Evaluation of bacteriologic culture of pooled fecal samples for detection of *Mycobacterium paratuberculosis*

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Objectives—To compare sensitivity of several methods of bacteriologic culture of pooled bovine fecal samples for detection of *Mycobacterium paratuberculosis* and evaluate homogeneity in number of *M paratuberculosis* in pooled fecal samples.

Sample Population—Feces from 10 dairy cows that shed *M paratuberculosis* at various concentrations and 1 dairy cow known to be free of infection with *M paratuberculosis*.

Procedure—5 fecal pooling methods, 2 culture methods, and 2 pool sizes were evaluated. Each pooled sample contained 1 infected sample and 4 or 9 uninfected samples.

Results—Sensitivity of detection of *M paratuberculosis* was greater with smaller pool size (5 vs 10 samples/pool). Detection sensitivity was also associated with concentration of bacteria in the infected sample. Results indicated that, compared with concurrent bacterial culture of individual infected samples, 37 to 44% of pooled samples with low bacterial concentrations yielded positive culture results and 94% of pooled samples with high bacterial concentrations yielded positive results.

Conclusions and Clinical Relevance—Bacteriologic culture of pooled fecal samples may provide a valid and cost-effective method of detecting *M paratuberculosis* infection in cattle herds. (*Am J Vet Res* 2002;63:1207–1211)

Johne's disease (paratuberculosis) is a chronic disease of cattle and other ruminants that is caused by *Mycobacterium paratuberculosis* (*M avium* subsp *paratuberculosis*). *Mycobacterium paratuberculosis* infects young calves and infection progresses slowly, with clinical signs most commonly appearing in cows 3 to 6 years of age. In dairy operations, associated economic losses include reduced milk production, premature culling, and reduced bodyweight in culled cows. The National Animal Health Monitoring System (NAHMS) has estimated that at least 22% of US dairy cattle herds have cows infected with *M paratuberculosis*[1] and US dairy farms with high prevalence of infection lose > $200/cow per year.[2] Due to the ongoing expansion of dairy herds nationwide and the widespread movement of dairy cattle, the disease continues to infect previously uninfected herds. In addition, concern persists that *M paratuberculosis* may be a cause of Crohn's disease in humans.

In response to these animal health and potential public health concerns, a **Voluntary Johne's Disease Herd Status Program (VJDHSP)** for identifying test-negative cattle herds has been developed.[3] The VJDHSP has been endorsed by the US Animal Health Association as a uniform model for state programs and implemented in several states. Other countries (including Australia and the Netherlands) have also recently developed national programs to identify test-negative herds and to help infected herds reduce losses associated with the disease.[4,5]

The approach used in the VJDHSP involves testing a statistical subset of the adult cattle in a herd by use of a serologic ELISA to detect antibodies against *M paratuberculosis*, followed by repeated annual herd tests with either the ELISA or individual-cow fecal bacteriologic cultures. This approach takes advantage of relatively low cost and quick turn-around of ELISA results initially in the program, despite low test sensitivity in subclinically infected cows (15%) and test specificity < 100%.[6] Within the VJDHSP, a correction is made for the imperfect specificity of the ELISA in that cows with positive ELISA results are tested to confirm positive status by use of bacteriologic culture of feces. Individual-cow bacteriologic culture of feces with sensitive techniques[7] provides advantages of increased test sensitivity and 100% test specificity[8,9] but at a higher cost and much longer turn-around time (as long as 16 weeks). An unfortunate consequence of this retesting is that herd sensitivity (the percentage of infected herds that yield positive results) of the ELISA testing strategy is reduced. While far from ideal, this program has been considered the best compromise between scientific validity and cost-benefit by use of validated tests that are presently available.

Other testing strategies are possible, however, including testing of pooled fecal samples. Christensen and Gardner[10] recently reviewed the theoretical basis for consideration of pooling in herd-level testing strategies. These authors concluded that the primary advantage of pooling is that a higher percentage of animals can be represented within a tested population for a certain fixed laboratory cost and that this advantage is maximized when within-herd prevalence is low (< 5%). This advantage, however, may be negated by logistical difficulties of pooling and potential loss of

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sensitivity relative to individual animal testing. Pooling of fecal samples is especially compelling for detection of herds infected with *M paratuberculosis*, because the low sensitivity of ELISA tests and low detectable within-herd prevalence of infection in infected herds has created a demand for sensitive yet low-cost test strategies for herd detection, especially in large herds.

