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Culture of strategically pooled bovine fecal samples as a method to screen herds for paratuberculosis

C. H. J. Kalis, J. W. Hesselink, H. W. Barkema, M. T. Collins

Abstract. Fecal samples from 733 cows in 11 dairy herds with a low prevalence of paratuberculosis were cultured for the presence of *Mycobacterium avium* subsp. *paratuberculosis* both individually and after combining (pooling) in groups of 5. The culture procedure was the modified Jørgensen method, which uses NaOH and oxalic acid for decontamination and modified Löwenstein-Jensen agar slants for cultivation. Pooling was performed by mixing fecal samples from 5 animals ordered by age, herein referred to as strategic pooling. Culture of individual fecal samples detected *M. a. paratuberculosis* infections in 43 of the 733 cows and 7 of 11 infected herds (herd sensitivity = 64%). Culture of pooled fecal samples detected *M. a. paratuberculosis* in 28 of 151 pooled samples representing 8 of the infected 11 herds (herd sensitivity = 73%). Feces of the 43 culture-positive cows was included in 32 pools: of these 32 pools, 26 were culture positive and 6 were culture negative. In addition to the 26 positive pools containing feces from cows that were found culture positive on individual fecal samples, another 2 pools were culture positive, although comprised of feces from cows with negative results after culture of individual fecal samples. From the total of 45 infected cows that were found (43 by individual fecal culture and an additional 2 by pooled fecal culture), individual fecal culture detected 43 of these 45 (96%), while pooled fecal culture detected 39 (87%). Culture of strategically pooled fecal samples using the modified Jørgensen method was equivalent in herd sensitivity to the culture of individual fecal samples and is significantly less expensive.

Paratuberculosis, or Johne's disease, in cattle is an infectious, chronic, granulomatous enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis*. This disease is characterized by a decrease in milk production, loss of body condition, and intermittent diarrhea without the general disease symptoms fever, depression, or reduced appetite. In the terminal phase, animals die in a cachectic state. The primary symptoms are caused by reduced nutrient absorption due to transmural granulomatous enteritis. The disease is widespread in cattle populations in almost all countries with a dairy industry and causes great economic losses, not only because of lower productivity but even more by loss of future income due to early culling.^{1,5,8,12} In addition, there is reported a potential association of *M. a. paratuberculosis* with Crohn's disease, a chronic, granulomatous ileocolitis of humans. The etiology of Crohn's disease is unknown, but it is suggested to be a disorder of the immune response to a persistent stimulus that could be an infectious agent such as *M. a. paratuberculosis*.²

Paratuberculosis is, apart from an individual animal

disease, more importantly a herd problem. Early detection of infected herds can lead to early intervention, thereby minimizing economic damage caused by the infection. Owners of cattle herds that are found non-infected are encouraged to pursue classification of their herds as being of low risk for paratuberculosis so that they may serve as safe sources of replacement animals. The diagnostic objective is to test herds of cattle to classify them as infected or presumptively noninfected with maximal accuracy and least cost. Some countries, such as Australia, have elected to use the absorbed ELISA for paratuberculosis as the principle diagnostic test for herd screening and classification.⁹ ELISA-based testing is readily automated and low cost; however, it is less sensitive and specific than fecal culture-based tests for paratuberculosis.^{3,13,14} A disadvantage of paratuberculosis diagnosis by fecal culture is cost. A previous report on use of pooled fecal samples for paratuberculosis diagnosis indicated >60% decrease in the sensitivity of detecting individual infected cattle.¹⁵ This study, however, employed a different culture method and pooling of samples in an age-dependent fashion, herein referred to as strategic pooling, in hopes of overcoming the problems with sensitivity. Paratuberculosis tends to cluster by age in groups of animals because exposure to *M. a. paratuberculosis* follows temporal patterns as multiple calves are exposed to infectious feces or milk from a shedding cow.

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This diagonal spread of the infection leads to age clustering of infections (unpublished results). Strategic pooling increases the likelihood of detecting an infected herd by increasing the likelihood of detecting an infected fecal pool through inclusion of more than 1 infected individual.

The objective of this study was to determine if culture of pooled samples would provide a cost-effective means of screening herds for paratuberculosis without significant loss of ability to detect infected herds.

