Enhanced Radiometric Detection of *Mycobacterium paratuberculosis* by Using Filter-Concentrated Bovine Fecal Specimens

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A commercial radiometric medium, BACTEC 12B, was modified by addition of mycobactin, egg yolk suspension, and antibiotics (vancomycin, amphotericin B, and nalidixic acid). Decontaminated bovine fecal specimens were filter concentrated by using 3-μm-pore-size, 13-mm-diameter polycarbonate filters, and the entire filter was placed into the radiometric broth. Comparison of the radiometric technique with conventional methods on 603 cattle from 9 *Mycobacterium paratuberculosis*-infected herds found that of 75 positive specimens, the radiometric technique detected 92% while conventional methods detected 60% ($P < 0.0005$). Only 3.9% of radiometric cultures were contaminated. To measure the effect of filter concentration of specimens on the detection rate, 5 cattle with minimal and 5 with moderate ileum histopathology were sampled weekly for 3 weeks. *M. paratuberculosis* was detected in 33.3% of nonfiltered specimens and 76.7% of filtered specimens ($P < 0.005$). Detection rates were directly correlated with the severity of disease, and the advantage of specimen concentration was greatest on fecal specimens from cattle with low-grade infections. Detection times were also correlated with infection severity: 13.4 ± 5.9 days with smear-positive specimens, 27.9 ± 8.7 days with feces from cows with typical subclinical infections, and 38.7 ± 3.8 days with fecal specimens from cows with low-grade infections. Use of a cocktail of vancomycin, amphotericin B, and nalidixic acid for selective suppression of nonmycobacterial contaminants was better than the commercial product PANTA (Becton Dickinson Microbiologic Systems, Towson, Md.) only when specimens contained very low numbers of *M. paratuberculosis*. Radiometric culture of filter-concentrated specimens generally doubled the number of positive fecal specimens detected over conventional methods, making it a useful tool for diagnosis and control of bovine paratuberculosis.

Bovine paratuberculosis (Johne's disease) affects at least 2.9% of the 10.3 million dairy cattle in the United States (23) and is equally prevalent in most other countries where laboratory diagnosis of the infection is possible (34). The prevalence of paratuberculosis in some states exceeds 15% (3, 4, 41). The national economic impact has been estimated to be as high as $1.5 billion annually (12).

The etiological agent, *Mycobacterium paratuberculosis*, is among the slowest growing of the cultivable mycobacteria, normally requiring 8 to 16 weeks to produce visible colonies on conventional agar media (40). It infects the terminal ileum of most ruminants and is excreted in the feces. Control of the disease in a dairy cattle herd involves hygienic measures to avoid exposure of calves to the organism or removal of infected animals from the herd (particularly those excreting *M. paratuberculosis*) or both (5, 10, 20, 29, 36).

Damato and Collins reported detection of *M. paratuberculosis* from ruminant fecal and tissue specimens by using mycobactin-supplemented radiometric broth media (7). Subsequently, specimen decontamination methods were simplified, and the radiometric medium was modified to improve recovery of this fastidious organism. A field study comparing *M. paratuberculosis* detection by conventional culture methods with that by the modified radiometric technique on 987

bovine fecal specimens demonstrated that the radiometric technique detected positive specimens faster but was not appreciably more sensitive (6, 22). Similar findings have been reported in studies on specimens from humans with mycobacterial infections (8, 21, 24, 27, 33, 35). In our estimation, two factors were adversely affecting radiometric *M. paratuberculosis* detection efficiency: the low numbers of organisms in fecal specimens from subclinically infected cattle and the commercial antibiotic cocktail (PANTA; Becton Dickinson Microbiologic Systems, Towson, Md.) used to control nonmycobacterial fecal microfloras. In this study, we describe a technique for concentrating mycobacteria from bovine feces prior to inoculation of radiometric broth media. In addition, we describe an alternative antibiotic cocktail for selective suppression of nonmycobacterial microfloras.