The Dutch Animal Health Service has applied a pooling approach to identify herds with negative results of testing for Johne's disease. Their program relies on bacteriologic cultures of age-clustered pooled fecal samples with samples from 5 cows/pool (samples are grouped on the basis of date of birth of cows). One study revealed 87% sensitivity for detecting *M paratuberculosis* infection, relative to bacteriologic culture of individual cow fecal samples at the same time,[11] indicating that age-clustered pooled fecal samples might be used to detect *M paratuberculosis* infection among herds of unknown status. Additional pooled samples also yielded positive culture results, although results for individual cultures were negative, indicating that some infected cows were detected in pooled samples alone. The pooled fecal bacteriologic cultures were performed at similar cost to individual fecal bacteriologic cultures, thereby reducing the cost per sampled cow by approximately a factor of 5. However, the Dutch study included only 11 cattle herds, and the fecal bacteriologic culture method used in that study (modified Jorgenson) is different from culture methods predominantly used in the United States.

Australian researchers have also evaluated the use of pooled fecal bacteriologic cultures in ovine Johne's disease surveillance. A recent report indicated that *M paratuberculosis* could be reliably detected in pooled samples that contained fecal pellets from 1 sheep with multibacillary paratuberculosis (defined as multifocal coalescing to severe diffuse intestinal lesions containing large numbers of acid-fast bacilli) and 49 uninfected sheep by use of a radiometric medium, and proposed that pooled fecal bacteriologic culture replace serologic testing for herd detection.[12] While these results appear promising, it is unclear whether pooled fecal bacteriologic cultures will work in US cattle populations as well as reports from the Netherlands and Australia suggest.

To begin to address this question, we conducted the study reported here to compare sensitivity of several pooled fecal bacteriologic culture methods to detect *M paratuberculosis* and evaluate homogeneity in numbers of *M paratuberculosis* in pooled fecal samples.

Materials and Methods

Fecal samples—Source of the fecal samples was a repository of fecal samples with known *M paratuberculosis* colony counts from the Johne's Disease Laboratory, New Bolton Center, University of Pennsylvania, which was collected during a period of several years from cattle known to be infected with *M paratuberculosis*; samples were stored in 15-g metal containers at −70 C. Ten samples were selected that represented a spectrum of shedding from light fecal shedding with < 10 colonies/tube (n = 6) to moderate fecal shedding with 10 to 50 colonies/tube (3) and a single sample representing heavy fecal shedding with > 50 colonies/tube. One known uninfected cow, with a history of no lifetime exposure to infected cattle and repeated negative results of fecal bacteriologic cultures, was selected as the source of noninfected fecal samples. The consistency of the feces from the known noninfected cow was termed loose, with a higher water content than may be expected for a typical dairy cow, because the cow was maintained on new spring grass pasture as the sole source of feed.

Study design—Different methods of pool preparation and bacteriologic culture, pool sizes, and colony count of each infected sample within each pooled sample were evaluated to compare differences in resultant sensitivity of bacteriologic culture. Each of 5 methods of pool preparation and bacteriologic culture was evaluated by use of 5 and 10 fecal samples within each pooled sample; 1 culture-positive fecal sample was included in each pool with either 4 or 9 culture-negative fecal samples from the uninfected cow. Two methods of pooling fecal samples were used at 1 laboratory that used a centrifugation culture procedure and 3 methods of pooling fecal samples were used at another laboratory that used a sedimentation culture procedure. Each culture was repeated to replicate the study results; therefore, 80 pooled fecal samples underwent bacteriologic culture at 1 laboratory and 120 pooled fecal samples underwent bacteriologic culture at the second laboratory. Each infected fecal sample was part of 20 fecal pools examined via culture. In addition, each laboratory performed bacteriologic culture on the 10 infected fecal samples individually in duplicate to provide a concurrent comparative culture result via the same technique used in that laboratory for bacteriologic culture of the pooled samples.