Materials and methods

Herds. Eleven dairy herds in the northern Netherlands with a low prevalence of paratuberculosis (yearly cumulative incidence of clinical disease <5%), confirmed by *M. a. paratuberculosis*-positive fecal culture, were used. Cows in these herds were housed during the winter in loose housing stalls with cubicles and were pastured during the summer. The diet of the cows was dried grass silage in winter and grass in summer, with commercial concentrates occasionally supplemented with corn silage or sugar beet pulp. Herd size ranged between 25 and 92 (mean 67) adult Holstein-Friesian and Dutch-Friesian crossbred cows.

Sampling procedure. Fecal samples were collected from the rectum of each animal in disposable plastic exam gloves. No lubricants were used during sample collection. After the fecal sample collection, the gloves were tied closed and identified with preprinted, self-adhesive labels that showed the bar code for each cow, as recorded in the Dutch Identification and Registration system.¹¹ Samples were kept at 4 C during transport and processed for isolation of *M. a. paratuberculosis* within 24 hr after arrival at the laboratory. The same sample was used for both individual and pooled culture.

Pooling procedure. Fecal samples of cows were ranked by age after numbering the bar coded identification and registration numbers according to birth date. Pooling was performed by weighing 2 g of feces from each of 5 cows into a sterile mortar. A small amount of sterile water (approximately 5 ml) was added to make it possible to thoroughly mix the 5 samples with a sterile pestle. After mixing, a 3-g aliquot (2 g feces and 1 ml water) was weighed out and transferred to a second sterile mortar.

Decontamination reagents. Three solutions were used in the decontamination procedure.⁷ 1) NaOH 4% in sterile water, 2) a mixture of oxalic acid and malachite green (OA-MG) in a concentration of 5 mg/ml oxalic acid and 1 mg/ml malachite green, and 3) a mixture of amphotericin B^d and neomycin,^c in a concentration of 50 mg/ml amphotericin B and 0.5 mg/ml neomycin.

Decontamination procedure. Decontamination was done as follows: from individual samples, a fecal sample weighing approximately 2 g (range 1.8–2.2 g) was thoroughly mixed with 8 ml of 4% NaOH in a sterilized pestle and mortar for 5–10 sec, depending on the consistency of the feces. The mixture was poured into a sterile 10-ml plastic centrifuge tube, which was then placed on its side in a mechanical rotary shaker and mixed for 15 min. The sample was centrifuged (15 min at 1,000 × g) and the supernatant was

poured off. Five milliliters of OA-MG solution were added to the pellet, which was resuspended using a sterile wooden stick. This suspension was placed in a mechanical rotary shaker for 15 min. The mixture was then centrifuged (15 min at 1,000 × g) and the supernatant was poured off. Four milliliters of antibiotic solution were added to the tube, and the pellet was resuspended using a sterile wooden stick and then vortexed. The mixture was then allowed to stand overnight at room temperature (20 C).

Culture medium. Modified Löwenstein-Jensen (LJ) medium⁶ was used. The concentrations of critical components were malachite green (component of the LJ base^a), 0.25 mg/ml; glycerin, 0.0075 ml/ml; pyruvate, 4.0 mg/ml; mycobactin,^b 2.0 µg/ml; cycloheximide,^c 0.75 mg/ml; chloramphenicol,^c 0.2 mg/ml; sodium penicillin G, 200 units/ml; and whole egg homogenate, 62.5%. Glass tubes (16-mm diameter × 15 cm) were filled with 7 ml medium, which was allowed to solidify at an angle such that the surface of the medium extended three fourths the way up the tube.

Medium inoculation and culture procedure. After the decontaminated suspension had settled overnight, there was a lower layer of particulate matter and an upper layer of clear, green solution. The clear upper layer just above the pellet was removed with a Pasteur pipette. Three or 4 drops of this solution were then distributed evenly over the surface of each of 4 tubes of modified LJ culture medium (all containing mycobactin). The inoculated tubes were loosely capped and left to stand for 24 hr at room temperature (20 C) such that the agar surface was horizontal in order to permit maximum absorption of the inoculum onto the surface of the medium. Then the tubes were tightly sealed with a rubber stopper, placed in racks, and incubated for 16 wk at 37 C. The tubes were inspected after 8, 12, and 16 wk for evidence of growth. Colonies thought to be *M. a. paratuberculosis* on the basis of their appearance and slow growth rate were stained by acid-fast stain. Tubes with 1 or more small, smooth to slightly rough, white to slightly yellow bacterial colonies of small acid-fast rod-shaped bacteria were presumptively considered culture positive, and then these bacteria were confirmed to be *M. a. paratuberculosis* by the use of IS-900 PCR.⁴ A sample was considered to be culture positive if 1 or more culture tubes were recorded as culture positive. The number of colonies of *M. a. paratuberculosis* in each tube was recorded. A culture tube was recorded as contaminated if fungal or bacterial growth other than *M. a. paratuberculosis* was found. A sample was considered to be contaminated if all 4 tubes were contaminated.

