Field testing of an enhanced direct-fecal polymerase chain reaction procedure, bacterial culture of feces, and a serum enzyme-linked immunosorbent assay for detecting *Mycobacterium avium* subsp *paratuberculosis* infection in adult dairy cattle

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Objective—To estimate the sensitivity (Se) and specificity (Sp) for an enhanced direct-fecal PCR procedure, bacterial culture of feces (BCF), and a serum ELISA for detecting *Mycobacterium avium* subsp *paratuberculosis* (MAP) infection in adult dairy cattle.

Sample Population—Fecal and serum samples were collected from 669 adult cattle randomly selected from a 4,000-cow dairy herd known to contain animals infected with MAP.

Procedures—Serum samples were evaluated for MAP-specific antibodies via ELISA. Fecal samples were evaluated by BCF and enhanced PCR methods (both gel-based [GB]-PCR and quantitative real-time [qRT]-PCR assays). Fecal samples also were pooled (5:1) and then subjected to GB-PCR assay. Bayesian statistical methods were used to estimate Se and Sp for each diagnostic test without knowledge concerning true MAP infection status.

Results—Adjusting for Se conditional dependence between serum ELISA and BCR, overall Se and Sp were estimated at 33.7% and 95.9%, 51.3% and 99.0%, and 32.2% and 100% for serum ELISA, qRT-PCR, and BCF, respectively. The GB-PCR assay yielded positive results for 38.3% of the pools known to contain feces from at least 1 cow that had positive GB-PCR results.

Conclusions and Clinical Relevance—Estimated Se values for the serum ELISA and BCF were slightly lower than those reported elsewhere. The enhanced qRT-PCR method offered relative improvements in Se of 52% and 59% over serum ELISA and microbial culture, respectively. Pooling of fecal samples and testing with the GB-PCR assay are not recommended. Additional studies with qRT-PCR and fecal pools are required. (*Am J Vet Res* 2007;68:236–245)

Paratuberculosis (Johne's disease) is a chronic infectious disease of the gastrointestinal tract of ruminants. It is caused by the bacterium MAP. Infection typically develops during the first few months after birth even though the first signs of disease may not appear for years.[1] Subclinical infection can lead to decreases in productivity and reproductive efficiency,[2,3] which in turn lead to an increased risk for culling.[4] Because there is a high rate of culling for production- and reproduction-based problems in modern dairy operations, few infected cattle ever develop clinical signs of the disease. This contributes to the considerable difficulties that exist in correctly determining infection status of dairy herds. In addition to causing substantial losses as a result of clinical and subclinical disease in cattle, MAP also has been implicated as a cause of Crohn's disease in humans.[5] Although this association remains unproven and contentious,[6] public perception of a causal link represents a potential economic risk to the dairy industry.

Similar to other infectious microorganisms, the risk of spread of MAP through a herd is directly related

Abbreviations

MAP	*Mycobacterium avium* subsp *paratuberculosis*
BCF	Bacterial culture of feces
Se	Sensitivity
Sp	Specificity
IS900	Insertion sequence 900
DIM	Days in milk
S:P	Sample-to-positive ratio
TVMDL	Texas Veterinary Medical Diagnostic Laboratory
GB	Gel-based
qRT	Quantitative real-time

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to the number of infected and susceptible cattle in the herd. There is no practical, effective treatment, so identification and culling of infected cows, in combination with on-farm biosecurity (including calving management, calf-rearing management, and purchase of replacements from herds with known MAP status), are vital to developing MAP-free herds.[7,8]

Two of the most common diagnostic tests for MAP used to determine herd status and for on-farm control programs (ie, serum ELISA and BCF) both have variable (as determined on the basis of method and stage of infection), though generally low, Se.[9-11] The ELISAs also have a slightly lower Sp (estimated between 96% and 99%) than BCF (which is often assumed to be 100%). The lower Sp value for the ELISAs can lead to misclassification of herd status when herd-level Sp is the concern.[12] Bacterial culture of fecal samples is considered the most commonly used referent test, but it requires considerable time and resources to perform and can quickly overburden dairy producers (eg, financially) and diagnostic laboratories (eg, time, space, and labor). Although advances in methods have considerably shortened culture times for samples with negative results on BCF (from 16 weeks to as little as 4 weeks), the poor Se for BCF remains an obstacle to widespread adoption of herd-status programs by beef and dairy producers. An ideal test would allow producers to test cattle for MAP once during an animal's lifetime and receive a definitive positive or negative status of that animal at modest cost. Pooling of fecal samples and then conducting BCF has the potential benefit of reducing the cost per animal tested in the pool and may be useful for monitoring or screening of low-risk herds.[13-18] However, it is not appropriately suited to within-herd control programs once MAP infection has been confirmed, and the length of time needed for confirmation is not improved over that of BCF for samples obtained from each animal.

Advances and developments in PCR techniques offer promising alternatives, particularly with regard to sample preparation and DNA extraction. Conducting PCR assays directly on fecal matter remains problematic; however, there are new and promising techniques that have been developed and still others that are almost ready for use.[19,20] The PCR assays have been used to improve Sp and confirm the identification of microorganisms, whereas traditional microbiologic methods (such as microbial culture) have limitations. The PCR assays also have limitations, particularly when the clinical samples contain substances that can inhibit PCR amplification.[20]

Methods to purify the DNA extract and remove inhibitory substances are crucial to the future success of these methods. An enhanced immunomagnetic bead separation coupled with bead beating and IS900 PCR technique was developed and found to be an effective procedure for the isolation, separation, and detection of MAP in spiked fecal samples obtained from cattle.[21,a] However, field validation for Se and Sp of that enhanced PCR method has yet to be confirmed for representative samples collected prospectively from a population of naturally infected commercial dairy cattle.