MATERIALS AND METHODS

Radiometric medium. Commercial 12B bottles were purchased from Becton Dickinson Microbiologic Systems. To each bottle was added 1.0 ml of egg yolk suspension (Difco, Detroit, Mich.), 0.1 ml of a 40-μg/ml mycobactin J solution (Allied Laboratories, Glenwood Springs, Colo.), and 0.1 ml of an antibiotic cocktail containing vancomycin, amphotericin B, and nalidixic acid, designated VAN. The final concentrations of these antibiotics in the radiometric broth were 10, 20, and 30 μg/ml, respectively. For studies comparing this cocktail to the commercial cocktail, PANTA, medium preparation was identical, with the exception of the cocktail

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used, and PANTA was used per the recommendations of the manufacturer.

Specimen processing for radiometric culture. Fecal specimens were collected directly from the rectum. Approximately 3 g was thoroughly mixed with 30 ml of a 1.0% solution of hexadecylcetylpyridinium chloride (HPC). The mixture was filtered through a two-ply thickness of sterile gauze into another tube and then allowed to stand undisturbed for 24 h at room temperature, permitting particulate matter to settle. Detection of *M. paratuberculosis* in nonconcentrated specimens was accomplished by transferring 0.1 ml from the surface of the fecal suspension into a bottle of radiometric culture medium (RCM) with a tuberculin syringe as previously described (22). Filter concentration was performed by drawing 10 ml of the fecal suspension into a 10-ml syringe fitted with an 18-gauge needle. The needle was then replaced with a sterile Swinex filter holder (Millipore Corp., Bedford, Mass.) containing a 13-mm-diameter, 3-μm-pore-size polycarbonate filter membrane (Nuclepore Corp., Pleasanton, Calif.). The fecal suspension was then slowly expressed through the filter, and the filtrate was discarded. Filters were rinsed with 2 ml of sterile distilled water followed by air to expel excess fluid. By using sterile forceps, the filter membrane was placed into the RCM, and the bottle was resealed with a 20-mm gray butyl rubber disk (West Corp., Phoenixville, Penn.), an aluminum ring (Sunbrokers, Inc., Wilmington, N.C.), and a hand-held crimper. Bottles were incubated at 35°C without agitation and read on a BACTEC 460 without CO_2. For studies 1 and 2 described below, bottles were read twice weekly for 4 weeks and then once weekly through week 10 of incubation. Bottles in study 3 were more heavily laden with *M. paratuberculosis* and thus were read three times each week. A positive bottle was defined as one with a growth index reading >30. The identity of isolates in all bottles was confirmed by subculture on plate media, as previously described (6, 22).

Conventional culture methods and media. Fecal specimens from dairy cattle in Wisconsin herds were cultured by the standard method used in the United States (38) by using 0.25% HPC as the decontaminant and Herrold egg yolk (HEY) agar as the medium (2 tubes with mycobactin and one tube without mycobactin). All cultures were examined monthly for 3 months. Bovine fecal specimens from Danish cattle were processed by the method of Beerwerth (1) and cultured as described by Jorgensen (13) by using modified Lowenstein-Jensen media. Cultures were examined weekly for 10 weeks.

Specimens. Three separate studies were conducted. Study 1 used fecal specimens from 603 dairy cattle in nine *M. paratuberculosis*-infected Wisconsin herds. Each was cultured by the conventional method at the Wisconsin Animal Health Laboratory, Madison, and in RCM by using filter-concentrated specimens in VAN-containing media at the University of Wisconsin—Madison. The specimens were fresh when processed by the Wisconsin Animal Health Laboratory but frozen at −70°C until processed for radiometric culture.

