Evaluation of microbial culture of pooled fecal samples for detection of *Mycobacterium avium* subsp paratuberculosis in large dairy herds

Saraya Tavornpanich, DVM, MPVM; Ian A. Gardner, BVSc, MPVM, PhD;
Randall J. Anderson, DVM, MPVM; Sang Shin, DVM; Robert H. Whitlock, DVM, PhD; Terry Fyock;
John M. Adaska, DVM, MPVM;Richard L. Walker, DVM, PhD; Sharon K. Hietala, PhD

Objective—To evaluate sensitivity of microbial culture of pooled fecal samples for detection of *Mycobacterium avium* subsp *paratuberculosis* (MAP) in large dairy herds and assess the use of the method for estimation of MAP prevalence.

Animals—1,740 lactating cows from 29 dairy herds in California.

Procedure—Serum from each cow was tested by use of a commercial ELISA kit. Individual fecal samples were cultured and used to create pooled fecal samples (10 randomly selected fecal samples/pool; 6 pooled samples/herd). Sensitivity of MAP detection was compared between Herrold's egg yolk (HEY) agar and a new liquid culture method. Bayesian methods were used to estimate true prevalence of MAP-infected cows and herd sensitivity.

Results—Estimated sensitivity for pooled fecal samples among all herds was 0.69 (25 culture-positive pools/36 pools that were MAP positive). Sensitivity increased as the number of culture-positive samples in a pool increased. The HEY agar method detected more infected cows than the liquid culture method but had lower sensitivity for pooled fecal samples. Prevalence of MAP-infected cows was estimated to be 4% (95% probability interval, 2% to 6%) on the basis of culture of pooled fecal samples. Herd-level sensitivity estimate ranged from 90% to 100% and was dependent on prevalence in the population and the sensitivity for culture of pooled fecal samples.

Conclusions and Clinical Relevance—Use of pooled fecal samples from 10 cows was a cost-effective tool for herd screening and may provide a good estimate of the percentage of MAP-infected cows in dairy herds with a low prevalence of MAP. (*Am J Vet Res* 2004;65:1061–1070)

Availability of rapid, low-cost, and accurate diagnostic tests and testing strategies is critically important for producers, veterinarians, and regulatory authorities involved in the detection and control of paratuberculosis (ie, Johne's disease) in cattle, a disease caused by ***Mycobacterium avium* subsp *paratuberculosis* (MAP)**. Existing antemortem tests for paratuberculosis in cattle are considered to have only low to moderate sensitivity, ranging from 15% to 50% in subclinically infected cattle, but high to very high specificity, ranging from 98% to 100%.[1,2] Although most researchers have focused on improving test accuracy, an alternative approach has been to develop better cost-effective ways to obtain information of similar diagnostic use. One promising approach is culture of pooled fecal samples, which has potential application for detection of infected herds, prevalence estimation, and initial screening to detect infected cattle.[3-5] Moreover, the method is cost-effective, particularly in large herds with a low within-herd prevalence. A simulation study[6] to estimate costs and benefits of whole-herd testing by use of pooled fecal samples revealed that pooling reduced costs by 43% in a 100-cow herd with a high prevalence of MAP and up to 71% in a 1,000-cow herd with a low prevalence.

Few field studies have evaluated culture of pooled fecal samples for detection of MAP-infected herds. A study[7] in Dutch dairy herds evaluated age-clustered samples of 5 individual fecal samples/pool as a pooling strategy. Samples were collected from 11 dairy herds with a low MAP prevalence (< 5%) and herd size that ranged from 25 to 92 cows. Analysis of results of that study revealed that culture of pooled fecal samples was a sensitive means of assessing herd status for infection with paratuberculosis. Herd sensitivity was estimated

Received September 22, 2003.
Accepted January 23, 2004.
From the Departments of Medicine and Epidemiology (Tavornpanich, Gardner, Hietala) and Pathology, Microbiology, and Immunology (Walker), School of Veterinary Medicine, University of California, Davis, CA 95616; the California Department of Food Agriculture, Animal Health Branch, 3800 Cornucopia Way, Ste F, Modesto, CA 95358 (Anderson); the New York State Animal Health Diagnostic Laboratory, Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853 (Shin); the Johne's Research Laboratory, New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, PA 19348 (Whitlock, Fyock); and the California Animal Health and Food Safety Laboratory System, 18830 Rd 112, Tulare, CA 93274 (Adaska).
Supported by a grant from the USDA—Animal and Plant Health Inspection Service (No. 02-9106-0722-GR); the California Dairy Research Foundation; and the Center for Food Animal Health, School of Veterinary Medicine, University of California, Davis.
Presented in part at the 7th International Colloquium on Paratuberculosis, Bilbao, Spain, July 2002.
The authors thank Dr. Beate Schikora, Dr. Pat McIntuff and Cynthia Young, and Jennifer Abate for technical assistance; Dr. Michael Collins for providing estimates of disease prevalence and test accuracy; and Dr. Nadine Sullivan for assistance with the microbial culture system.
Address correspondence to Dr. Tavornpanich.

as 73% by use of pooled fecal samples, compared with 64% for culture of individual fecal samples. However, that study was conducted in small dairy herds and used a modified Jorgensen culture method, which differs from culture techniques widely used in the United States. In a study[8] conducted in the United States, culture of 5 fecal samples/pool revealed a sensitivity of 94% for detection of MAP-infected dairy herds with 100% specificity. The herds in that study were of small to moderate size (35 to 423 cattle/herd); hence, cost savings may not be sufficiently large for herd screening in large dairy herds with a low within-herd prevalence. Analysis of results in another study[9] indicated that culture of pooled fecal samples that used 10 samples/pool also had good sensitivity, compared with culture results for individual fecal samples, when samples were handled in an optimal manner. In that study, sensitivity estimates for culture of pooled fecal samples ranged from 22% to 100% and were dependent on the number of colonies per tube in fecal samples from MAP-infected cows. Nevertheless, results from that study may not be representative of test accuracy in naturally infected populations with variable infection prevalence and cows shedding varying numbers of MAP. In addition, typical delays in culture of fecal samples associated with transportation to and storage of samples at laboratories may decrease the sensitivity.