The primary objective of the study reported here was to use a strategic random-sampling scheme and apply Bayesian statistical methods (without knowledge of true MAP infection status) to estimate the Se and Sp of each of BCF, a direct-fecal enhanced PCR assay, and a commercially available serum ELISA for diagnosis of MAP infection in adult dairy cattle and determine whether these estimates varied by season, number of DIM, or lactation number. The secondary objective was to examine the effect of strategic pooling of fecal samples on the Se for the direct-fecal enhanced PCR test.

Materials and Methods

Study population—Collection of samples was performed on a dairy farm from October 2003 through August 2004. At that time, the producer had a herd of approximately 4,000 lactating Holstein cows, with approximately 60% in the first lactation, 20% in the second lactation, and 20% in the third or higher lactation. In the year before the study, the annual culling rate was 35% to 40%. Mean milk production for the herd was 11,400 kg/y (mature-equivalent milk for milking 3 times/d). Cows were housed in freestall barns and drylot facilities with a special-needs barn providing a maternity area separate from the sick pen. In terms of reproduction, the voluntary waiting period was 70 days for all cows. For cows calving in 2002, the mean first-breeding pregnancy rate was 34%, and the overall pregnancy rate for all breedings was 21%.

During 2000, it was evident that a number of cows had clinical signs of paratuberculosis. In early 2001, the owner implemented a program to minimize exposure of neonatal calves to MAP. Until that time, calves had been receiving pooled colostrum and had been housed in a calf area in the maternity barn for the first 24 hours after birth. Beginning in April 2001, cows in their fourth month of gestation were tested by use of a commercial serum ELISA[b] for serologic responses against MAP antibody. In March 2003, the testing protocol was changed such that the cows were near the end of lactation when samples were obtained for testing by use of the serum ELISA.

At any given time, approximately 100 adult cows in the herd record system[c] were seropositive for MAP (as determined on the basis of a cutoff for the S:P of ≥ 0.25 for the commercial serum ELISA[b]). Culling decisions for the herd were not based on S:P values or interpreted results; instead, culling decisions were based on factors such as reproduction, production, and clinical illness that may well have been impacted by MAP status. The ELISA results for each cow were derived from the owner-initiated and owner-funded MAP testing protocol, which formed the basis for the sampling scheme used in the study.

Sample selection and processing—Strategic samples of blood and feces were obtained from cows with the highest S:P values (ie, cows with positive test results determined within the past year) in the herd for each of 4 seasons from October 2003 through August 2004. In addition, samples were obtained from a subset of cows (chosen by use of a random-number generator) stratified into quartiles (same time frame) on the basis of the ordered ELISA negative S:P values (ie, < 0.25). All cows with serum ELISA results from the dairy's existing

sampling strategy (serum samples obtained at or near the end of lactation) during the 3 months preceding the start of our study served as the sampling frame. Cattle eligible for inclusion in the study were identified by use of the herd's computerized records.[c]

Records were sorted on the basis of S:P values; this automatically included all cows with S:P values ≥ 0.25 (cutoff value indicated in the manufacturer's instructions[b]). The quartile cutoff points were determined on the remaining cows with negative results on the ELISA in each seasonal sampling frame, and samples were obtained from an equal number of cows within each quartile so that the total (including cows with positive results) was approximately 150 cows/season. Additional cows (5/category) also were listed in the sample to account for any cattle that were not available or not restrained in self-locking head gates on the date of sample collections. For example, if during July, August, and September of 2003 there were 30 cows that had positive results for MAP antibodies based on the ELISA, serum and feces were collected from all 30. Then, the remaining cattle with test results for those months were sorted by use of the ELISA S:P value, and an equivalent number (ie, 30 cows) were randomly selected from each S:P quartile so that the seasonal quarterly sample size was 150. Any cow eligible during a given quarterly seasonal test period was not considered eligible for selection at a later time. This stratified sampling scheme optimized the distribution of ELISA test results, when compared with results for BCF and direct-fecal enhanced PCR assay, while ensuring the highest probability of identifying cows in the herd that shed MAP in the feces. Blood and fecal samples were shipped on ice to the TVMDL in College Station, Tex, and were processed in accordance with standard laboratory operating procedures.

For the second objective, fecal samples were strategically pooled (ordered on the basis of age) before submission to the TVMDL. Fecal material was pooled by personnel involved with obtaining samples from the cattle. A 5-g aliquot of feces from each cow was mixed with equal aliquots from each of 4 other cows (total amount of each pooled sample was 25 g).

Serum ELISA for the diagnosis of MAP infection—All blood samples were centrifuged. Serum was decanted into serum tubes. A commercial ELISA kit[b] was used to evaluate serum antibodies against MAP. All samples were analyzed in duplicate wells at the TVMDL in accordance with the manufacturer's instructions. Results were recorded directly into an automated database recorder. Optical density, which was measured by use of a 650-nm filter, was used to calculate S:P values; interpretation of results (S:P ≥ 0.25 was considered a positive result) was in accordance with the manufacturer's recommendation. The ELISA results were treated as dichotomous variables.

BCF for the diagnosis of MAP infection—The BCF procedure generally was performed as described elsewhere.[22] Three grams of feces was placed in a 50-mL centrifuge tube; distilled water was added to achieve a final volume of 35 mL. Samples were mixed on a rotating mixer for 30 minutes. Samples were allowed to stand undisturbed for 30 minutes, and the supernatant was then decanted into a new 50-mL tube and centrifuged at 1,700 × *g* for 20 minutes.