Herd sensitivity analysis. The ability of culture of strategically pooled fecal samples to detect infected herds was evaluated for 11 low prevalence herds. All fecal samples were cultured both individually and as pooled samples. If a sample or a pool of samples was contaminated, both the pool of samples and its 5 matched samples were excluded from the analysis. A positive pool was defined as a) one from which *M. a. paratuberculosis* was isolated or b) one that contained feces from an animal found to be infected by isolation of *M. a. paratuberculosis* from the fecal sample cultured individually. Herd sensitivity was defined as the ability to detect at least 1 infected animal in an infected herd. McNemar's chi-square test and the kappa statistic were used

Table 1. Number of *Mycobacterium avium* subsp. *paratuberculosis*-positive cultures in the 11 herds with simultaneous culturing of pooled and individual bovine fecal samples.

Herd	No. of animals (individual samples)	No. of culture-positive animals	No. of pools	No. of culture-positive pools
1	58	0	12	0
2	65	8	13	4
3	63	0	13	1
4	66	2	14	1
5	25	0	5	0
6	45	3	9	3
7	78	4	16	2
8	92	10	19	7
9	92	15	19	9
10	82	0	17	0
11	67	1	14	1
Total	733	43	151	28

to compare agreement of results on the herd level for culture of individual and pooled fecal samples.¹⁰

Results

Culture of individual fecal samples. In the 11 herds, 733 animals were sampled and *M. a. paratuberculosis* was isolated from 43 individual fecal samples (5.9%) from cattle in 7 herds (Table 1). Of the culture tubes for individual fecal samples, 113 (3.9%) were contaminated after 8 weeks of incubation, increasing cumulatively to 175 (6.0%) after 16 weeks of incubation. In 5 samples (0.7%), all 4 tubes were contaminated.

Culture of pooled fecal samples. In the 11 herds, 151 pooled fecal samples were cultured and *M. a. paratuberculosis* was isolated from 28 pools (18.5%) representing cattle in 8 herds. For cultures of pooled fecal samples, 18 culture tubes (3.0%) were contaminated after 8 weeks of incubation, increasing cumulatively to 30 (5.0%) after 16 weeks. In one pool (0.7%), all 4 tubes were contaminated.

Herd sensitivity of culture of pooled and individual fecal samples. One hundred eleven pools were culture negative and so were the involved 555 individual fecal samples. Two pools were culture positive in spite of failure to detect *M. a. paratuberculosis* in the 10 individually cultured fecal samples. Most likely in each of these 2 pools, feces from 1 cow yielded a false-negative culture result while feces from the other 4 cows in the pool were true negative. Six pools were culture negative in spite of presence of 1 culture-positive sample in each of them, as was demonstrated by culture of individual samples. Twenty-six culture-positive pools each contained culture-positive individual samples. From these 26 *M. a. paratuberculosis*-positive pools, 17 pools included 1 each of the infected

cows detected by individual samples, 7 pools included 2 each individually detected cows, and 2 pools included 3 each of these cows (Table 2). From the 43 culture-positive cows, feces from 37 cows (86%) were culture positive in individual fecal samples and were included in culture-positive fecal pools. The remaining 6 culture-positive cows were part of culture-negative pools.

On the 11 *M. a. paratuberculosis*-infected low prevalence herds in the study, culture of pooled fecal samples detected 8 (herd sensitivity = 73%) while culture of individual fecal samples detected 7 (herd sensitivity = 64%). Chi-square analysis showed no difference in herd detection rate between culture of pooled and individual fecal samples and excellent agreement in results ($\kappa = 0.79$).