Study 2 involved weekly sampling of 10 clinically normal *M. paratuberculosis*-infected dairy cattle housed at the University of Wisconsin for a 3-week period. Each sample was tested with and without filter concentration and in RCM with VAN or with the PANTA antibiotic cocktail. Prior to the study, ileum biopsies were performed on the cows and each was classified as to the severity of paratuberculosis on the basis of the degree of histopathology and the number of

TABLE 1. Comparison of *M. paratuberculosis* detection by radiometric and conventional media in a field study with nine dairy herds (study 1)

Herd	No. tested	No. positive	% positive	No.[a]			
				RCM (+) HEY (+)	RCM (+) HEY (−)	RCM (−) HEY (+)	RCM (−) HEY (−)
A	84	2	2.4	0	2	0	82
B	90	3	3.3	0	2	1	87
C	68	3	4.4	3	0	0	65
D	45	2	4.4	0	2	0	43
E	61	5	8.2	0	5	0	56
F	58	7	12.1	0	5	2	51
G	61	10	16.4	5	4	1	51
H	103	26	25.2	19	6	1	77
I	33	17	51.5	12	4	1	16
Low prev[b]	348	15	4.3	3	11	1	333
High prev[c]	255	60	23.5	36	19	5	195
All herds	603	75	12.4	39	30	6	528

[a] RCM, RCM with filter-concentrated specimens; HEY, HEY agar medium with standard specimen processing.
[b] Low prev, Low paratuberculosis prevalence herds (A, B, C, D, and E).
[c] High prev, High paratuberculosis prevalence herds (F, G, H, and I).

mycobacteria observed on Ziel-Neelsen acid-fast stained tissue sections.

Study 3 was conducted in Copenhagen. Fourteen bovine fecal specimens and six lymph nodes which were smear positive by acid-fast staining were selected from those submitted to the National Veterinary Laboratory, Copenhagen, Denmark, for routine isolation of *M. paratuberculosis*. The specimens were processed while fresh by the method of Beerwerth (1) (decontamination with oxalic acid and sodium hydroxide) and inoculated onto conventional media (modified Lowenstein-Jensen) (13). They were then frozen at −20°C until processed for culture by the radiometric technique. Tissues were ground in a mortar with a pestle prior to decontamination. Both concentrated and nonconcentrated specimens were tested in RCM with VAN. In addition, the remainder of the HPC-decontaminated specimen suspensions were inoculated onto modified Lowenstein-Jensen media. All positive bottles were subcultured onto these same media.

Data analysis. *M. paratuberculosis* detection rates for each method were compared by using the McNemar test for correlated proportions (30). Differences in detection success between groups of cows were compared by binomial proportions analysis (30). The time until *M. paratuberculosis* was detected in radiometric media was defined as the number of days of incubation until a growth index reading of >30 units occurred, until acid-fast staining of a sample of the medium showed organisms resembling *M. paratuberculosis*, and until no growth was observed after subculture from the bottle on blood agar. For conventional media, the time until detection was defined as the number of incubation days until visible growth consistent with the colonial morphology of *M. paratuberculosis* was observed. Days to *M. paratuberculosis* detection were expressed as mean ± standard deviation and were compared by using Student's *t* test (30).

RESULTS

Study 1. Table 1 summarizes the findings of study 1. Among the 603 fecal specimens from nine dairy herds, 75 were found positive by using either RCM or HEY agar. Radiometric culture of filter-concentrated specimens de-

TABLE 2. Comparison of *M. paratuberculosis* detection by four radiometric culture techniques on feces of
cattle with mild or moderate intestinal pathology (study 2)

Infection severity	No. of cows	Total no. of samples	No. (%) positive specimens by method[a]:				
			A	B	C	D	Combined
Mild	5	15	1 (5.6)	12 (80.0)	0 (0.00)	7 (46.7)	20 (33.3)
Moderate	5	15	9 (60.0)	11 (73.3)	8 (53.3)	12 (80.0)	40 (66.7)
Combined	10	30	10 (33.3)	23 (76.7)	8 (26.7)	19 (63.3)	60 (50.0)

[a] Method A, Direct inoculation of specimens to radiometric media with VAN antibiotics; method B, inoculation of filter-concentrated specimens to radiometric media with VAN antibiotics; method C, direct inoculation of specimens to radiometric media with PANTA antibiotics (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin); method D, inoculation of filter-concentrated specimens to radiometric media with PANTA antibiotics.

tected 69 of 75 (92%) of the positive specimens, which was significantly greater than the 45 of 75 (60%) detected by using HEY agar ($P < 0.0005$). Thirty (40%) specimens were positive only on RCM, in contrast with six (8%) that were positive only on HEY agar. Nineteen RCM cultures (3.9%) and no HEY cultures were contaminated (a contaminated culture was defined as contamination of both tubes inoculated).