Supernatant was discarded, and the pellet was resuspended in 30 mL of 0.9% cetylpyridinum chloride:1.9% brain-heart infusion. Samples were incubated overnight at 37°C as a decontamination step and then centrifuged at 1,700 × *g* for 20 minutes. Supernatant was discarded, and the pellet was resuspended in 1 mL of sterile water with 50 µg of amphotericin B/mL, 100 µg of vancomycin/mL, and 100 µg of nalidixic acid/mL. Samples were again incubated overnight at 37°C and then inoculated onto Herrold egg yolk medium (0.2 mL/tube) in 5 tubes (4 tubes contained mycobactin J,[d] and 1 tube was without mycobactin J). Tubes were placed in a slanted position with caps loosened and incubated at 37°C. One week later, samples were checked for contamination, caps were tightened, and tubes were placed in an upright position and incubated for up to 15 additional weeks. Tubes were checked weekly for appearance of MAP. When no growth was visible after 16 weeks of incubation, samples were considered to have negative results. When colonies typical of MAP were observed at 16 weeks (ie, those growing on tubes containing mycobactin J only), they were stained by use of a cold acid-fast stain. Results for BCF were not quantified; rather, results were dichotomized as positive or negative for growth. Mycobactin J–dependent acid-fast organisms from suspect colonies were considered to be positive for MAP via confirmation by use of PCR testing.

Direct-fecal enhanced PCR assay for the diagnosis of MAP infection—In accordance with a technique described elsewhere,[21] we used a common extraction and enhancement technique followed (initially) by a qualitative GB-PCR assay and then a qRT-PCR assay. During the study period, the TVMDL made a transition from use of the GB-PCR assay to the qRT-PCR assay. Beginning in 2006, all PCR assays were being conducted by use of the qRT-PCR method. However, for the purposes of comparison of direct-fecal PCR assay of fecal samples for each animal, both PCR tests were performed during the study. In contrast, pooled fecal samples were compared only by use of the GB-PCR method.

Extraction of DNA—The MAP DNA was extracted by use of a technique described elsewhere.[21] Briefly, supermagnetic beads[e] were coated with rabbit polyclonal anti-MAP antibodies.[f] After washing, the beads were incubated with fecal samples to immunocapture the MAP organisms. A magnetic separator[g] was used to harvest immunocaptured bacteria. After washing, the immunocaptured bacteria were lysed by use of a bead beater. Finally, the samples were incubated with proteinase K[h] and RNase A[i] and then extracted with an equal volume of phenol-chloroform-isopropanol solution. The DNA was precipitated with isopropanol, sodium acetate, and polyacyl carrier. The pellet was then washed with 70% ethanol and redissolved in 100 µL of DNase and RNase water.

GB-PCR assay—We used IS900 primers to amplify MAP bacterial DNA (forward primer, 5′–GGC GTT GAG GTC GAT CGC CCA–3′; reverse primer, 5′–CCG CTA ATT GAG AGA TGC GAT T–3′). The PCR master mix solution contained final concentrations of 10× PCR

buffer, 0.25mM each of forward and reverse primer, 0.25mM of dinucleoside triphosphates, 2.5mM $MgCl_2$, and 2 units of DNA polymerase.[j] With a 2.5-µL template, the PCR assay was performed with a total reaction volume of 25 µL. Amplification was conducted in a programmable thermocycler[k] for the following conditions: 1 cycle of denaturation at 94°C for 5 minutes; followed by 40 cycles at 94°C for 1 minute, 65°C for 30 seconds, and 72°C for 1 minute; followed by a final extension at 72°C for 10 minutes. The PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide, and developed under UV light to reveal a band corresponding to 287 bp.

qRT-PCR assay—The qRT-PCR assay was conducted by use of IS900 primers (forward primer,[l] 5′–CGG GCG GCC AAT CTC–3′; reverse primer,[m] 5′–CCA GGG ACG TCG GGT ATG–3′) and a probe[n] (5′–FAM TTC GGC CAT CCA ACA CAG CAA CC TAMRA–3′) to amplify an 84-bp product in the genome of MAP. The qRT-PCR was conducted in a quantitative PCR system[o] by use of a total volume of 25 µL that contained 5 µL of template, 12.5 µL of universal master mix,[p] 900nM each of forward and reverse primer, and 250nM of probe. Reaction conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and a final step of 60°C for 1 minute. The threshold cycle was defined as the cycle at which the fluorescence was significantly higher than the mean SD of the preceding cycles and the sequence detection application began to detect the increase in signal associated with an exponential growth of the PCR product.

This method (ie, enhanced GB- and qRT-PCR assays)[21] was used to correctly identify the 30 unknown samples provided for the fecal proficiency test program.[a] Results of GB- and qRT-PCR assays were treated as dichotomous variables.

Statistical analysis—The Se and Sp for each test (serum ELISA, enhanced PCR [analysis restricted to the qRT-PCR assay], and BCF) were estimated by use of a latent class analysis and Bayesian methods without knowledge concerning true MAP infection status.[23-26] Values for Se and Sp could not be estimated directly[27,28] because available information only included the number of cattle with each of the 8 possible combinations of positive and negative test results (eg, positive or negative for serum ELISA, qRT-PCR assay, and BCF, respectively). Total count for each test pattern was the sum of the unobserved (latent) number of infected and uninfected cattle for each category.

The basic statistical model was constructed for a single population and assumed that BCF had perfect Sp (ie, Sp = 1) but was allowed to have imperfect Se (ie, Se ≤ 1). Prior probability distributions used in this analysis were determined (**Appendix**). Infection prevalence was modeled by use of a prior probability distribution centered at 13% because this was the sample proportion that had positive results for 1 or both of the qRT-PCR and BCF tests. A distribution with a large variance was chosen for the sample MAP infection prevalence because it was based on these results. Prior distributions for the Se and Sp of the serum ELISA and Se of traditional BCF were obtained from another study.[18] The unweighted mean of the reported prior probability distributions from both experts cited in that study was used for the analysis. Noninformative prior probabilities (ie, uniform distributions in which all values between 0 and 1 are equally likely) were used for the Se and Sp of the qRT-PCR assay because limited information has been published concerning this assay and its evaluation was the primary objective of the study reported here.