Two evaluations of the use of culture of pooled fecal samples for estimation of MAP prevalence in cattle have been reported.[8,10] In one of those studies,[8] investigators found a high correlation between prevalence of MAP determined by culture of pooled fecal samples and culture of individual fecal samples. Investigators in the other study[10] used pools consisting of 3 individual fecal samples to detect MAP infection and estimate animal-level prevalence of MAP. However, these prevalence estimates were calculated on the basis of apparent prevalence rather than the true prevalence of culture-positive fecal pools and hence failed to account for the imperfect sensitivity of pooled fecal samples. Moreover, the pool size used in the latter study[10] was unusually small and unlikely to be practical in large herds with a low MAP prevalence. Bayesian estimation of prevalence from testing results of pooled samples (20 samples/pool) when the test sensitivity is imperfect has been used to determine egg-level prevalence of *Salmonella* spp.[4] The method takes into account prior knowledge, which is expressed as a probability distribution, and then updates the estimates on the basis of test results. The posterior distribution from a Bayesian analysis has a direct probability interpretation.[11]

Objectives of the study reported here were to evaluate sensitivity for culture of pooled fecal samples for the detection of MAP in large dairy herds and assess the use of culture of pooled fecal samples for estimation of overall animal-level prevalence. In addition, sensitivity of a new liquid culture method[a] was evaluated and compared with that of traditional culture of fecal samples by use of **Herrold egg yolk (HEY)** agar. The liquid culture technique was based on detection of pressure changes within the space above the broth medium attributable to gas consumption of the bacteria, and it decreased diagnostic time from 16 weeks down to 5 weeks. The method has been used for detection of *Mycobacteria* organisms in specimens obtained from humans[12] and was the standard method currently in use for MAP culture at the New York State Animal Health Diagnostic Laboratory.[13] However, we are not aware of any studies that have evaluated use of this culture method for detection of MAP in pooled fecal samples obtained from cattle.

Materials and Methods

Study population—During the fall of 2001, 29 dairy herds in California with no or minimal data on their status with regard to MAP infection were included in the study. Dairies were identified by veterinarians who were members of 1 dairy practice. On the basis of histories provided by the owners and veterinarians, many herds were expected to be infected with MAP because of the lack of use of specific control measures for paratuberculosis. Information on general herd characteristics, management practices, and purchase of cattle was obtained during interviews. In each herd, 60 lactating cows were randomly selected from among the lactating cows available on the date of that herd visit. For each cow, data were collected that included tag number, date of birth, lactation number, and number of days in milk.

Collection of samples—A blood sample was collected from the coccygeal vein and a fecal sample (approx 100 g of feces) was collected from the rectum of each cow in the study. Samples were placed on cold packs and transported on the day of sample collection to the **California Animal Health and Food Safety Laboratory (CAHFSL)** at the University of California, Davis. After arrival at the laboratory, blood samples were centrifuged; sera were harvested, divided into aliquots, and stored frozen at –20°C until tested. For each fecal sample, 3 aliquots (15 g/aliquot) were placed in ointment tins and screw top containers and stored at –70°C until tested.

ELISA—All sera were tested by use of an ELISA kit[b] optimized by use of a plate reader equipped with a 650-nm filter. The method was the standard procedure used to detect antibody to MAP in bovine serum at the CAHFSL. Briefly, sera were diluted in sample diluent containing *M phlei* antigens to remove cross-reacting antibodies. The MAP antibody in diluted samples bonded to MAP antigen attached to the walls of 96-well plates. After incubation for 30 minutes at 25°C, unbound materials were removed by washing. Horseradish peroxidase was added to each well, and it bonded to the antigen-antibody complex. In the final step, enzyme substrate was added and plates were incubated for another 15 minutes at 25°C. Amount of color was measured spectrophotometrically at the specified wavelength. Serum samples were assayed along with positive- and negative-control samples, and **optical density (OD)** values were transformed to **ratios of sample value to positive value (S/P)** by use of the following equation:

$$S/P = (\text{OD of unknown sample} - \text{OD of negative-control sample}) / (\text{OD of positive-control sample} - \text{OD of negative-control sample})$$

Serum samples were initially assayed in a single well. When the S/P value for a sample was < 0.2, the result for the sample was considered negative. Samples with S/P values ≥ 0.2 were reassayed in duplicate. When the mean S/P value for the duplicates was ≥ 0.25, the result was considered positive; a mean S/P value of < 0.25 for the duplicates was considered a negative result.

Pooling method—We submitted 1,740 fecal samples (60 samples/herd) to the CAHFSL. Individual and pooled fecal samples were cultured. For each herd, fecal samples

were randomly assigned an identification number at the time of submission; 6 pooled samples were then created by use of the identification numbers of the individual fecal samples. Pool size was selected as 10 fecal samples/pool because of cost considerations and because the use of 10 samples/pool yielded good sensitivity in another study.[9] Individual fecal samples were thawed for 2 hours at 25°C. An aliquot (2 g of feces) was obtained from each sample by use of a wooden tongue depressor and weighed on a clean weight boat. Aliquots from 10 cows from the same herd were pooled. The 20 g of feces in each pool were mixed and placed into a 50-mL plastic tube. Each tube were labeled, and pooled samples were submitted for culture on the same day that the pools were created.

Microbial culture—Fecal samples were cultured by use of 2 methods (conventional culture on HEY agar and a liquid culture method[a]). Because of the large number of samples and limited capacity in each laboratory, samples were cultured at 3 approved laboratories (the **Johne's Disease Research Laboratory [JDRL]** at the University of Pennsylvania, the CAHFSL at Davis, and the CAHFSL at Tulare, Calif). Culture of all individual and pooled fecal samples by use of the liquid culture method was performed at the CAHFSL at Davis. Culture of all pooled fecal samples by use of the HEY agar method was performed at the JDRL, and culture of individual fecal samples by use of the HEY agar method was performed at all 3 laboratories. All laboratories used a centrifugation culture procedure, which is a modification of the Cornell method and is currently recommended as the national screening test for Johne's disease by the National Veterinary Services Laboratory in Ames, Iowa.[14]

The protocol used by personnel at the CAHFSL in Davis and Tulare was identical, but it differed slightly from the protocol used by personnel at the JDRL. At all laboratories, fecal samples were processed through 4 steps (measurement, mixing, sedimentation, and decontamination). Samples were then inoculated onto HEY agar and liquid media. Briefly, 2 g of feces was measured and placed into 35 mL of sterile distilled water in a sterile 50-mL centrifuge tube. Contents were mixed by use of a mechanical rocker for at least 30 minutes, and each tube was then placed upright to allow fiber contents in the suspension to settle for 30 minutes. A 5-mL sample was transferred from the top of the suspension into a second 50-mL tube that contained 25 mL of 0.9% hexadecylpyridium chloride in brain-heart infusion broth. Each sample was incubated at 37°C for 18 to 24 hours, followed by centrifugation. Supernatant was discarded. For the decontamination phase, the pellet was resuspended in an antimicrobial solution that contained amphotericin B, vancomycin, and nalidixic acid in brain-heart infusion broth. Samples were then incubated at 35° to 37°C for another 24 hours.