The prevalence of paratuberculosis in the herds sampled influenced the *M. paratuberculosis* detection rates. In low prevalence herds (<10% of cows fecal culture positive), 15 of 348 (4.3%) specimens were positive; the RCM detected *M. paratuberculosis* in 14 (93.3%) of these, while HEY agar detected the organism in only 4 (26.7%) ($P < 0.025$). In the higher prevalence herds (>15% of cows fecal culture positive), 60 of 255 (23.5%) specimens were positive, and RCM detected *M. paratuberculosis* in 55 (91.7%) while HEY agar detected the organism in 41 (68.3%) ($P < 0.01$).

Study 2. Ten *M. paratuberculosis*-infected but clinically normal Guernsey cows were the subjects of this study. Ileum biopsies indicated that 5 of the 10 cows had a moderate degree of pathology, much less than is normally seen in clinically affected animals, characterized by thickening of the mucosa, infiltration with mononuclear inflammatory cells, and readily evident acid-fast small rod-shaped bacteria typical of *M. paratuberculosis*. The other 5 cows had very mild histopathological reactions, with occasional giant cells and few or no acid-fast organisms in the tissue sections examined. Diagnosis of paratuberculosis was established in these animals by radiometric culture of the biopsy tissues.

All 10 cows were sampled weekly for 3 weeks. From each sample, while fresh, two 3-g portions were suspended in 30 ml of HPC and then coarse filtered as described above. After 24 h of decontamination, the first suspension was inoculated (0.1 ml) directly into RCM containing VAN (method A). The 10 ml was filtered, and the filter was placed into RCM with VAN (method B). The second fecal suspension was treated in the same way, but with RCM containing PANTA (0.1 ml of the suspension for direct inoculation, method C; the filter-concentrated specimen, method D). On five occasions, none of the four methods detected *M. paratuberculosis*: twice among cows with a mild infection and three times among cows with moderate infections. Intermittent excretion of the organism is considered a feature of paratuberculosis (5). The overall *M. paratuberculosis* detection rate, combining all methods on all weeks, was greater with feces from animals with moderate ileum histopathology, 66.7%, than with feces from those with mild histopathology, 33.3% ($P < 0.0001$). Similarly, the mean time to detect positive specimens from cows with moderate pathology, 27.9 ± 8.7 days, was significantly less than that for cows with mild infections, 38.7 ± 3.8 days ($P < 0.001$).

Table 2 summarizes the isolation success of each method on each group of cows. No difference was found in detection rates for three of the methods used on samples from cows with moderate infections; only in PANTA-containing bottles was the isolation rate with filtered specimens (80.0%) significantly greater than that for specimens directly inoculated into the bottles, 53.3% ($P < 0.05$).

Detection rates of *M. paratuberculosis* in feces of cows with mild infections were significantly affected by the culture method employed. Regardless of the antibiotic cocktail used in the medium, the organism was detected in filter-concentrated specimens far more often than in nonfiltered specimens ($P < 0.005$). This effect of filter concentration was also found when isolation rates for all cows in the study were combined ($P < 0.005$). With feces from cows with mild infections, the *M. paratuberculosis* detection rate obtained by using filter-concentrated specimens in VAN-containing media (method B) (80%) was higher than that obtained by using filtered specimens in PANTA-containing bottles (method D) (46.7%) ($P < 0.10$).

Considering all 10 cows tested, the detection rate for method C was lowest (26.7%), but this was not significantly lower than that for method A (33.3%). Filter concentration of specimens significantly ($P < 0.0001$) increased isolation success to 63.3% in PANTA-containing bottles and to 76.7% in VAN-containing bottles; these two rates were not significantly different from each other, however. The effect of filter concentration of specimens on mean time to detect *M. paratuberculosis* was calculated for bottles inoculated with the same fecal specimen when both the nonfiltered and the filtered samples became positive. Detection times were 30.1 ± 5.8 days with nonfiltered specimens and 24.3 ± 6.4 days for filtered specimens ($P < 0.01$). None of the 120 radiometric cultures in this study were contaminated.