A model was created to evaluate conditional dependence between pairs of diagnostic test results. Separate covariance terms were fitted for infected (Se covariance) and uninfected (Sp covariance) cattle.[25,29,30] The dataset was artificially separated into 2 populations on the basis of the median number of DIM (228 days), which created 2 populations that were expected to have infection prevalences that differed. All possible pairwise Se and Sp covariance terms were included in this model. The Se and Sp of the tests were assumed to be equal in both of these population subsets for the evaluation of conditional dependence.

Stratified analyses were performed to estimate Se and Sp of the 3 tests based on season of testing, lactation number, and DIM. Season of testing was dichotomized into hot months (samples obtained in August and October) and cool months (samples obtained in February and May). Cows in their first or second lactation were grouped to form 1 category, and all cows in their third or higher lactation formed a second category. The variable DIM was divided into 3 categories (< 180 days, 180 to 359 days, and ≥ 360 days). All models, including the complete unstratified dataset, were implemented independently to allow for the estimation of stratum-specific values of diagnostic test accuracy.

Diagnostic test accuracy was estimated by Markov chain Monte Carlo methods by use of available software.[q] Computer code was designed so that stratum-specific models were implemented independently but were nested within a single set of instructions so that at each iteration of the Monte Carlo procedure, values were generated for all variables in all strata. Sample infection prevalences, Se, and Sp were generated within each iteration. Stratum-specific measures of accuracy were subtracted from each other within each iteration for all diagnostic test variables. The 95% intervals of the differences that did not include zero were considered statistically probable (which is analogous to the term statistically significant that is often used when reporting frequency statistics). No attempt was made to adjust for multiple comparisons.

Plots of iterates for each model variable (eg, Se and Sp) were monitored for trends in successive iterations to determine when convergence was achieved. Before convergence, iterate plots will have an increasing or decreasing linear trend during successive iterations. Alternating increasing and decreasing trends (wavelike effect) also indicates lack of convergence. Convergence is achieved when the variable values (iterates) do not have a linear trend and thus appear to be fluctuating randomly above and below a hypothetic regression line through the points. Convergence was visually assessed by use of iterate plots and confirmed by calculating the Gelman-Rubin statistic.[q] Values obtained before reach-

ing convergence (termed the burn-in phase of the analysis) were not used for making inferences. After a burn-in phase of 900,000 iterations, the following 200,000 iterations were used for inferences. Median values and percentiles provided point estimates and probability intervals, respectively.

Simple cross tabulation, which was limited to GB-PCR results for fecal samples obtained from each animal and for pooled fecal samples, was performed by use of standard statistical software[r] to estimate the relative Se of strategic pooling, compared with GB-PCR results for each cow. In addition, GB-PCR results for pooled fecal samples were compared with the sum of the number of fecal samples from each of the 5 cows that had positive GB-PCR results when tested separately that were included in each pooled sample, thereby providing an estimate of the probability of a result for a pooled sample being positive on the basis of the number of GB-PCR–positive cows that contributed to the pooled sample.

Results

Diagnostic test performance—Fecal samples and sera were obtained from 694 cows. Of these, 669 had records that included complete and verifiable information concerning the 3 test results as well as pertinent covariate information (**Table 1**). Both qRT- and GB-PCR methods were performed on fecal samples obtained from each cow. Of the 669 cattle, 74 had positive results by use of the GB-PCR method, whereas an additional 20 had positive results by use of the qRT-PCR method. None of the 575 cattle that had negative results by use of the qRT-PCR method had positive results for the GB-PCR method. Bayesian analyses were restricted to serum ELISA, direct-fecal qRT-PCR, and BCF results.

The 2-population conditional-dependence model had a statistically probable Se covariance between the serum ELISA and BCF tests on the basis that the median value was positive and the 95% probability interval did not include zero (**Table 2**). The BCF was assumed to have a Sp of 100%; therefore, by definition, it was independent of the other tests (conditional on the animal being uninfected with MAP). The single possible Sp covariance term between the serum ELISA and qRT-PCR method was not probable. Unstratified and stratified analyses (on the basis

Table 1—Joint frequency patterns for diagnostic test results for 3 tests used to detect MAP in commercial adult dairy cattle in which the true infection status was unknown.

Test result pattern	Serum ELISA	qRT-PCR	BCF	No. of cattle
1	N	N	N	526
2	N	N	P	8
3	N	P	N	43
4	N	P	P	4
5	P	N	N	32
6	P	N	P	9
7	P	P	N	12
8	P	P	P	35
Total	**NA**	**NA**	**NA**	**669**

N = Negative result. P = Positive result. NA = Not applicable.

Table 2—Estimates of conditional-dependence terms by use of Bayesian methods based on a 2-population model to evaluate Se and Sp of 3 diagnostic tests (BCF, serum ELISA, and an enhanced direct-fecal qRT-PCR assay) for detection of MAP infection among commercial adult dairy cattle in which the true infection status was unknown.

Variable	Test 1	Test 2	Median	95% Probability interval*
Se covariance	Serum ELISA	BCF	0.065	0.044, 0.098
	Serum ELISA	qRT-PCR	0.027	–0.041, 0.060
	qRT-PCR	BCF	0.023	–0.010, 0.043
Sp covariance	ELISA	qRT-PCR	0.003	–0.001, 0.023

*Probability intervals that do not include zero are considered statistically probable.