For cultures on HEY agar at the CAHFSLs, 4 tubes of HEY agar with iron-chelating protein (mycobactin[c]) were inoculated with 0.15 mL of the antimicrobial-containing suspension and incubated at 37°C in a slanted position with the caps loosely positioned to allow the surface to dry. After 1 to 2 weeks, caps were tightened and tubes were placed in an upright position. Tubes were examined weekly for 12 weeks to detect growth and contamination. At the JDRL, approximately 0.25 mL of the antimicrobial-containing suspension was inoculated into 4 tubes that contained HEY agar with mycobactin.[c] Tubes were incubated with the caps loosely positioned and subsequently incubated with the caps tightened and the tubes in an upright position. Tubes were evaluated to detect growth and contamination at 2-week intervals for 16 weeks. Each culture with positive results was evaluated for mycobactin-dependent characteristics by subculturing in an additional tube of HEY agar with and without mycobactin.[c] Ziehl-Neelsen acid-fast staining was performed on any colonies with typical morphologic features of MAP to allow the assessment of acid-fast status. Colonies of MAP usually are buff colored, and cells are short rods. Isolates that were mycobactin-dependent and acid-fast and had colony and microscopic morphologic characteristics consistent with MAP were reported as positive results. The number of colonies in each tube also was counted and recorded as very low (mean, < 1 CFU/tube), low (mean, ≥ 1 to 9.9 CFUs/tube), moderate (mean, 10 to 49.9 CFUs/tube), or heavy (mean, ≥ 50 CFUs/tube).

For culture in liquid medium, sample preparation and culture were performed in accordance with the manufacturer's recommendations. The liquid culture was an automated system that consisted of a culture system instrument and bottles that contained broth. The bottles contained liquid broth and 4 reagents (a growth supplement, mycobactin,[c] egg yolk, and antimicrobials) that helped optimize recovery of MAP from bovine fecal samples. Briefly, after the previously described decontamination process was completed, 1 mL of the antimicrobial-containing suspension was added to the broth bottles; contents were mixed thoroughly before bottles were placed into an incubator. The system monitored samples by use of a nonradiometric, pressure-detection system that measured changes in gas pressure in the space of the culture bottles at 20-minute intervals; it then generated a signal and transferred results directly to a computer.

Bovine fecal samples were assayed along with positive- and negative-control samples. Bottles were incubated for 42 days at 37°C. When no growth was evident in the bottles by the end of that incubation period, the culture was considered to have negative results. All samples with positive results, as determined by measurement of gas pressure in the bottles, were examined to detect microscopic morphologic and acid-fast characteristics consistent with MAP, which was then confirmed by use of a **polymerase chain reaction (PCR)** assay.[15] Results were reported as system-negative or -positive and the day at which the culture was detected with positive results. For analytic purposes, a cow that had positive results for culture with HEY agar or liquid medium was considered to be an MAP-infected cow because both assays were considered to have a specificity of 100%. Similarly, a pooled sample that had positive culture results or contained ≥ 1 culture-positive individual sample used to create the pooled sample was considered an MAP-positive pooled sample. Detection of ≥ 1 MAP-infected cow in a herd by culture of individual or pooled fecal samples was sufficient to classify the herd as an MAP-infected herd.

Sensitivity for culture of pooled fecal samples relative to culture of individual fecal samples—Sensitivity for culture of pooled fecal samples among all herds was estimated as the proportion of pooled samples with positive results, given that there was at least 1 culture-positive cow in the pool detected by culture of individual fecal samples. The ability for culture of pooled fecal samples, compared with culture of individual fecal samples, to detect MAP-infected herds was estimated as the number of herds that had positive results for culture of pooled fecal samples divided by the number of herds that had positive results for culture of pooled or individual fecal samples. A 95% **confidence interval (CI)** was calculated by use of exact binomial methods. The McNemar χ^2 test was used to compare the sensitivity for culture with HEY agar and liquid medium, and values of $P < 0.05$ were considered significant.

Bayesian methods for estimation of prevalence and test accuracy—A Bayesian approach was used to estimate true prevalence of MAP-infected cows (animal-level prevalence) on the basis of results for culture of individual fecal samples, animal-level prevalence on the basis of data for cul-

ture of pooled fecal samples, and herd-level sensitivity for culture of pooled fecal samples. The method was based on the principle that prior knowledge about test characteristics and infection prevalence (which can be acquired from experts or published experiments) can be incorporated into the model and used to update inferences for these variables. Three models were used for analysis.

Estimate of animal-level prevalence on the basis of individual test results (model 1)—Prevalence was estimated as described elsewhere[16] by use of results of the ELISA and culture of individual fecal samples. In the model, the **unknown animal prevalence (π_i) in the *i*th herd from which blood and fecal samples were randomly selected from n cows** was defined. There were 4 possible outcomes for each cow (positive for both tests [11], positive for the ELISA but negative for culture [10], negative for the ELISA but positive for culture [01], and negative for both tests [00]). The probabilities of cows having test results in 1 of these 4 categories were p_{11}, p_{10}, p_{01}, and p_{00}, respectively. The model specified the probability density for the number of cows (x_i from the *i*th herd) that had 1 of the 4 possible test outcomes. The values of x_i have the following multinomial distribution:

$$x_i \sim \text{multinomial}\ (n_i, [p_{i11}, p_{i10}, p_{i01}, p_{i00}])$$

For this distribution, the following equations can be solved:

$$p_{i11} = (\pi \times [Se_E \times Se_{IC} + Covse]) + ([1 - \pi] \times [1 - Sp_E] \times [1 - Sp_{IC}])$$

$$p_{i10} = (\pi \times ([Se_E \times \{1 - Se_{IC}\}] - Covse)) + ([1 - \pi] \times [1 - Sp_E] \times Sp_{IC})$$

$$p_{i01} = (\pi \times ([\{1 - Se_E\} \times Se_{IC}] - Covse)) + ([1 - \pi] \times Sp_E \times [1 - Sp_{IC}])$$

$$p_{i00} = (\pi \times ([\{1 - Se_E\} \times \{1 - Se_{IC}\}] + Covse)) + ([1 - \pi] \times Sp_E \times Sp_{IC})$$

where π is the animal-level prevalence; Se_E and Sp_E are sensitivity and specificity for the ELISA, respectively; Se_{IC} and Sp_{IC} are sensitivity and specificity for culture of individual fecal samples, respectively; and Covse is the sensitivity covariance. The Covse was included in the model to account for the possible effect of conditional dependence between the ELISA and culture of individual fecal samples. The magnitude of Covse is affected by the magnitude of test sensitivities, and limits of Covse were defined as follows[17]:

$$\text{maximum}(-[1 - Se_E] \times [1 - Se_{IC}], -Se_E \times Se_{IC}) \le Covse \le \text{minimum}([Se_E \times \{1 - Se_{IC}\}], [\{1 - Se_E\} \times Se_{IC}])$$

Because culture of fecal samples was considered to have specificity of 100%, then by definition, the test specificities were conditionally independent. Hence, inclusion of a specificity covariance was unnecessary. This model was used to estimate within-herd prevalence for each herd. All cattle were combined into a single population to estimate the overall animal-level prevalence and then were categorized into 2 populations on the basis of lactation number (ie, low [≤ 2 lactations] or high [> 2 lactations]) for estimation of MAP prevalence in these 2 groups.