Fecal pooling methods and bacteriologic culture—One laboratory used a centrifugation culture procedure and 2 methods of pooling the fecal samples. In centrifugation pool method A, 2 g of feces from each sample within each pool was mixed in a 50-ml plastic conical tube with a sterile wooden stick. The composite sample was vortexed for 10 to 15 seconds until it appeared homogeneous. Two grams of the resultant fecal mixture was mixed with 35 ml of water, inverted on a rocker for 30 minutes, and allowed to settle for 30 minutes. Five milliliters from the upper third of the tube was transferred to a second tube that contained 25 ml of 0.9% **hexadecylpyridium chloride (HPC)** and half strength **brain heart infusion broth (BHI**; prepared with 18.5 g/L of distilled water). The sample was incubated for 18 to 24 hours at 37 C, centrifuged at 900 × g for 30 minutes, and the supernatant was decanted. The pellet was resuspended in 1 ml of half strength BHI with antimicrobials, and the sample was incubated for 18 to 24 hours at 37 C. Two hundred microliters (7 drops) was inoculated on the surface of each of 4 tubes of **Herrold's egg yolk medium (HEYM)**. The inoculated tubes were placed in an incubator at 37 C. The tubes were left in the horizontal position for 7 to 14 days with the cap loosened to allow the water to evaporate from the surface of the media. The caps were then tightened, and the tubes were placed upright for the remainder of the 16-week incubation period. In centrifugation pool method B, each sample was processed individually as described for method A, and prior to inoculation of the media, the resuspended pellets from each individual fecal sample within the pool were mixed together in a 50-ml plastic conical tube and vortexed. Two hundred microliters of the mixture (7 drops) was plated onto 4 tubes of HEYM, and the sample was vortexed again.

The other laboratory used a sedimentation culture procedure[13] with 72 hours of sedimentation prior to inoculation of tubes that contained HEYM. In sedimentation pool method A, 2 g of feces from each sample that composed each pooled sample was placed in a disposable petri plate and mixed thoroughly with a sterile wooden stick.

After mixing, a 2-g sample of the pooled feces was placed in a tube with 40 ml of distilled water and placed on a horizontal shaker for 30 minutes. Tubes were left standing upright for 1 hour. The upper 5 ml of supernatant was transferred to a tube with 35 ml of 0.9% HPC and left on a counter top for approximately 72 hours for sedimentation. After sedimentation, 2 ml of sediment was removed from the bottom of the tube by use of a sterile Pasteur pipette and mixed with 0.1 ml of amphotericin B (50 mg rehydrated in 10 ml of distilled water). From this mixture, 200 µl (7 drops) was inoculated on the surface of 4 tubes of HEYM. The inoculated tubes were placed in a 37 C incubator for 7 to 14 days with the cap loosened to allow the excess moisture from the inoculum to dry on the surface of the media. The caps were then tightened, and the tubes were placed upright in a 37 C incubator for the remainder of 16 weeks. In sedimentation pool method B, each fecal sample was processed as an individual sample. After the 72-hour sedimentation, 2 ml of the sediment from each sample in the pool was placed in a 50-ml plastic conical tube and vortexed for 10 to 15 seconds. A 2-ml portion of the pooled sediments was mixed with 0.1 ml of amphotericin B. The pooled sample was used to inoculate media as described for method A. In sedimentation pool method C, samples were pooled and processed in the same way as for method B except the pooled sediments were allowed to sediment a second time for 72 hours. A 2-ml sample was taken from the sediment of the pooled sample, combined with 0.1 ml of amphotericin B, and inoculated and incubated as in methods A and B.

At each laboratory, each processed fecal sample was plated on 4 tubes of HEYM that contained mycobactin J. Each laboratory made a batch of HEYM to be divided equally between the 2 laboratories. This procedure allowed each laboratory to inoculate 2 tubes of media of the same lot from each laboratory, which eliminated differences in recovery attributable to differences in media quality. Isolates grown on HEYM were subcultured on 2 tubes of HEYM, 1 that contained mycobactin J and 1 without mycobactin J, for confirmation of mycobactin dependency. Each isolate was stained with Ziehl-Neelsen acid-fast stain, and the morphologic characteristics were considered compatible with *M paratuberculosis*.

Statistical analyses—The presence of detectable *M paratuberculosis* was compared among pooled sample sizes (5 or 10 samples), culture and sample pool preparation methods, and mean **colony forming units (CFU)** per tube for infected samples by use of multivariable logistic regression modeling. The logistic model included bacteriologic culture status of pooled samples as the dependent variable. Independent variables included size of pooled fecal samples (5 or 10 samples), culture and pool preparation method (using a 5-level class variable), log of mean colony count from concurrent culture of individual infected samples, and replicate number (to control for replication). The repeatability of culture results of pooled fecal samples to detect *M paratuberculosis* as a measure of within-sample homogeneity of numbers of colonies isolated was evaluated by use of Spearman correlation and the κ statistic. A commercial statistical program[a] was used for all data analyses. A value of $P \leq 0.05$ was considered significant.

Results

Each of the 10 infected fecal samples was individually culture-positive on at least 1 tube of media in the 2 replicates (8 tubes total) in each laboratory, although several of the mean colony counts were low. One fecal sample yielded positive culture results on only 1 of 4 tubes in 1 replicate at each laboratory.