Table 3—Values for Se, Sp, and prevalence estimates for 3 diagnostic tests for detection of MAP infection among commercial adult dairy cattle in which the true infection status was unknown, as determined by use of Bayesian methods for the entire sample population and various strata generated on the basis of covariate information.

	Serum ELISA		qRT-PCR		BCF*	
Population	**Se†,‡**	**Sp†,‡**	**Se†,‡**	**Sp†,‡**	**Se†,‡**	**Prevalence†,‡**
Sample population	33.7 (27.8, 40.1)	95.9 (94.1, 97.5)	51.3 (41.8, 62.4)	99.0 (96.1, 100)	32.2 (24.7, 41.6)	23.2 (17.8, 29.2)
Season§						
1	30.5 (24.0, 37.7)	95.9 (93.9, 97.5)	56.1 (41.6, 72.1)	97.8 (93.1, 99.9)	28.1 (19.3, 39.7)	20.3 (13.4, 28.3)
2	31.4 (25.1, 38.3)	96.1 (94.1, 97.7)	49.2 (37.3, 63.5)	98.8 (95.3, 100)	36.4 (26.8, 48.4)	23.5 (16.9, 31.4)
Lactation No.						
1 and 2	30.0 (23.8, 36.8)	95.7 (93.6, 97.4)	48.1 (36.4, 62.0)	97.8 (93.1, 99.9)	30.7 (22.2, 42.2)	23.2 (15.8, 31.5)
≥ 3	32.1 (25.4, 39.3)	96.4 (94.5, 97.9)	57.5 (42.9, 73.5)	98.9 (95.5, 100)	34.7 (24.6, 47.0)	20.6 (14.6, 28.0)
DIM						
< 180	32.3 (25.8, 39.5)	95.4 (93.2, 97.3)	56.1 (41.8, 73.8)	98.5 (94.0, 99.9)	34.1 (24.2, 46.9)	24.0 (16.8, 32.9)
180 to 359	29.0 (22.6, 36.1)	96.3 (94.3, 97.9)	45.1 (32.2, 59.4)	97.1 (91.4, 99.9)	31.6 (22.5, 43.5)	23.3 (15.2, 32.6)
≥ 360	26.8 (19.8, 34.6)	96.7 (94.7, 98.1)	70.4 (39.2, 97.0)	98.0 (92.8, 99.9)	34.4 (21.3, 49.8)	11.1 (5.7, 19.0)

Values reported represent percentage (95% probability interval). All models include a conditional dependence term for Se between the serum ELISA and BCF.

*The BCF was assumed to have perfect Sp without error. †Point estimate is the median value of the posterior distribution. ‡Probability interval represents values from the 2.5 to 97.5 percentiles. §Samples for season 1 were obtained in August and October, whereas samples for season 2 were obtained in February and May.

Table 4—Results for pooled fecal samples* tested by use of an enhanced direct-fecal GB-PCR assay on the basis of the number of cows with positive results when fecal samples were tested individually that were included in each pooled fecal sample.

No. of cows with positive results within each pooled sample†	Pooled fecal samples		Positive results		Negative results	
	No.	%	No. positive/No. tested	%	No. negative/No. tested	%
0	78	56.5	11/78	14.1	67/78	85.9
1	45	32.6	11/45	24.4	34/45	75.6
2	15	10.9	12/15	80.0	3/15	20.0
3	0	0	NA	NA	NA	NA
4	0	0	NA	NA	NA	NA
5	0	0	NA	NA	NA	NA
Total	**138**	**100**	**34/138**	**24.6**	**104/138**	**75.4**

*Pooled fecal samples consisted of samples from each of 5 cows, which were strategically grouped on the basis of age (from youngest to oldest) within each season of sample collection. †Represents the number of cows with positive results for the GB-PCR assay when fecal samples were tested individually.
See Table 1 for remainder of key.

of season, DIM, and lactation number) that incorporated the term for Se dependence between the ELISA and BCF converged, as determined by use of the Gelman-Rubin statistic (**Table 3**). The overall Se and Sp were estimated at 33.7% and 95.9%, 51.3% and 99.0%, and 32.2% and 100% (fixed by analysis) for serum ELISA, the enhanced qRT-PCR method, and BCF, respectively. Point estimates for Se and Sp did not appear to be affected by season. The Se of the qRT-PCR and BCF tests was higher in cows with a higher number of lactations. Estimates also appeared to change slightly for values of DIM, with the most noticeable difference being the higher Se of the qRT-PCR and BCF tests with increasing time since parturition. However, no pairwise comparisons between Se and Sp were probable in the stratified analysis, and differences could simply have represented random variation.

Pooling of fecal samples—A total of 138 pooled fecal samples (strategically ordered on the basis of age) were subjected to testing by use of the GB-PCR test; the qRT-PCR method was not conducted on pooled fecal samples. The 138 pooled samples represented 690 adult cattle from which valid fecal samples were collected (4 cows were excluded because their pool did not include samples from 5 cattle). Of 60 pooled samples that included at least 1 sample from an adult cow that had positive results when tested at the individual level by use of the GB-PCR method, only 23 (38.3%) subsequently had positive results (**Table 4**). In addition, 11 of 78 pooled samples that included samples only from those cattle that had negative results when tested individually also had positive results for the GB-PCR method. Of the 75 cattle that had positive results for MAP infection when tested individually by use of the direct GB-PCR method, only 35 were detected when diluted in pooled samples consisting of fecal samples from 5 cows (test Se relative to direct individual testing, 46.7%). The probability of a pooled sample yielding a positive result was a dose-dependent event, with pooled samples that contained feces from 2 cows with positive results when tested individually having a higher probability (80.0%), compared with the probability for those that contained feces from only 1 cow that had a positive result when tested individually (24.4%).