Estimate of animal-level prevalence on the basis of results for culture of pooled fecal samples (model 2)—Overall π was estimated by use of a Bayesian model for a single test that was used in 1 population.[4] The model specified a probability density for the **number of pooled samples with positive results (y)** out of the **total number of pooled samples tested (m)**. This density has the following binomial distribution: y ~ binomial (m, ap), where ap represents the apparent prevalence of MAP in pooled samples. The **prevalence of pooled samples truly positive for MAP (tp)** was obtained by adjusting ap for **sensitivity for culture of pooled fecal samples (Se_{PC})** and **specificity for culture of pooled fecal samples (Sp_{PC})**. The Se_{PC} was defined as *P*(pooled sample has positive results | pooled sample truly is positive for MAP), Sp_{PC} was defined as *P*(pooled sample has negative results | pooled sample truly is negative for MAP), and tp was calculated as follows: $tp = [ap + Sp_{PC} - 1] / [Sp_{PC} + Se_{PC} - 1]$. Overall animal-level prevalence was derived from the relationship between π and tp by the following equation:

$$\pi = 1 - (1 - tp)^{1/k}$$

where k is the number of individual fecal samples in a pool, as described elsewhere.[4] In addition, the second model was nested in the first model to derive a 95% **probability interval (PI)** for the difference between estimates calculate on the basis of data for pooled and individual fecal samples.

Estimate of herd-level sensitivity for culture of pooled fecal samples (model 3)—Herd sensitivity (Hse) of a pooled test with k samples per pool and m pooled samples per herd is a function of π and the accuracy of the pooled test as described by the following equation[3]:

$$Hse = 1 - ([(1 - \{1 - \pi\}^k) \times \{1 - Se_{PC}\}] + [\{1 - \pi\}^k \times Sp_{PC}])^m$$

Therefore, overall π estimated from the first model and Se_{PC} and Sp_{PC} obtained from the second model can be used as input variables for deriving an estimate of the Hse for culture of pooled fecal samples.

Prior information about test characteristics and MAP prevalence was obtained from expert opinion and published studies. Uncertainty about these values can be represented by use of beta (α, β) distributions where the values of α and β determine the shape of the distribution. In the study reported here, another expert[d] and 1 of the coauthors (RHW) provided their opinion about Se_E, Sp_E, Se_{IC}, Sp_{IC}, and the within-herd prevalence of MAP in US dairy herds. Prior information about Se_{PC} was obtained from another study[9] in which the value for Se_{PC} was estimated to be 0.37 (37 pooled samples had positive results out of 100 pooled samples containing MAP that were tested) when a pooled sample contained a single fecal sample with a low colony count (< 10 CFUs/tube) and 0.94 (75 pooled samples had positive results out of 80 pooled samples containing MAP that were tested) when a pooled sample contained an individual fecal sample with a moderate or high colony count. In general, α was equal to the number of test-positive samples + 1, and β was equal to the number of test-negative samples + 1. Therefore, on the basis of that other study,[9] 2 beta distributions ([38, 64] when a pooled sample contained feces from a cow that was a low shedder of MAP and [76, 6] when a pooled sample contained feces from a cow that was a high shedder of MAP) were used for Se_{PC}. In addition, we assumed that 70% of cows were low shedders of MAP. We used a weighted mean of these 2 sensitivities (0.52 with a beta distribution [50, 46]) for the overall Se_{PC}. Elicitations of the prior information for all variables in the models and the corresponding beta distributions were determined (**Table 1**). The model was used by incorporating differing prior distributions, including an uninformative beta (1,1) to determine how sensitive the variable estimates were to the prior specification.

Posterior distributions from a Bayesian analysis are conditional densities of the variables of interest (animal prevalence, test sensitivity, and test specificity) given the observed data. Generally, posterior densities are not recognizable distributions and too complicated to be calculated analytically. Therefore, Gibbs sampling and Markov-chain Monte Carlo simulations were used to generate a solution.[18,19] In Gibbs sampling, samples

Table 1—Elicited prior estimates and corresponding beta (α, β) distributions for prevalence of *Mycobacterium avium* subsp *paratuberculosis* (MAP) and accuracy of tests.

Variable	Expert 1				Expert 2			
	Mode	LL*	UL†	Beta distribution	Mode	LL[a]	UL[b]	Beta distribution
Animal-level prevalence	0.05	NA	0.3	(1.5, 10.8)	0.30	NA	0.95	(1,1)
ELISA								
Sensitivity	0.25	NA	0.3	(58.8, 174.5)	0.20	NA	0.40	(4.5, 14.8)
Specificity	0.98	0.96	NA	(272.4, 6.5)	0.96	0.94	NA	(384, 17)
Culture of individual fecal samples								
Sensitivity	0.35	NA	0.5	(11.8, 21.00)	0.30	NA	0.45	(10.2, 22.5)
Specificity	0.995	0.99	NA	(1,137.5, 6.7)	1.00	0.99	NA	(370.6, 1.3)
Culture of pooled fecal samples								
Sensitivity								
Overall	0.52	0.49	NA	(50, 46)	NA	NA	NA	NA
Containing feces from a single cow that was a low shedder of MAP	0.37	0.34	NA	(38, 64)	NA	NA	NA	NA
Containing feces from a single cow that was a high shedder of MAP	0.94	0.90	NA	(76, 6)	NA	NA	NA	NA
Specificity	0.995	0.99	NA	(1,137.5, 6.7)	NA	NA	NA	NA

*Value considered by the expert (with 95% confidence) to be the lowest possible value for that variable. †Value considered by the expert (with 95% confidence) to be the highest possible value for that variable.
NA = Not applicable; only the lower limit (LL) or upper limit (UL) is needed for estimation of beta parameters.

from the posterior densities are generated iteratively. After several thousand iterations, the median can be used as a point estimate and the 2.5 and 97.5 percentiles can be used as a 95% PI for each variable. All Bayesian models were analyzed by use of a software program.[e] We performed 55,000 iterations of each model, with the first 5,000 iterations discarded as a period during which any dependence on the starting values for the Markov-chain Monte Carlo iterations was lost (ie, a burn-in period). Median and 95% PI of the variables of interest were reported.

Results

Sample population—The number of lactating cows in participating herds ranged from 285 to 2,233 (mean, 898 lactating cows). Of the 29 herds included in the study, 21 consisted of only Holstein cattle, 3 consisted of only Jersey cattle, and 5 consisted of cows of both breeds. Twenty-one herds housed their lactating cows in freestalls, 2 herds housed their cows completely in drylots, and 6 herds used a combination of both housing styles; no other types of housing were used in these 29 herds. Most herds (25/28) had a history of at least 1 cow with nonresponsive diarrhea and persistent weight loss during the preceding 2 years.