Study 3. For the 20 smear-positive tissue and fecal specimens tested, *M. paratuberculosis* was isolated from 70% by the standard method used in Denmark (13), from 85% when HPC decontamination was used prior to inoculation of the same media, and from all 20 (100%) by the radiometric technique whether or not filter concentration of the specimens was used. Contamination rates were 30, 15, and 0% for the three techniques, respectively. Whether decontamination was done with oxalic acid and sodium hydroxide or with HPC, 56 days of incubation were required before colonies were visible on modified Lowenstein-Jensen medium. Mean times to detect positive cultures in radiometric media were 13.4 ± 5.1 and 15.8 ± 5.9 days for filter-concentrated and directly inoculated specimens, respectively. One specimen was detected after only 3 days of incubation. All *M. paratuberculosis* isolates grew faster on conventional media after subculture from positive bottles than they did on conventional media primary cultures. On average, 26 ± 5 days were

required for *M. paratuberculosis* to grow on modified Lowenstein-Jensen medium when subcultured from positive radiometric primary cultures at the time the grown index reading was >300. Frequently, the specimen was found to be positive by radiometric culture, and *M. paratuberculosis* was isolated by subculture on conventional media before the parallel primary culture on modified Lowenstein-Jensen medium showed growth.

DISCUSSION

Filter concentration of specimens significantly increased the rate of detection and decreased the time for radiometric detection of *M. paratuberculosis* in bovine fecal specimens. This is consistent with the mathematical model of the relationship between the number of *M. paratuberculosis* inoculated into a radiometric broth and the rate of $^{14}CO_2$ production (19). From cows at one end of the clinical spectrum of paratuberculosis (advanced disease), 20 of 20 smear-positive specimens yielded *M. paratuberculosis* in 13.4 ± 5.1 days. At the other extreme, five cows with very low-grade infections gave positive RCM cultures on 80% of 15 culture attempts in 38.7 ± 3.8 days. The field study of nine infected herds showed that RCM with filter-concentrated specimens detected 92% of infections in cattle excreting *M. paratuberculosis* in their feces, while conventional culture on HEY agar detected only 60%. Since the fecal specimens were cultured when fresh by conventional methods but were frozen before processing for RCM culture, some loss in *M. paratuberculosis* viability may have occurred, and thus the difference in detection rates could have been greater (31). In general, the radiometric technique would most likely detect the majority of infections in cattle shedding *M. paratuberculosis*, with a single bottle of medium, in roughly 4 weeks with filter-concentrated specimens, and cultures could be reliably defined as negative for presence of the organism if no growth was detected after 7 weeks of incubation.

For conventional methods, it has been recommended that four tubes of media be inoculated with nonconcentrated bovine fecal specimens and that they be observed for 16 weeks (23, 40). Centrifugal concentration of bovine fecal specimens was shown to shorten somewhat the incubation time required; 98% of positive cultures were detected in 12 weeks on HEY agar with mycobactin (42). Centrifugation did not increase the isolation rate of *M. paratuberculosis* from fecal specimens, however, when compared to the standard sedimentation method. Kim et al. (17) compared these two methods for specimen processing and also compared conventional HEY media with and without miconazole to control fungal contaminants. The sources of specimens were cattle clinically suspected of having paratuberculosis. Centrifugation did not significantly increase *M. paratuberculosis* isolation rates, nor did miconazole significantly decrease recovery of the organism. Isolation rates for both specimen processing methods on media with and without miconazole ranged from 38.8 to 49.6% of positive specimens. These values are in the range reported for the diagnostic sensitivity of conventional fecal culture for *M. paratuberculosis* (5, 14). Contamination rates reported by Kim et al. on all mycobactin-containing HEY media ranged from 4.1 to 10.8% (17). Whitlock et al. reported similar results: 9.6% of 4,688 bovine fecal specimens contaminated (reset) by using centrifuge concentration, amphotericin B mixed with inocula, and four tubes of HEY agar per specimen (42). The contamination rate of 3.9% in RCM with filter concentrated specimens compares favorably with these values.

