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

MICHAEL T. COLLINS, KARI B. KENEFFICK, DONALD C. SACKETT, RANDALL S. LAMBERT, J. MCDONALD, AND BERG JORGENSEN

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin 53706-1182, and National Veterinary Laboratory, Copenhagen, Denmark

Received March 1990; Accepted 24 August 1990

A commercial radiometric medium, BACTEC 12B, was modified by addition of mycobacin, egg yolk suspension, and antibiotics (vancomycin, amphotericin B, and nalidixic acid). Decontaminated bovine fecal specimens were filter concentrated by using 3-μm-pore-size, 35-mm-diameter polycarbonate filters, and the entire filter was placed into the radiometric broth. Comparison of the radiometric technique with conventional methods on 661 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 mild and 5 with moderate to severe histopathology were sampled weekly for 3 weeks. M. paratuberculosis was detected in 33.3% of unfiltered specimens and 76.7% of filtered specimens (P < 0.006). 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: 12.4 ± 5.9 days with negative-positive specimens, 27.9 ± 8.7 days with feces from cows with typical subclinical infections, and 38.5 ± 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, Tewson, 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 (Johnes' disease) affects at least 2.9% of the 10.3 million dairy cattle in the United States (22) 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 (46). It infects the terminal ileum of most ruminants by ingestion in 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 excrting M. paratuberculosis) or both (5, 10, 30, 39, 36).

Damaio and Collins reported detection of M. paratuberculosis from ruminant fecal and tissue specimens by using mycobacin-supplemented radiometric broth media (15). Subsequently, specimens 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 98

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, 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, Tewson, Md.) used to control nonmycobacterial fecal microflora. 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 microflora.

MATERIALS AND METHODS

Radiometric medium. Commercial 12B broths 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 mycobacin J solution (Allied Laboratories, Glensford 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, media preparation was identical, with the exception of the cocktail.
used. and PANTA was used per the recommendations of the manufacturer.

Specimen processing for radiometric culture. Fecal speci-
mens were collected directly from the rectum. Approxi-
mately 3 g was thoroughly mixed with 30 ml of a 1.0% solution of hexadecylpyridinium 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 non-
centrifuged 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 tubeuculin syringe as previously described (22). Filter concentration was performed by transferring 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 5-ml filter holder (Milli-
apore 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 resterilized with a 25-mm gray butyl rubber disk (West Corp., Phoenixville, Penn., an aluminum ring/Sum-
brokers, Inc., Wilmington, N.C.), and a hand-held clip. Bottles were incubated at 35°C without agitation and read on a BACTEC 460 with CO2. For stools 1 and 2 described below, bottles were read twice weekly for 4 weeks and then once weekly through week 16 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 ≥3.6. 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 methods of the United States (38) by using a 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 Berer (17) and cultured as described by Jorgensen (13) by using modified Lowenstein-Jensen media. Cultures were examined weekly for 16 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 cul-
tured by the conventional method as in Wisconsin Animal Health Laboratory, Madison, and in RCM by using filter-
centrated specimens in VAN-containing media at the University of Wisconsin—Madison. The specimens were fresh when procured by the Wisconsin Animal Health Laboratory but frozen at −70°C until processed for radio-
metric 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 0.2% HPC with PANTA's antibiotic cocktail. Prior to the study, slenium 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

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, Copen-
hagen, Denmark, for routine isolation of M. paratubercu-
losis. The specimens were processed while fresh by the method of Berer (17) (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 radiomet-
ric technique. Tissues were ground in a mortar with a pestle prior to decontamination. Both concentrated and noncon-
centrated specimens were tested in RCM with VAN. In addition, the remainder of the HPC-decontaminated speci-
men suspensions were inoculated onto modified Lowen-
stein-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 (38). Differences in detection success between groups of cows were compared by binomial propor-
tions analysis (38). 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. paratubercu-
losis detection were expressed as mean ± standard deviation and were compared by using Student's t test (40).

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>
<thead>
<tr>
<th>Herd</th>
<th>No. of Cows Tested (No. of Positive)</th>
<th>RCM (1)</th>
<th>RCM (2)</th>
<th>RCM (3)</th>
<th>RCM (4)</th>
<th>HEY (1)</th>
<th>HEY (2)</th>
<th>HEY (3)</th>
<th>HEY (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>84</td>
<td>2,4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>82</td>
<td>76</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>B</td>
<td>96</td>
<td>3,3</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>87</td>
<td>87</td>
<td>87</td>
<td>87</td>
</tr>
<tr>
<td>C</td>
<td>68</td>
<td>3,4</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>D</td>
<td>61</td>
<td>5,8</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>56</td>
<td>52</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>E</td>
<td>61</td>
<td>5,8</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>56</td>
<td>52</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>F</td>
<td>58</td>
<td>7,12</td>
<td>0</td>
<td>5,2</td>
<td>0</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>G</td>
<td>61</td>
<td>10,16</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>H</td>
<td>51</td>
<td>26,25</td>
<td>2</td>
<td>9</td>
<td>1</td>
<td>77</td>
<td>77</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>I</td>
<td>17</td>
<td>51,15</td>
<td>2</td>
<td>8</td>
<td>1,0</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

*Low prev.* = 348/45 × 100 = 77.7%

**High prev.** = 253/60 × 100 = 42.2%

†RCM, RCM with filter-concentrated specimens; HEY, HEY agar medium with standard specimens processing.

‡Low prev., Low paratuberculosis prevalence herd (A, B, C, D, and E).