For each herd, 6 pooled fecal samples were created (174 pooled fecal samples for the 29 herds). All 174 pooled fecal samples were cultured by use of HEY agar. However, only 87 pooled fecal samples and all individual fecal samples used to create the pools (ie, 870 individual fecal samples) were cultured by use of both methods. An additional 104 individual fecal samples that had ELISA S/P values > 0.2 were cultured by use of HEY agar. Hence, there were 974 individual fecal samples that were cultured by use of HEY agar and 108 pooled fecal samples for which the culture status of all individual fecal samples used to create the pooled samples was determined or for which at least 1 individual sample in a pool had positive culture results.

Sensitivity for culture of pooled fecal samples relative to culture of individual fecal samples—Of the 974 cows for which we cultured individual fecal samples, 50 (5.1%) had positive results for the HEY agar or liquid culture method. Feces of the 50 culture-positive cows were distributed in 36 pooled samples; the number of culture-positive individual fecal samples in a pooled sample ranged from 1 to 3. Culture of pooled fecal samples detected MAP in 27 pooled samples (25 pooled samples from 36 pooled samples that contained MAP, and 2 pooled samples in which all fecal samples used had negative culture results). Assuming that tests for culture of fecal samples had a specificity of 100%, sensitivity for culture of pooled fecal samples relative to culture of individual fecal samples among all herds was estimated to be 0.69 (25 pooled samples with positive results/36 pooled samples that truly contained MAP), with a 95% CI of 0.52 to 0.84. Sensitivity increased as the number of culture-positive individual samples in a pooled sample increased; it ranged from 0.64 when there was at least 1 culture-positive sample in a pool to 1.0 when there were 3 culture-positive individual samples in a pool. Of the 50 cows that had culture-positive individual samples, 8 (15%) were very low (mean, < 1 CFU/tube), 19 (38%) were low (mean, ≥ 1 to 9.9 CFUs/tube), 7 (14%) were moderate (mean, ≥ 10 to 49.9 CFUs/tube), and 16 (32%) were high (mean, ≥ 50 CFUs/tube) shedders. Of 25 pools that contained at least 1 culture-positive individual sample, pooled fecal culture detected MAP from 1 of 5 (20%) very low, 5 of 8 (63%) low, and 10 of 11 (91%) moderate and high shedders.

Comparison of sensitivity for culture of pooled fecal samples between HEY agar and liquid media—We cultured 87 pooled fecal samples by use of both methods. However, 6 pooled samples prepared for

culture with HEY agar were excluded from evaluation because of an error in handling of the samples. Use of HEY agar detected MAP in 7 of 20 pooled samples (7/19 [37%] pooled samples that contained ≥ 1 culture-positive individual sample, as determined by culture with HEY agar or liquid medium, and 1 additional pool in which all individual fecal samples had negative culture results). Use of liquid medium detected MAP in 10 of 20 (50%) pooled samples that contained at least 1 culture-positive individual sample, as determined by culture with HEY agar or liquid medium(**Table 2**). For culture of individual fecal samples, use of HEY agar detected more cows shedding MAP organisms in feces than were detected by use of liquid culture. Of 870 individual fecal samples that were cultured by use of HEY agar and liquid medium, 32 (3.7%) had positive results for either method, 27 (3.1%) had positive results for HEY agar only, and 20 (2.3%) had positive results for liquid culture only. However, the sensitivities for culture with HEY agar and liquid medium did not differ significantly (P = 0.145). For 20 culture-positive samples (as determined by use of liquid medium), 17 (85%) were detected in < 32 days (mean time to detection, 24 days).

Estimate of animal-level prevalence on the basis of results of ELISA and culture of individual fecal samples—We were able to obtain data on lactation number for 865 cows that were tested by use of the ELISA and culture of fecal samples. Of the 865 cows, 13 (1.5%) had positive results for both tests, 31 (3.6%) had positive results for ELISA only, 18 (2.1%) had positive results for culture of fecal samples only, and 803 (92.8%) had negative results for both tests (**Table 3**). Bayesian analyses were performed to estimate the true prevalence of cows infected with MAP (animal-level prevalence). Use of prior information from 1 expert yielded an overall posterior median for animal-level prevalence of 0.09 (95% PI, 0.05 to 0.16). Of the 865 cows tested, 536 (62%) were lactation > 2, and the prevalence of MAP in this subpopulation was 0.11 (95% PI, 0.06 to 0.19), which was slightly higher than the median prevalence of MAP in cows that were lactation ≤ 2 (median, 0.07; 95% PI, 0.03 to 0.13). Within-herd prevalence for each of the 29 herds ranged from 0.05 (95% PI, 0.001 to 0.10) to 0.29 (95% PI, 0.14 to 0.50).

Table 2—Frequencies for results of pooled fecal samples obtained from cattle in 29 dairy herds in California and cultured by use of Herrold egg yolk (HEY) agar and a liquid culture system.

Culture method	Result for pooled fecal samples	No. of culture-positive samples*			
		0	1	2	3
HEY agar†	+	1	2	3	2
	–	62	8	2	1
Liquid culture	+	0	3	4	3
	–	67	8	2	0

*Each pooled sample consisted of 10 randomly selected individual fecal samples. †We excluded 6 pooled samples prepared for culture by use of HEY agar from the evaluation because of an error in sample preparation.
+ = Positive culture result. – = Negative culture result.

Table 3—Paired results for an ELISA and culture of individual fecal samples for cows in 29 dairy herds in California on the basis of lactation number.

ELISA	Culture of individual fecal samples	Lactation No.		
		≤ 2	> 2	Total
+	+	3	10	13
+	–	9	22	31
–	+	6	12	18
–	–	311	492	803
Total		**329**	**536**	**865**

See Table 2 for key.

Table 4—Posterior median and 95% probability interval (PI) for animal-level prevalence determined on the basis of individual test results and sensitivity and specificity of an ELISA, culture of individual fecal samples, and culture of pooled fecal samples for 29 California dairy herds.

Variable	Expert 1		Expert 2	
	Median	95% PI	Median	95% PI
Animal-level prevalence	0.09	0.05, 0.16	0.11	0.06, 0.21
ELISA				
Sensitivity	0.25	0.20, 0.31	0.19	0.10, 0.34
Specificity	0.97	0.96, 0.99	0.96	0.95, 0.98
Culture of individual fecal samples				
Sensitivity	0.34	0.21, 0.49	0.30	0.17, 0.46
Specificity	0.994	0.989, 0.997	0.997	0.988, 0.999
Culture of pooled fecal samples				
Sensitivity				
Overall	0.51	0.41, 0.61	NA	NA
Containing a sample from a single cows that was a low shedder of MAP	0.36	0.27, 0.46	NA	NA
Containing a sample from a single cow that was a high shedder of MAP	0.93	0.86, 0.97	NA	NA
Specificity	0.994	0.990, 0.998	NA	NA

NA = Not applicable.