Discussion

The primary objective of the study reported here was to assess the diagnostic test accuracy of an enhanced direct-fecal IS900 PCR assay for MAP infection in commercial dairy cattle in field conditions. In this case, field conditions included collecting samples from cattle at a large commercial dairy in Texas and the use of fee-for-service MAP testing at a veterinary diagnostic laboratory. On the basis of analysis of our findings, the enhanced direct-fecal PCR method,[21] when used in a real-time platform, yielded a relative improvement in test Se of 52% and 59%, compared with the serum ELISA and BCF, respectively. The absolute Se was estimated to be 51.3% in the model, which accounted for conditional dependence of BCF and the serum ELISA. Not unexpectedly, the Sp of the enhanced qRT-PCR assay was estimated to be extremely high (99.0%) in the conditional model. This also exceeded our estimate of Sp for the serum ELISA (95.9%), but it was less than the assumed Sp of 100% for BCF.

Our estimate of Sp for the commercial ELISA is extremely similar to that reported in another study.[31] The enhanced qRT-PCR assay appears to offer an advantage over the serum ELISA and BCF in terms of Se and a time advantage over BCF (results in a few days for the qRT-PCR method, compared with up to 16 weeks for BCF); however, there is a cost disadvantage when the qRT-PCR assay is compared with BFC and the serum ELISA (the price charged by the TVMDL at the time of the study was $25.00 for the qRT-PCR test, compared with $15.00 and $5.00 for BCF and the serum ELISA for in-state submissions, respectively). In addition, the labor required for DNA extraction and qRT-PCR processing and reagent costs for the qRT-PCR method exceeded costs for BCF and the serum ELISA; however, similar to other areas of technologic advances, costs may be expected to decrease during the years after introduction. In addition, whereas laboratory bottlenecks for BCF typically relate to space (eg, incubators and length of time needed for bacterial culture), those of the qRT-PCR

method typically are labor (laboratory personnel), availability of the test, and hardware costs.

It is important to mention that of the 56 samples that had positive results for MAP by use of BCF, 17 (30.3%) had negative results for the qRT-PCR method (Table 1). The reasons for this discrepancy are unknown. Possibilities include variation in the distribution of MAP organisms within a fecal sample and increased capacity for an extremely small number of organisms to reproduce during several weeks in BCF, as opposed to their capacity to increase during 30 to 40 cycles of a qRT-PCR assay. It is also important to understand that laboratory assay Se is not the same as clinical diagnostic Se, which is much more relevant to our analysis.

The lower laboratory assay Sp of the enhanced qRT-PCR method, when compared with that for BCF, could pose some problems when results are interpreted on a herd basis.[12] However, use of the enhanced qRT-PCR assay as a screening test, with BCF used subsequently as a confirmatory test, would improve herd Sp to 100%.

The other component of the primary objective was to determine whether season, DIM, and lactation number affected accuracy estimates for paratuberculosis tests. Season and DIM did not affect the accuracy for the 3 paratuberculosis tests evaluated. The Se of the enhanced qRT-PCR assay was estimated to be 70.4% for DIM ≥ 360; although the Se was higher than that for other DIM categories, this was not statistically probable. The Se values of tests for paratuberculosis are considered to increase with the stage of infection and therefore are expected to be higher in older cattle. Lactation number was used as a surrogate for the amount of time in the herd, and tests performed on cattle in lactation 3 or higher had higher overall Se estimates, but these were not statistically probable. It is not clear whether a larger dataset that included test results from multiple herds would document expected biological differences.

Lack of a perfect criterion-referenced test for detecting MAP infection in susceptible host species poses numerous difficulties for developing and implementing MAP-control programs in herds.[8] Although BCF (with or without PCR confirmation) is the standard confirmatory test (on the basis of an assumed Sp of 100%), it is recognized that usefulness of BCF as a screening test is suboptimal, given the Se of the test for detecting infected animals varies among laboratories (ie, assay variability) as well as among herds with differences in management characteristics and animals of varying productivity, age, and stage of infection. The BCF may perform especially poorly in young, recently infected cattle or those in preclinical stages of the disease.[32] Many factors affect the probability of a truly infected animal having a positive result for BCF. These can be allocated into host factors (eg, age, stage of infection, and intermittent shedding), fecal factors (eg, number of organisms per gram of feces and uneven distribution of MAP organisms throughout fecal material), and test factors (eg, culture medium and decontamination reagents).[33]

Selection of an assay (eg, among serum ELISA, BCF, and PCR assay) is not straightforward and will depend on such factors as cost; laboratory capacity, performance, and efficiency; and diagnostic test accuracy. Despite apparent relative improvements in Se and Sp for the qRT-PCR assay over values for the serum ELISA and Se for the qRT-PCR assay over that for BCF, other factors may be important when considering the appropriate test for a given herd situation and diagnostic laboratory. For example, although the enhanced direct-fecal qRT-PCR assay performed best for the commercial herd described here, other herds that contain many more young and preclinical (eg, nonshedders or low shedders) cattle or cattle of unknown infection status may not be suitable for testing with this assay. A large herd with unknown MAP infection status would be more suited to screening by use of the serum ELISA, with follow-up confirmation of positive results by use of BCF, rather than to initiate testing with a relatively expensive alternative such as the direct qRT-PCR assay. On the other hand, in a herd of known infection status that contains older cows and in which culling decisions need to be made in a timely manner, the direct-fecal qRT-PCR assay may play a more important role. Adoption of direct-fecal qRT-PCR techniques in herd, state, national, and international control programs requires considerable discussion before implementation.

