.0 %
Serum ELISA B	358 / 359	99.7%[a]	98.5 – 100.0 %
Milk ELISA	359 / 360	99.7%[a]	98.5 – 100.0 %

* Different letters indicate differences in specificity ($p<0.05$)

Source for ELISA specificity estimates: (Collins MT et al, 2005)

Table 2. Description of apparent test prevalence in *M. paratuberculosis* infected herds by test result

Test method	Cows tested	% test positive	% test heavy fecal shedder[a]
At least one culture test positive	1553	25.4%	5.3%
All 3 culture tests positive	1777	5.0%	NA
Culture using centrifugation	1808	18.4%	5.6%
Culture using sedimentation	1807	10.1%	3.9%
Culture using BACTEC	1481	9.6%	4.5%
Fecal PCR	1808	7.6%	NA
Serum ELISA A	1706	10.5%	NA
Serum ELISA B	1704	7.9%	NA
Milk ELISA	1576	7.9%	NA

NA = not applicable

[a] Heavy fecal shedder defined as 3+ to 4+

Table 3. Percent of fecal PCR assay and serologic assays that were positive by fecal shedding level based on average of 3 bacterial culture methods

	All fecal shedders	Light to moderate fecal shedders[a]	Heavy fecal shedders[a]
Fecal PCR	91/395 (23.0%)	10/244 (4.1%)	60/79 (76.0%)
Serum ELISA A	102/367 (27.8%)	29/234 (12.4%)	56/77 (72.7%)
p-value[b]	0.04	0.002	0.72
Serum ELISA B	101/367 (27.5%)	31/234 (13.2%)	53/77 (68.8%)
p-value[b]	0.06	0.001	0.34
Milk ELISA	84/327 (25.7%)	27/202 (13.4%)	44/66 (66.7%)
p-value[b]	0.31	0.002	0.059

[a] Light to moderate fecal shedders defined as 1+ to 2+, heavy fecal shedder defined as 3+ to 4+

[b] p-value from McNemar's test to detect differences in *M. paratuberculosis* detection between fecal PCR assay and other assays

Table 4. Prior distribution estimates (elicited from experts) and Bayesian posterior distribution estimates for sensitivity and specificity of fecal PCR, fecal culture using centrifugation (CENT), serum ELISA A, and serum ELISA B.

	Prior distribution estimates			Posterior distribution estimates[a]	
Sensitivity	**Mode**	**UL[b]**	**Beta**	**Median**	**95% PI**
Fecal PCR	0.30	0.50	(6.28, 13.32)	0.29	(0.24, 0.35)
CENT	0.60	0.80	(7.04, 5.03)	0.75	(0.66, 0.83)
ELISA A	0.30	0.50	(6.28, 13.32)	0.27	(0.23, 0.32)
ELISA B	0.30	0.50	(6.28, 13.32)	0.26	(0.22, 0.31)
Specificity	**Mode**	**LL[b]**	**Beta**	**Median**	**95% PI**
Fecal PCR	0.995	0.990	(1137.51, 6.71)	0.993	(0.986, 0.997)
CENT	0.999	0.995	(919.87, 1.91)	0.998	(0.994, 0.999)
ELISA A	0.960	0.940	(384.13, 16.96)	0.949	(0.938, 0.960)
ELISA B	0.990	0.970	(212.12, 3.13)	0.980	(0.971, 0.987)

[a] Posterior median with 95% probability interval (PI).
[b] Value considered by the experts (with 95% confidence) to be the highest (sensitivity) or lowest (specificity) possible value for that variable.