Use of prior information from the second expert resulted in a slight increase for overall animal-level prevalence of 0.11 (95% PI, 0.06 to 0.21). Median prevalence of MAP in the population of cows that were lactation ≤ 2 and lactation > 2 was 0.09 (95% PI, 0.03 to 0.21) and 0.15 (95% PI, 0.07 to 0.28), respectively. However, the difference in prevalence estimates determined on the basis of opinion of the 2 experts was minimal (the 95% PI for the difference between the 2 experts included a value of 0). The within-herd prevalence estimated on the basis of the second expert's opinion was not calculated because the prior information about prevalence for that expert was not suitable for the model. Posterior estimates for sensitivity of ELISA and culture of individual fecal samples varied depending on changes in the prior values, whereas specificity of both tests was extremely high (≥ 95% for the ELISA and > 99% for culture of fecal samples) and more stable than for the sensitivity values (**Table 4**). There was minimal dependence between the sensitivity of ELISA and culture of individual fecal samples; median sensitivity covariance between the 2 tests was 0.07 (95% PI, 0.01 to 0.13).

Estimate of animal-level prevalence on the basis of results of culture of pooled fecal samples—Of 174 pooled samples that were cultured, 27 (15.5%) yielded positive results. When prior modes of 0.52 and 0.995 were used for sensitivity and specificity, respectively, for culture of pooled fecal samples, true prevalence of MAP-positive pooled samples was 0.31 and the overall animal-level prevalence was 0.04 (95% PI, 0.02 to 0.06). True prevalence estimated on the basis of results for a subset of 3 pooled samples/herd was 0.3 (95% PI, 0.1 to 0.6), which was similar to the estimate (0.31) determined on the basis of results for 6 pooled fecal samples/herd.

Sensitivity estimate for culture of pooled fecal samples was higher in a pool that included feces from a cow that was a high shedder of MAP than in a pool that included feces from a cow that was a low shedder of MAP (sensitivity ranged from 0.36 to 0.93; Table 4). Animal-level prevalence was estimated to be 0.02 (95% PI, 0.01 to 0.03) for a pooled sample with a high sensitivity and 0.06 (95% PI, 0.03 to 0.10) for a pool with a low sensitivity. Overall prevalence estimated on the basis of data for pooled fecal samples was lower than the overall estimate determined on the basis of data for individual fecal samples (95% PI for the difference between pooled and individual fecal samples, –0.01 to –0.13).

Estimate of herd sensitivity for culture of pooled fecal samples—Of the 29 herds, 2 (6.9%) had samples that had negative results when tested by use of the ELISA, culture of individual fecal samples, and culture of pooled fecal samples (**Table 5**). Culture of individual and pooled fecal samples detected MAP in 21 and 17 herds, respectively, from this population. Herd-level sensitivity for culture of pooled fecal samples relative to culture of individual fecal samples was 0.81 (17/21) ,and the 95% CI was 0.58 to 0.95. Using Bayesian methods, herd-level sensitivity for culture of pooled fecal samples was obtained by incorporating posterior information about animal-level prevalence determined on the basis of results for individual fecal samples and various posterior sensitivities for culture of pooled fecal samples. For each herd with 6 pooled samples consisting of 10 individual samples/pooled sample, estimates of herd sensitivity for culture of pooled fecal samples ranged from 0.90 (95% PI, 0.79 to 0.96) when pooled samples contained feces from a cow that was a low shedder of MAP to 0.999 (95% PI, 0.997 to 1) when pooled samples contained feces from a cow that was a high shedder.

Table 5—Number of MAP-positive samples for an ELISA, culture of individual fecal samples, and culture of pooled fecal samples for cattle in 29 dairy herds in California.

Herd	ELISA*	Culture of individual fecal samples†	Culture of pooled fecal samples‡
1	0	0	0
2	0	0	0
3	0	1	0
4	0	2	0
5	1	1	0
6	1	1	1
7	1	1	1
8	1	2	1
9	2	0	0
10	2	0	0
11	2	0	0
12	2	0	0
13	2	1	1
14	2	1	1
15	2	2	1
16	2	3	1
17	3	2	1
18	3	2	1
19	3	2	2
20	4	0	0
21	4	1	0
22	4	3	2
23	5	6	2
24	6	3	2
25	6	3	2
26	6	8	4
27	7	0	0
28	13	2	2
29	13	2	2
Total	**97**	**50**	**28**

*Based on 60 samples/herd. †Positive results for culture by use of HEY agar or a liquid culture system on 30 to 45 samples/herd. ‡Positive results for culture by use of HEY agar or a liquid culture system on 6 pooled samples/herd.

Discussion

The goals of the study reported here were to evaluate the sensitivity for culture of pooled fecal samples for detection of MAP in large dairy herds and assess the use of culture of pooled fecal samples for estimation of animal-level prevalence when there were multiple pooled samples cultured for each herd. The rationale inherent in use of a pooled testing strategy is that when MAP prevalence is extremely low, testing of pooled samples will provide data similar to that for testing of samples from individual cattle but at a substantially lower cost, providing that the sensitivity of culture for individual and pooled samples is comparable. The cost-effectiveness for culture of pooled fecal samples increases as herd size increases. For the herd sizes in the study reported here, testing all cows (assumed expense of $20/culture) would cost $5,700 for the herd with 285 cows and $44,660 for the herd with 2,233

cows. By use of a strategy for testing of pooled samples, the cost is reduced in proportion to the number of fecal samples in the pool. For pooled samples consisting of 10 individual fecal samples/pool, a dairy producer will save $5,120 on initial screening for a herd with 285 cows and $40,180 for a herd with 2,233 cows. When it is desired to determine an animal-level diagnosis by testing each individual sample in pools with positive results, the additional cost will depend on the proportion of pooled samples with positive results, which theoretically is an increasing function of within-herd prevalence. The lower the prevalence, the lower the number of additional culture tests required. The worst-case scenario would be an extremely high proportion (> 90%) of pooled samples with positive culture results. This would require retesting of almost every cow in the herd to identify the infected cows that had contributed to the positive culture results of pooled samples. In this scenario, the cost of culturing pooled fecal samples and then retesting individual samples in the positive pools would exceed the cost of culturing individual samples only. This scenario, however, is unlikely to happen because the prevalence of paratuberculosis in cattle is rarely extremely high. In this study in which 6 pooled samples were cultured per herd, almost all herds that had positive results (16/17) had only 1 or 2 pooled samples that yielded positive cultures and that would then have required retesting of those individual fecal samples. Therefore, when all individual samples in positive pools need to be cultured, the total cost for culture of pooled fecal samples followed by retesting feces from cows in MAP-positive pooled samples for the 29 dairy herds would range from $120/herd (0 pooled samples with positive results) to $920/herd (4 pooled samples with positive results). The mean cost of $313/herd was approximately one fourth the cost ($1,200) for culturing individual fecal samples from 60 cows/herd. Disadvantages of culture of pooled fecal samples, compared with culture of individual fecal samples, were a potential loss of sensitivity through dilution of samples and additional time required for retesting of individual fecal samples when a pooled sample yielded positive results.