Performance of BCF (in terms of Se) is linked to the herd pattern of serum ELISA results.[10] An important conditional dependence between these tests was identified in the study reported here. Therefore, interpretation of test accuracy estimates derived from our study must be considered with respect to the context of our sample pattern (not the herd pattern) of serum ELISA results. In other words, each sample of cows from the herd was divided evenly 5 ways among ELISA-positive cows and the ranked S:P quartiles of ELISA-negative cows; thus, this is the hypothetic herd pattern to which the results are likely to be most generally applicable. However, our results did not reveal a statistically probable conditional dependence between the enhanced qRT-PCR assay and either the serum ELISA or BCF.

The estimated Se for the commercial serum ELISA was at values slightly lower than those reported elsewhere,[9] whereas our estimate of Sp was extremely similar to that reported in another study.[31] However, as has been pointed out, the Se for many serum ELISA and BCF approaches may be variable and dependent on the laboratory as well as the herd pattern of infected animals' stages of disease, parities, and stages of lactation.[9,10,33] Diagnostic test evaluations have often been based on sets of banked samples (serum or feces) derived from cattle known to be infected with MAP (through experimental challenge or positive results on BCF) or that come from cattle raised on farms with an extremely low probability of being infected with MAP.[9,11] Although this approach offers several obvious advantages, the findings may not be generally applicable to field settings because the relative infective dose and disease progression among research cattle and characteristics of herds at low risk for MAP may not extend to typical commercial settings. In particular, among known herds with endemic infections and that contain infected cattle, these approaches to diagnostic test evaluation may cloud interpretation in a field setting.

In the past few years, Bayesian approaches to diagnostic test evaluation have become more common.[23-26]

These offer several advantages, including the ability to assess Se and Sp without knowledge concerning true infection status. In addition, the samples may be derived from field settings (such as commercial dairy farms) where MAP is known to be endemic, as opposed to artificially constructing sets of MAP-positive and -negative cattle from a mixture of sources.

Analyses in which knowledge concerning true infection status is lacking are becoming more common in the veterinary literature and have an advantage in situations when a perfect criterion-referenced test is not available. Bayesian methods based on Markov chain Monte Carlo sampling are relatively easy to implement and can solve problems involving complicated likelihood functions when integrative calculus is not possible. The ability to incorporate prior knowledge in the form of density functions is another advantage of the Bayesian approach because this information can act to guide the analysis in the appropriate direction (ie, consistent with current knowledge). These statistical methods can provide biologically useful results, but they also have weaknesses.

A weakness of the current analysis could have been the fact that the prior probability distribution for sample prevalence was based on data collected in the study reported here. This conflicts with the definition of prior; however, it was necessary because the sampling method did not represent the herd structure, and it was uncertain how this sampling would affect within-herd prevalence. A distribution with a large variance was used, and Se analysis was performed on the prevalence by use of a noninformative prior probability distribution. The noninformative prior did not qualitatively change results, and therefore, the more precise results from the model that incorporated the informative prior probability distribution were reported.

Bayesian statistical models may not converge, and this problem is often encountered in situations involving complicated likelihood functions. It is not possible to make valid inferences on the basis of results from a model that has not converged. Lack of convergence can result when the assumptions of the statistical model are violated. These assumptions include conditional independence of test results, equal Se and Sp among populations, and differences in infection prevalences among evaluated populations. Incorporated prior probability distributions are further assumed to be a valid representation of the investigators' prior knowledge of the problem. Noninformative prior probability distributions can be substituted to better investigate the magnitude of effect of particular priors on the study results. Conditional dependence terms can be incorporated into models to relax the assumption of conditional dependence. Lack of convergence can also be a problem when the number of unknown variables estimated exceeds the number of degrees of freedom in the data. Convergence and model misspecification are serious problems that must be addressed for the valid interpretation of results from these analyses.

The inappropriate inclusion or exclusion of conditional dependence terms can lead to misspecification of the model and biased results. Conditional dependence is a statistical property that can often be explained by the underlying biological characteristics of tests. Positive test results for assays that measure the same analyte would be expected to be correlated within infected animals. Statistical models must be used to estimate dependencies, and these are expected to vary among datasets. Statistical tests can only determine whether the addition of dependency terms creates a model that better fits the data. It was expected that the qRT-PCR assay and BCF would be conditionally dependent within infected cattle because the feces must contain specific DNA for the organism to be cultured successfully. Serum ELISA and BCF tests do not measure the same analyte, but they are both correlated with later stages of MAP infection; this is the likely cause of dependency between the tests. However, the inclusion and exclusion of specific dependency terms were based on statistical considerations and not the expected biological correlation between tests.

At the time the study reported here was conducted, we were not aware of any studies in which investigators considered pooling of fecal samples and subsequent testing by use of direct-fecal PCR methods. In contrast, strategic approaches to pooling of fecal samples and testing by use of BCF have been investigated in the past few years as a legitimate means to reduce the costs associated with herd testing while maintaining a reasonable test Se at the herd level.[13-18] The objective for the study reported here was not to directly compare results of the GB-PCR assay on pooled fecal samples to results for BCF on pooled samples, but rather to investigate the relative loss of Se resulting from pooled samples tested by use of the GB-PCR assay, compared with that for the GB-PCR assay performed on individual fecal samples. Because of the transition to the qRT-PCR assay from the GB-PCR assay during the study, we were unable to compare the loss of Se in the pooled fecal samples tested by use of the GB- and qRT-PCR methods.

The pooling approach for testing by use of BCF appears to be appropriately suited to establishing infection status of herds (eg, herd certification programs). There is generally some loss of Se expected for pooled fecal samples when tested by use of BCF, relative to that for individual fecal samples (eg, 94% and 88% from high and moderate shedders, respectively[16]; 69% overall[18]), probably as a result of the fact that fecal matter from an individual animal that yields positive results for MAP is diluted among 4 or 9 other samples (based on pools of 5 and 10 fecal samples, respectively). Even with the expected loss in Se, pooling of fecal samples and testing by use of BCF may be useful and offer considerable cost savings in disease-control programs.[15,17] However, when a positive result is detected for a pooled sample, identification of the affected animal can be problematic. Follow-up culture of samples from each of the cattle in the pooled sample will be required.