Effect of pooling on contamination and colony counts. The 1 contaminated pool contained feces from 5 cows found culture negative on individual fecal samples. The 5 contaminated individual samples were matched to 5 culture-negative pools. In total, 6 pools and 30 matched individual samples were excluded from the comparison. Mean number of colonies counted on all 4 culture tubes from the 28 culture-positive pooled fecal samples and the 6 culture-negative pools containing culture-positive individuals was 15.8. The mean number of the colonies counted on all 4 tubes of the individually sampled culture-positive animals was 31.4. Colony counts appeared most strongly influenced by the number of *M. a. paratuberculosis* per gram of feces in an individual sample rather than the number of culture-positive individuals per fecal pool (Table 2).

Discussion

Fecal sample pooling logically results in dilution of the number of *M. a. paratuberculosis* in any one sample comprising the pool. However, the observed reduction in colony counts on pooled samples was not as great as expected. Instead, colony counts on pooled samples were around 50% of those of the total of individual samples. We attribute this to concentration of *M. a. paratuberculosis* during the sample processing and decontamination procedure. With a perfect concentration technique, dilution of fecal samples should not influence the number of colonies at all.

Fecal culture results for paratuberculosis are classified as either positive, negative, or contaminated. Isolation of 1 or more colonies of *M. a. paratuberculosis* is sufficient to define a sample as positive. Detection of a single infected cow is sufficient to classify the herd as infected. Colony counts are not as important when culture is used for herd screening as when it is used in a paratuberculosis control program.

There were 45 *M. a. paratuberculosis*-infected cows (43 detected by individual cultures plus another 2

Table 2. Comparison of number of *Mycobacterium avium* subsp. *paratuberculosis* colonies in individual and matched pooled fecal samples.

	No. of pools (<i>n</i> = 145)	Culture-positive individual fecal samples per pool	Mean total colonies per pool (range)	Mean total colonies from individual fecal samples (range)
Pool neg.* + individuals neg.	111	0	0	0
Pool pos.* + individuals neg.	2	0	5 (1–10)	0
Pool neg. + 1 individual pos.	6	1	0	7 (1–20)
Pool pos. + 1 individual pos.	17	1	21 (5–150)	33 (1–400)
Pool pos. + 2 individuals pos.	7	2	15 (2–30)	36 (5–116)
Pool pos. + 3 individuals pos.	2	3	28 (2–54)	244 (9–480)
Average number in positive† pools (<i>n</i> = 34)		1.3	15.8	31.4

* Neg. = *M. a. paratuberculosis*-negative culture; pos. = *M. a. paratuberculosis*-positive culture.

† Pools with *M. a. paratuberculosis*-positive culture (including positive pools with only culture-negative individuals) and pools with *M. a. paratuberculosis*-negative culture but containing culture-positive individuals.

found in culture-positive pools). On an individual cow basis, individual fecal culture detected 43 of these 45 (96%), while pooled fecal culture detected 39 (87%). This drop in detection rate is less than that found by Vialard et al.¹⁵ and could be attributable to differences either in the pooling method or culture technique. The positive effect of strategic pooling on sensitivity is demonstrated by the 1.3 culture-positive individual samples in positive pools of 5 animals (45 culture-positive animals related to 34 positive pools; Table 2). In case culture-positive samples should be divided randomly, 0.3 culture-positive animal per pool (45 detected animals from a total of 733 animals) can be expected.

Dilution of 1 *M. a. paratuberculosis*-containing (positive) fecal sample with 4 other fecal samples not containing *M. a. paratuberculosis* reduced the CFU of *M. a. paratuberculosis* per gram below the detection threshold in 6 of 34 (17.6%) pools (Table 2). By contrast, culture of individual fecal samples missed detection of infected cows in 2 of these 34 (5.9%) pools. This illustrates that there is an element of chance apart from the element of dilution related to detection of *M. a. paratuberculosis* in feces, particularly when samples contain low numbers of the organism and the bacteria are not uniformly distributed in the fecal samples.

The primary purpose of using pooled fecal samples is to screen herds that are presumed to be not infected with *M. a. paratuberculosis* rather than to identify infected individuals for culling in a paratuberculosis control program. Thus, the most critical concern is whether pooling of fecal samples affects herd sensitivity. Herd sensitivity of pooled fecal culture (73%) was not different from that of individual fecal culture (64%). In conclusion, the culture method we employ using strategically pooled fecal samples can be used to screen herds for paratuberculosis with equivalent herd sensitivity but at significant cost savings.

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