Sensitivity for culture of pooled fecal samples, compared with that of culture of individual fecal samples, and, hence, its use is dependent on factors such as the detection limit of the pooling method, prevalence in the population, distribution of MAP-positive individual samples in the pools, and number of organisms shed in feces of MAP-infected cows. Sensitivity of culture of pools consisting of 10 fecal samples in the study reported here was comparable to that for pools consisting of 5 fecal samples in other studies.[7,8] We found that sensitivity varied depending on the number of culture-positive samples in the pool and the number of MAP organisms shed in feces of infected cows, which is similar to findings of other studies. The distribution of MAP-positive individual fecal samples in the pooled samples was not uniform, which was expected for a population of herds with variable but low prevalence. In pools that contained feces from MAP-positive cows, 25 of 36 (69%) had 1 culture-positive individual sample. Sensitivity for culture of pooled fecal samples that contained 1 culture-positive sample was approximately 38%, which was lower than that estimated in another study[8] (58%) in which a pool consisting of 5 samples was used. Sensitivity for culture of pooled fecal samples was greatly affected by the number of organisms shed by MAP-positive cows whose feces were included in a pool. The technique detected 20% of the pools that included feces from a cow that was a very low MAP shedder (< 1 CFU/tube) but 91% when pools contained feces from a cow that was a moderate or high shedder. When there was > 1 culture-positive individual sample in a pooled sample, sensitivity for culture of the pooled fecal sample was considerably increased and the amount of shedding had minimal effect on the detection of MAP. The pooling strategy of 10 randomly selected individual fecal samples/pool may be inadequate for a high probability of detection for cows that are very low shedders of MAP. However, approximately 15% of the infected cows in this population were very low shedders, and cows shedding few MAP organisms may not be important sources of environmental contamination at the time of testing. In addition, these cows may be readily detected if shedding in the feces increases with time and pooled testing is repeated every 6 to 12 months. Other strategies such as pooling feces from cows that have a higher probability of being infected into the same pooled sample may also improve sensitivity for culture of pooled fecal samples.

Specificity for culture of pooled fecal samples is considered to be perfect or almost perfect (≥ 99.5%). However, pooled samples can yield positive results when all individual samples in that pool have negative culture results. This phenomenon has been reported in other studies[7-9] and most likely is attributable to lack of homogeneity of MAP in fecal samples. The assumption of a specificity of 100% for the HEY agar culture method was determined on the basis of the nature of the test and consistent results from other studies.[1,2,8,20] Specificity of 100% for the liquid culture method was determined on the basis of confirmation with the PCR assay. Although we assumed specificity to be 100%, uncertainty about the specificity value for the test was allowed in the Bayesian model to estimate the true prevalence of MAP-infected cows. When specificity was 100%, the prevalence estimate (true prevalence) was higher than the apparent prevalence because of the imperfect sensitivity. However, when specificity was < 100%, the true prevalence would be lower than the apparent prevalence in herds with an extremely low prevalence but higher than the apparent prevalence in herds with a high prevalence. This also depended on the value for the test sensitivity.

The liquid culture method appears to be a promising alternative to traditional culture by use of HEY agar because sensitivities of the 2 methods for individual fecal samples were comparable and the liquid culture method substantially reduced the interval from onset of culture to detection of MAP. The rapid turnaround time for the liquid culture method probably will have important practical advantages for control of paratuberculosis, especially when culture of pooled fecal samples is used as a screening test and additional time is required for retesting of feces from cows included in MAP-positive pools. In the study reported here, the mean time to

detection by use of the liquid culture method was 24 days, which was approximately 2 to 3 months less than the time to detection by use of the HEY agar culture method. We were unable to determine whether the liquid culture method was more sensitive than the HEY agar method for testing of pooled samples because of the small number of pooled samples available; therefore, additional studies with a larger sample size are necessary to determine whether the decreased diagnostic time for the liquid culture method, compared with that for HEY agar, translates into improved control of paratuberculosis. Other factors such as false-positive results, processing costs, and laboratory facilities are factors that should be considered before routine use of the liquid culture method is implemented.

Estimation of within-herd prevalence also is important for selection of specific management practices that can be implemented to control MAP transmission in a dairy herd. The sampling scheme was not initially optimized for prevalence estimation but allowed us to assess the use of culture of pooled fecal samples for estimation of MAP prevalence. Our study used Bayesian methods to estimate animal-level prevalence on the basis of results for culture of individual and pooled fecal samples. Bayesian methods account for uncertainty in test sensitivity and specificity and allow prior information or expert knowledge to be incorporated into the analysis. In this study, prevalence estimation determined on the basis of results for culture of pooled and individual fecal samples was estimated in parallel and the difference in estimates was measured. Prevalence estimates were within the range of prior information provided by the first expert, who considered the most likely value to be 5% with 95% confidence that the prevalences for similar untested herds would be < 30%. Culture of pooled fecal samples provided a good estimate of the proportion of infected cows that were shedding MAP in the feces. The prevalence estimate determined on the basis of culture of pooled fecal samples was 4%, which was similar to the estimate of the number of positive results for culture of individual fecal samples (5%). However, the prevalence estimate determined on the basis of data for culture of pooled samples was lower than the prevalence estimate determined on the basis of results of 2 tests on individual samples (ELISA and culture of individual fecal samples) because culture of pooled fecal samples detected only the infected cows that were shedding MAP in feces, whereas the ELISA results included cows that had antibodies against MAP. Antibody-positive cows included cows that were not shedding MAP in feces at the time of sample collection. The ratio of these 2 estimates would yield an estimate of the proportion of infected cows that are shedding MAP at a single time point.