The detection limit of radiometric culture was previously shown to be 3 organisms (19). With the concentration technique employed, this theoretically equates to 3 *M. paratuberculosis* organisms per g of feces. Even if the filter concentration technique was not 100% efficient at trapping *M. paratuberculosis* from decontaminated bovine feces, the detection limit of the procedure is probably significantly lower than the detection limit of 10^2 organisms per g of feces of the conventional culture or a new gene probe (39) and explains the greater sensitivity of the method.

Filters of various pore sizes and compositions were tested in earlier studies and 3-μm-pore-size polycarbonate filters (13-mm diameter) were found optimal; smaller-pore filters became too easily plugged, and larger-pore filters failed to trap *M. paratuberculosis* as efficiently. Polycarbonate filters resisted tearing better than cellulose acetate filters, and 13-mm-diameter filters were easier to get into radiometric culture bottles than 25-mm-diameter filters.

The most common contaminant found when culturing bovine feces for *M. paratuberculosis* is *Bacillus* spp. (37). By using 3-μm-pore-size filters, these contaminants and their spores easily pass through the filter. By contrast, light and scanning electron microscopy reveal that *M. paratuberculosis* is normally found in feces as large clusters of cells often adhered to cellular debris (unpublished data). Filter concentration of fecal suspensions trapped these organisms. It also permitted washing the decontaminant away from the organism, which diminished the likelihood of HPC inhibition of *M. paratuberculosis* growth.

Egg yolk, in our experience, is essential for growth of *M. paratuberculosis* from many bovine clinical specimens, particularly when low numbers of the organism are present. Kononov et al. described the same observation with *Mycobacterium tuberculosis* (18). The antibiotic cocktail employed in this study, VAN, was marginally superior to the commercial preparation, PANTA, in terms of *M. paratuberculosis* detection and growth rates and was less expensive.

Serological methods for diagnosis of paratuberculosis have been used for many years but have lacked diagnostic sensitivity and specificity (5). However, new techniques are being employed that markedly improve serological test accuracy (25, 26, 43). An advantage of culture methods or gene probes over immunological tests for paratuberculosis is that they identify animals that are excreting the organism and that are infectious for other animals in the herd. Control of paratuberculosis in a herd involves regular testing and selective culling of infectious animals (28, 32). Those that may be infected and have an antibody titer may not be infectious and may in fact be immune (5, 9). Benedictus et al. calculated the average total farm loss per culled animal with paratuberculosis, but without clinical signs of the disease, to be roughly $800. All production losses in these animals occurred in the last two lactations prior to culling, and this coincided with the period of fecal shedding of *M. paratuberculosis* (2). This provides another reason why diagnostic tests based on detection of the organism in feces are advantageous.

Cost effectiveness of diagnostic methods in agriculture is of paramount importance. The cost of the radiometric technique described was $2.84 for expendables and required 6 min per sample, including media and filter preparation. These figures make detection of *M. paratuberculosis* by radiometric culture more economical than by conventional methods, which use four tubes of medium, or by gene probes. Since culture methods are 100% specific, the cost to herd owners of a false-positive test result (approximately

$500 for replacement of a culled adult cow) is avoided. In light of the chronic nature of paratuberculosis and since the disease is transmitted primarily from cows to calves at the time of parturition, rapid diagnosis (24 to 48 h) is not essential, provided cows are tested prior to calving. Radiometric culture of filter-concentrated fecal specimens detected over 50% more positive specimens compared with conventional culture methods and thus provides the most sensitive and cost-effective method for detection of infectious animals with paratuberculosis. The methods described might also be useful for improved detection of Mycobacterium avium from pigs (11) or M. avium from patients with acquired immunodeficiency syndrome (15, 16).

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