§High prev., High paratuberculosis prevalence herd (G, H, and I).
**Table 2:** Comparison of *M. paratuberculosis* detection by four radiometric culture techniques on feces of cattle with mild or moderate intestinal pathology (study 2)  

<table>
<thead>
<tr>
<th>Infection</th>
<th>No. of cows</th>
<th>Total m. of sample</th>
<th>No. (%) positive specimens by method*</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>20</td>
<td>20</td>
<td>1 (5.0)</td>
<td>1 (5.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (5.0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Moderate</td>
<td>15</td>
<td>15</td>
<td>9 (60.0)</td>
<td>0 (0.0)</td>
<td>1 (6.7)</td>
<td>0 (0.0)</td>
<td>10 (66.7)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>Combined</td>
<td>35</td>
<td>35</td>
<td>12 (33.3)</td>
<td>2 (5.7)</td>
<td>8 (23.1)</td>
<td>1 (2.9)</td>
<td>21 (60.0)</td>
<td>35 (100)</td>
</tr>
</tbody>
</table>

*Method A: Direct inoculation of specimens to radiometric media with VAS antibodies; method B: inoculation of filter-concentrated specimens to radiometric media with VAS antibodies; method C: direct inoculation of specimens to radiometric media with PANTA antibodies; method D: inoculation of filter-concentrated specimens to radiometric media with PANTA antibodies.*

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 VANC-containing media (method B) 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 for method B (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 VANC-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 both inoculated with the same fecal specimen when both the unfiltered and the filtered samples became positive. Detection times were 30.1 ± 3.8 days with nonfiltered specimens and 14.3 ± 6.4 days for filtered specimens: this difference was statistically significant (P < 0.001).

For the 20 sterile-positive tissue and fecal specimens tested, *M. paratuberculosis* was isolated from 70% by the standard method used in Denmark (13), from 85% when HIS decontamination was used prior to inoculation of the same media, and from all 28 (100%) by the radiometric technique whether or not filter concentration of the specimens was used. Contamination rates were 30.1, 15.9, and 6.0% 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 13.7 ± 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 ± 3 days were...
Filter concentration of specimens significantly increased the rate of detection and decreased the time for radiometric detection. In a study comparing the general in the Modified Lowenstein-Jensen method, the growth index in the filar primary culture on modified Lowenstein-Jensen medium showed growth.

Especies

Filter concentration of specimens significantly increased the rate of detection and decreased the time for radiometric detection. In a study comparing the general in the Modified Lowenstein-Jensen method, the growth index in the filar primary culture on modified Lowenstein-Jensen medium showed growth.

The detection limit of radiometric culture was previously shown to be 1 organism (19). With the concentration technique employed, this threshold is equivalent to 5 M. parauberculosi s organisms per g of feces. Even if the filter concentration technique was not 100% efficient at trapping M. parauberculosi s from decontaminated bovine feces, the detection limit of the procedure is probably significantly lower than the detection limit of 1 organism 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. parauberculosi s efficiently. Polycarbonate filters resisted clogging 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. parauberculosi s is Bacillus sp. (37). By using 3-μm-pore-size filters, these contaminates and their spores easily pass through the filter. By contrast, light and scanning electron microscopy reveal that M. parauberculosi s 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 was also permitted washing the decontaminant away from the organism, which diminished the likelihood of HPC inhibition of M. parauberculosi s growth.

Egg yolk, for example, is essential for growth of M. parauberculosi s from many bovine clinical specimens, particularly when low numbers of the organism are present. Konovoz et al. described the same observation with Myco- tuberculosi s paratuberculosis (41). The methodology employed in this study, VAN, was marginally superior to the commercial preparation, PANTA, in terms of M. parauberculosi s 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 specific antigens, such as those that are infectious for other animals in the herd. Control of paratuberculosis in a herd involves regular testing and selective culling of infected animals (9, 38, 32). Those may be infected and have an antibody titer may not be infectious and may in fact be immune (5). Benecletus et al. calculated the average total farm loss per culled animal with paratuberculosis, but without clinical signs of the disease, to be roughly S3000. 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. parauberculosi s (2). This provides another reason why diagnostic tests based on detection of the organism in feces are advantages.

Cost effectiveness of diagnostic methods in agriculture is of paramount importance. The cost of the radiometric technique described was S2.84 for expendables and required 6 min per specimen, including media and filter preparation. These figures make detection of M. parauberculosi s 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. Radio-
microscopic culture of filter-concentrated fecal specimens de-
tected over 50% more positive specimens compared with conventional culture methods and thus provides the most sensitive and cost-effective method for detection of infec-
tious animals with paratuberculosis. The methods described might also be useful for improved detection of Mycobac-
terium avium from pigs (11) or M. avium from patients with acquired immunodeficiency syndrome (15, 16).

ACKNOWLEDGMENTS

This work was supported in part by the University of Wisconsin
Applied Research Program and the Danish Veterinary Research
Council. The assistance of A. J. Cooley with biopsy evaluations is grate-
fully acknowledged. We also thank J. Bemowski, Department of
Mycobacteriology, Statens Seruminstitut, Copenhagen, Denmark,
for use of a BACTEC 460 instrument; and the Wisconsin Animal
Health Laboratory for performing the conventional cultures.

LITERATURE CITED

1. Beerworth, W. 1967. The ziehlneelsen stain from the
Kor Haastre and their Bedeutung der Epidemiologie der


ereum paratuberculosis in radiometric, Middlebrook and enri-


nosis and management. CSIRO, Melbourne, Australia.


cation and experience with vaccination. Commission of the European Communities, Copenhagen.

15. Kibens, T. E., and R. Camarada. 1986. Laboratory diagnosis of mycobacterial infections in patients with immunodefi-


23. Meredith, G. Z., Beggadi, and W. D. Tigges. 1977. Au-


30. Richards, W. D., and C. O. Thoms. 1977. Effect of freezing on the viability of Mycobacterium paratuberculosis in bovine tes-


26-1225-1226.