We are unaware of any studies that have evaluated such pooling strategies for use with direct-fecal PCR methods. To explore this approach, we used the same set of samples as for the 3-test evaluation study. However, only the GB-PCR assay was used for this secondary objective. In our study, there was a marked loss of Se with pooling of fecal samples, as determined on the basis of results for the enhanced GB-PCR assay when

comparing results for the pooled samples with results for each of the individual samples that comprised the pool. Only 38.3% of pooled fecal samples known to contain feces from a cow that had positive results when tested individually by use of the GB-PCR assay actually had positive results for the GB-PCR method. The probability improved to 80% for those pooled samples that contained feces from 2 cattle with positive results when tested individually. Conversely, of the 75 cattle that had positive results when tested individually by use of the GB-PCR assay, only 35 were in pooled samples that yielded positive results when tested by use of the GB-PCR assay, and 24 of those were in pooled samples that also contained feces from another MAP-infected cow.

The reasons for this apparently disproportionate loss of assay Se for pooled fecal samples tested by use of direct-fecal GB-PCR assay, as compared with losses reported elsewhere[13-18] for BCF, are likely multifactorial. We speculate that traditional BCF methods offer a greater opportunity for detection of an extremely small number of MAP bacteria (even when diluted 5- or 10-fold) because they allow the MAP to reproduce over time and achieve minimum detectable limits, which results in a positive result for the pooled sample (especially during a 16-week growth period). On the other hand, despite improvements in enhanced recovery of DNA by use of immunomagnetic techniques and bead beating, the probability is likely greater that fecal dilution would drive DNA concentrations below minimum detection limits for PCR assays. These results must not be assumed to be constant among PCR assays (eg, may not apply to the qRT-PCR assay), because our results are limited to only the GB-PCR assay.

By use of Bayesian statistical methods, we evaluated the enhanced IS900 qRT-PCR method and determined that it offered a relative improvement in Se over serum ELISA and BCF for the detection of MAP. The Sp of the qRT-PCR method approached the Sp generally accepted for BCF; thus, the qRT-PCR method may be selected by clinicians when greater Sp is desired than is available with ELISA tests but more rapid reporting of results is required than is available with BCF. The increased cost of the enhanced qRT-PCR method, compared with that for the ELISA or BCF, must be considered as well. Finally, on the basis of analysis of our study findings, pooling of fecal samples for MAP detection by use of an enhanced IS900 GB-PCR method cannot be recommended at this time. However, because test characteristics can change among populations on the basis of age, disease prevalence, and other factors, additional studies should be performed to determine the applicability of enhanced PCR assays for use in dairy cattle.

a. Johne's disease fecal proficiency test, National Veterinary Services Laboratories, Ames, Iowa.
b. HerdChek, IDEXX Laboratories Inc, Westbrook, Me.
c. DairyComp 305, Valley Agricultural Software, Tulare, Calif.
d. Mycobactin J, Becton-Dickinson, Sparks, Md.
e. BioMag goat anti-rabbit immunoglobulin IgG, Polysciences Inc, Warrington, Pa.
f. Laboratory of Dr. I. R. Grant, Department of Food Microbiology, Queen's University of Belfast, Belfast, UK.
g. Multi-32 microcentrifuge tube separator, Polysciences Inc, Warrington, Pa.
h. Proteinase K, Promega, Madison, Wis.
i. RNase A, Qiagen Inc, Valencia, Calif.
j. Tag Gold DNA polymerase, Roche Molecular Systems, Branchburg, NJ.
k. Biometra thermocycler, Whatman Biometra Inc, Goettingen, Germany.
l. IS900 forward primer, Sigma Genosys, Sigma-Aldrich, St Louis, Mo.
m. IS900 reverse primer, Sigma Genosys, Sigma-Aldrich, St Louis, Mo.
n. IS900 probe, Sigma Genosys, Sigma-Aldrich, St Louis, Mo.
o. ABI 7000 real-time PCR, Applied Biosystems, Foster City, Calif.
p. TaqMan universal master mix, Applied Biosystems Inc, Foster City, Calif.
q. WinBUGS, version 1.4, MRC Biostatistics Unit, Cambridge, UK.
r. SPSS, version 12.0, SPSS Inc, Chicago, Ill.

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Appendix

Prior probability distributions for variables in the Bayesian model used to estimate Se and Sp for 3 diagnostic tests (BCF, serum ELISA, and an enhanced direct-fecal qRT-PCR assay) used to detect MAP infection among commercial adult dairy cattle in which the true infection status was unknown.

Variable	Test	Prior probability distribution (β)	Mode	Median	95% Probability interval
Prevalence*	NA	2.9, 18.0	0.10	0.13	0.030, 0.282
Se	Serum ELISA	31.7, 94.7	0.25	0.25	0.180, 0.330
Sp	Serum ELISA	328.2, 11.8	0.97	0.97	0.943, 0.982
Se	qRT-PCR	1.0, 1.0	NA†	0.50	0.025, 0.975
Sp	qRT-PCR	1.0, 1.0	NA†	0.50	0.025, 0.975
Se	BCF	11.0, 21.8	0.32	0.33	0.187, 0.503
Sp	BCF	—	1.0‡	—	—

*Represents the prevalence for the sample population, which does not represent herd prevalence.
†Uniform (noninformative) prior probability distribution in which all values between 0 and 1 are equally likely.
‡Bacterial culture of feces is assumed to have perfect Sp without variability.
NA = Not applicable. — = Not determined.