The mean CFU per tube was calculated for each group of pooled samples for each infected sample by culture and pooling method. For pooled samples with mean concurrent individual sample bacterial culture results < 2.5 CFU/tube, mean CFU per tube ranged from 0 to 1.2. For pooled samples with mean concurrent individual sample bacterial culture results from 10 to 15 CFU/tube, mean CFU per tube ranged from 0.5 to 4.7. Mean CFU per tube for pooled samples with concurrent individual sample bacterial culture results of 70 CFU/tube (maximum concentration counted) ranged from 66 to 70.

From logistic regression analysis, variables associated with pool culture status (using the Wald χ^2 test statistic) included pool size ($P = 0.03$), bacterial culture and pool preparation method ($P = 0.04$), and $\log_{10}$ of mean CFU per tube from concurrent bacteriologic culture of infected fecal samples ($P < 0.001$). Interactions among these variables were not significant. The likelihood ratio χ^2 statistic for the overall model was 111 with 7 *df* ($P < 0.001$).

The odds ratio for detection of *M paratuberculosis* in pools with 10 samples relative to that for pools with 5 samples was 0.42 (95% **confidence interval (CI)**, 0.19 to 0.94), indicating a greater probability of detection in pools of 5 samples. Individual odds ratios for method of bacterial culture and pool preparation (relative to centrifugation method A) included centrifugation method B (0.83; 95% CI, 0.24 to 2.79), sedimentation method A (0.46; 95% CI, 0.14 to 1.58), sedimentation method B (0.21; 95% CI, 0.06 to 0.75), and sedimentation method C (1.48; 95% CI, 0.43 to 5.05). While an overall difference was observed across methods of bacterial culture and pool preparation in the logistic model, the only method statistically different from the reference category (centrifugation pooling method A with direct mixing of fecal samples) was sedimentation pooling method B (odds ratio indicated lower probability of detection). This indicated that combining fecal samples directly provided similar or higher sensitivity, compared with pooling of processed inoculum samples. Replicate number was not significant in the logistic model.

Pooled samples that contained a single sample with a high colony count were more likely to be detected than those that contained a sample with a low colony count, and the odds ratio for an increase of 1 $\log_{10}$ in mean CFU per tube was 2.92 (95% CI, 2.18 to 3.90). The percentage of pooled samples that yielded positive culture results increased from 34 (pools of 5 samples) or 22% (pools of 10 samples) for pooled samples that included a sample with < 1 CFU of *M paratuberculosis* per tube to 100% for pooled samples that included a sample with at least 50 CFU/tube (**Fig 1**).

For pooled samples of either size (5 or 10 samples) that contained an infected sample with a low colony count from concurrent bacteriologic culture (mean < 2.5 CFU/tube), *M paratuberculosis* was detected in 37% (37/100) and 44% (39/88), by replicate, of pooled samples. In addition, the pooled samples yielded *M paratuberculosis* in 35% (7/20) and 16% (5/32) of pooled samples (by replicate) in which negative results of culture were obtained for the concurrent bacterial culture of

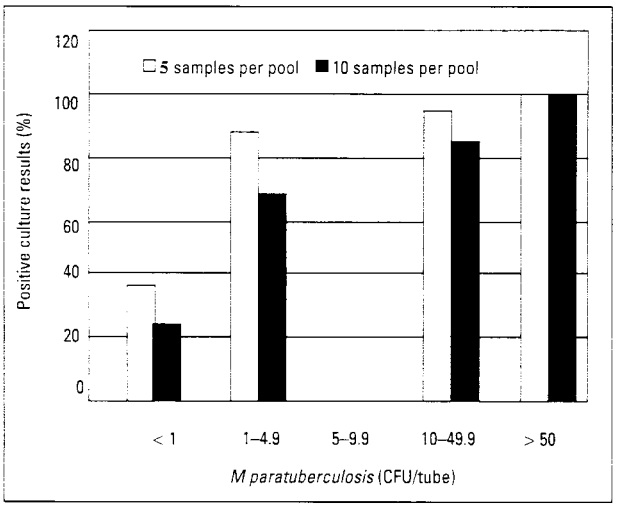

Figure 1—Percentage of pooled bovine fecal samples that contained various concentrations of *Mycobacterium paratuberculosis* (mean colony forming units [CFU] per tube) and yielded positive results for bacteriologic culture.

infected sample. For the pooled cultures that contained an infected sample with a moderate to high colony count, (mean > 10 CFU/tube), the sensitivity of the pooled fecal method was 94% (75/80 in each replicate).