Herd-level diagnosis by use of 6 pooled samples, each of which consisted of 10 individual fecal samples/pool, appears to be a cost-effective and practical testing strategy. For the scenario with a low animal-level prevalence, culture of pooled fecal samples detected at least 81% of herds known to have cattle infected with MAP that were detected by culture of individual fecal samples, but culture of pooled fecal samples was at substantially lower cost. The technique may be a useful strategy for level 1 herds in the Voluntary Johne's Disease Herd Status Program,[21] a classification that is assigned on the basis of results for ELISA tests conducted on sera obtained from 30 cows, followed by culture of fecal samples from cows with positive ELISA results. Herd-level sensitivity of the testing strategy used in level 1 herds ranges from 33% to 84%[22] and is dependent on the percentage of cows with positive result for culture of MAP. Estimates of herd sensitivity determined by use of Bayesian methods revealed that when pooled samples contained feces from a cow that was a moderate or high shedder of MAP, sensitivity for culture of pooled fecal samples increased to 100%. Theoretically, estimates for herd-level sensitivity should be based on the true MAP status of study herds and not solely on results for tested cattle. For the worst-case scenario in which cattle in all 29 herds were infected with MAP, our estimates of herd-level sensitivity would be lower by approximately 30%. The 29 study herds were selected on the basis of large herd size and no or minimal testing for MAP. Although most herds reported a history of cows with clinical signs consistent with paratuberculosis, 2 herds had no cows that had positive results by use of the ELISA or culture of fecal samples, and it is possible that these herds were truly not infected. Misclassification of noninfected herds as infected herds would decrease herd-level test sensitivity.

The study reported here was designed to evaluate sensitivity for culture of pooled fecal samples relative to that for culture of individual fecal samples. Although some individual fecal samples in pools were selected on the basis of a high ELISA S/P value (ie, > 0.2), this would increase the probability of detecting MAP-infected cows in culture of individual and pooled fecal samples to a similar degree. A potential limitation that may have affected interpretation of findings was the differences in processing of fecal samples in the 3 laboratories. Although all laboratories used the same culture method, sensitivity for culture of individual fecal samples may have differed among the laboratories and could explain why 2 pooled fecal samples had positive culture results despite the fact that all individual fecal samples in those pools had negative culture results. However, sensitivity for culture of pooled fecal samples in the study was defined as the probability of detecting MAP-positive pools, given that there was at least 1 culture-positive individual fecal sample in the pool. Therefore, false-negative results for culture of individual fecal samples were not included in the estimation.

[a]ESP para-JEM culture system II, TREK Diagnostic Systems Inc, Sun Prairie, Wis.

[b]HerdCheck, IDEXX Laboratories Inc, Westbrook, Me.

[c]Mycobactin-J, Allied Monitor Inc, Fayette, Mo.

[d]Collins M. Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wis: Personal communication, 2002.

[e]WinBUGS 1996–2001, version 1.4, Imperial College & MRC, London, UK. Available at: www.mrc-bsu.cam.ac.uk/bugs. Accessed Apr 12, 2004.

References

1. Whitlock RH, Wells SJ, Sweeney RW, et al. ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. *Vet Microbiol* 2000;77:387–398.

2. Sweeney RW, Whitlock RH, Buckley CL, et al. Evaluation of a commercial enzyme-linked immunosorbent assay for the diagnosis of paratuberculosis in dairy cattle. *J Vet Diagn Invest* 1995;7:488–493.

3. Christensen J, Gardner IA. Herd-level interpretation of test results for epidemiologic studies of animal diseases. *Prev Vet Med* 2000;45:83–106.

4. Cowling DW, Gardner IA, Johnson WO. Comparison of methods for estimation of individual-level prevalence based on pooled samples. *Prev Vet Med* 1999;39:211–225.

5. Munoz-Zanzi CA, Johnson WO, Thurmond MC, et al. Pooled-sample testing as a herd-screening tool for detection of bovine diarrhea virus persistently infected cattle. *J Vet Diagn Invest* 2000; 12:195–203.

6. van Schaik G, Stehman SM, Schukken YH, et al. Pooled fecal culture sampling for *Mycobacterium avium* subsp *paratuberculosis* at different herd sizes and prevalence. *J Vet Diagn Invest* 2003; 15:233–241.

7. Kalis CHJ, Hesselink JW, Barkema HW, et al. Culture of strategically pooled bovine fecal samples as a method to screen herds for paratuberculosis. *J Vet Diagn Invest* 2000;12:547–551.

8. Wells SJ, Godden SM, Lindeman CJ, et al. Evaluation of bacteriologic culture of individual and pooled fecal samples for detection of *Mycobacterium paratuberculosis* in dairy cattle herds. *J Am Vet Med Assoc* 2003;223:1022–1025.

9. Wells SJ, Whitlock RH, Lindeman CJ, et al. Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis*. *Am J Vet Res* 2002;63:1207–1211.

10. Sorensen O, Rawluk S, Wu J, et al. *Mycobacterium paratuberculosis* in dairy herds in Alberta. *Can Vet J* 2003;44:221–226.

11. Carlin BP, Louis TA. *Bayes and empirical Bayes methods for data analysis*. 2nd ed. Boca Raton, Fla: Chapman & Hall/CRC, 2000;121–191.

12. Tortoli E, Cichero P, Chirillo MG, et al. Multicenter comparison of ESP Culture System II with BACTEC 460TB and with Lowenstein-Jensen medium for recovery of *Mycobacteria* from different clinical specimens, including blood. *J Clin Microbiol* 1998; 36:1378–1381.

13. Shin SJ. A new liquid-culture method, the TREK ESP Culture System II, for rapid detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples. *Proc Annu Meet US Anim Health Assoc* 2000;104:392–393.

14. Stabel JR. An improved method for cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples and comparison to three other methods. *J Vet Diagn Invest* 1997;9:375–380.

15. van der Giessen JW, Haring RM, Vauclare E, et al. Evaluation of the abilities of three diagnostic tests based on the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in cattle: application in a control program. *J Clin Microbiol* 1992; 30:1216–1219.

16. Joseph L, Gyorkos TW, Coupal L. Bayesian estimation of disease prevalence and the parameters of diagnostic tests in the absence of a gold standard. *Am J Epidemiol* 1995;141:263–272.

17. Gardner IA, Stryhn H, Lind P, et al. Conditional dependence between tests affects the diagnosis and surveillance of animal diseases. *Prev Vet Med* 2000;45:107–122.

18. Gelfand AE, Smith AFM. Sampling based approaches to calculating marginal densities. *J Am Stat Assoc* 1990;8:398–409.

19. Spiegelhalter DJ, Best NG, Gilks WR, et al. Hepatitis B: a case study in MCMC methods. In: Gilks WR, Richardson S, Spiegelhalter DJ, eds. *Markov chain Monte Carlo in practice*. Boca Raton, Fla: Chapman & Hall, 1998;21–43.

20. Stabel JR, Whitlock RH. An evaluation of a modified interferon-γ assay for the detection of paratuberculosis in dairy herds. *Vet Immunol Immunopathol* 2001;79:69–81.

21. Bulaga L. US voluntary Johne's disease herd status program for cattle. *Proc Annu Meet US Anim Health Assoc* 1998;102:420–433.

22. Wells SJ, Whitlock RH, Wagner BA, et al. Sensitivity of test strategies used in the Voluntary Johne's Disease Herd Status Program for detection of *Mycobacterium paratuberculosis* infection in dairy cattle herds. *J Am Vet Med Assoc* 2002;220:1053–1057.