Results from repeatability analyses (as a measure of homogeneity) of the 100 pools replicated indicated 81% agreement between the replicates. The κ statistic was 0.62, indicating moderate to substantial agreement between replicates.[14] The Spearman correlation coefficient that estimated the correlation between replicates (mean CFU per tube) was 0.74 ($P < 0.001$).

Discussion

Since effective control strategies against Johne's disease are focused at the herd level,[15,16] it is critical that each cattle producer determines the Johne's disease infection status of his or her herd. Herd-level diagnosis is presently problematic, especially in low prevalence herds, using available test strategies.[17] An alternative diagnostic method involves culture of pooled fecal samples from groups of cows within a herd, which provides advantages of bacterial culture (increased sensitivity and specificity, relative to ELISA) at a fraction of the cost of whole-herd individual-cow culture of feces.

Strengths of this study included a randomized study design that involved 2 bacterial culture methods and laboratories, with replication. The same media was used in both laboratories, although the laboratories used different culture methods. Previously examined fecal samples were used for the infected samples, and these samples were recultured in replicate at each laboratory for standardization purposes.

A single infected fecal sample was included in each pooled fecal sample, which limited generalizations from the results because more than a single infected cow is expected in infected herds, especially if age-clustered methods of collecting pooled fecal samples are used, as by the Dutch Animal Health Service.[11] Our study design, however, provided a challenging model to evaluate various fecal pooling methods, because several of the *M paratuberculosis*-infected fecal samples had low colony counts. We used results from concurrent bacteriologic cultures as the basis of comparison rather than the previous culture results, because viability of some of the organisms may have been lost during storage.

Fecal homogeneity with regards to number of *M paratuberculosis* is a concern with use of bacteriologic culture of pooled fecal samples. A small study[5] that evaluated daily fecal shedding in 3 infected cows (2 of which developed clinical disease during the study) revealed cyclic variability in fecal shedding. Despite this variability, however, fecal shedding was detected on at least 80% of the sampling days. Our study evaluated variability of culture results in samples collected at a single point in time, to evaluate homogeneity within a sample. While some variability of culture results across replicated pooled culture results was noted, results from the repeatability analyses indicated good agreement, consistent with adequate homogeneity within fecal samples collected at a single point in time for detection of infection at the pool level. The effect of cyclic variability in shedding needs to be further studied, however, to evaluate its effect on detection of infected cattle.

These results indicate that *M paratuberculosis* can be detected in pooled cattle fecal samples, even when only 1 infected sample in the pool contained a small number of organisms. Approximately 40% of pooled samples that contained a single sample with few mycobacteria and 94% of pooled samples with a single sample with moderate to high numbers of mycobacteria yielded positive culture results. In addition, several pooled samples were culture-positive although concurrent culture of the infective sample yielded negative culture results. This apparent discrepancy was also reported in the Dutch study,[11] although reasons for it are unclear. Because specificity of culture is considered to be 100%, we interpreted a positive result for a pooled sample as a true positive result and a further indication that use of pooled samples is adequate for detection of light fecal shedding with *M paratuberculosis*. Satisfactory results were achieved by use of either sedimentation or centrifugation culture methods. Similar sensitivity resulted from mixing fecal samples directly or after further processing, indicating that relatively simple methods could be used for pooling fecal samples.

Higher sensitivity was achieved by use of pooled samples that contained 5 samples, compared with pooled samples that contained 10 samples, which indicated a dilution effect. For use of pooled samples in the field, economic considerations need to be evaluated because larger pool sizes would certainly cost less and could be satisfactory for herds with infected cows shedding *M paratuberculosis* at high concentrations. An important consideration is the expected distribution of infected cattle within a herd. Extrapolating from a study of 10 Pennsylvania dairy herds,[9] we expect about 70% of infected cows to be light fecal shedders (< 10 CFU/tube), 10% to be moderate fecal shedders (10 to 50 CFU/tube), and 20% to be high shedders (> 50 CFU/tube). In herds with at least 1 high fecal shedder, it might be possible to detect *M paratuberculosis* in

even larger fecal pools than those evaluated in this study.

A limitation of this study was the inclusion of only 10 infected fecal samples. Repeated testing of these samples was useful for evaluating differences in pool size and bacterial culture and pool preparation method, but limited generalizations that could be made from the study results. A necessary step prior to widespread use of pooled fecal samples is an evaluation of the sensitivity and utility of this method to detect groups of cattle infected with *M paratuberculosis* in field studies. With further confirmation, we expect that bacteriologic culture of pooled fecal samples could be used as a herd screening method in the United States, similar to strategies used elsewhere.

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