Cultivation of *Mycobacterium paratuberculosis* from Bovine Fecal Samples by Using Elements of the Roche MB Check System

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Components of a commercially available, nonradioometric, biphasic (liquid medium then solid medium) system for the detection of *Mycobacterial* species, Roche MB Check, were adapted for the isolation of *Mycobacterium paratuberculosis* from bovine fecal specimens. A two-stage culture procedure was developed in which processed fecal samples were incubated in modified commercial liquid medium and then subcultured onto Herrold’s egg yolk medium with mycobactin. By using known culture-positive samples and/or samples from animals clinically affected with paratuberculosis, it was found that visible colonial growth on solid media could be obtained after 4 weeks of incubation in liquid medium containing egg yolk and mycobactin followed by 8 weeks of incubation on Herrold’s egg yolk medium. In the second part of the study, conventional fecal culture (sample sedimentation in hexadecylcetylpyridinium chloride followed by incubation on Herrold’s egg yolk medium) was compared with the two-stage system using a two-step centrifugation technique for sample preparation. One hundred fecal samples from clinically normal but absorbed-enzyme immunosay-positive cattle were used for the comparison. Conventional culture yielded a sensitivity of 16.5%, whereas the sensitivity of the two-stage system was 29.4%. When used in parallel, the tests detected 36.5% of the samples. There was no significant difference between the two methods in the time taken to obtain visible colonies. These results indicate that the two-stage method is a sensitive method for isolation of *M. paratuberculosis* from fecal samples obtained from cattle with clinical paratuberculosis. In addition, the two-stage system is more sensitive than conventional culture for the isolation of *M. paratuberculosis* from subclinically infected cattle.

Paratuberculosis (Johne’s disease) is a chronic incurable condition of ruminants characterized by granulomatous enteritis, diarrhea, and emaciation. The causative organism, *Mycobacterium paratuberculosis*, is a gram-positive, acid-fast, aerobic, nonmotile, non-spore-forming bacillus. It is distinguished from other mycobacterial species by its extremely slow growth and its strict requirement for the iron-chelating agent mycobactin when cultivated on artificial media (24, 25).

Diagnosis of the condition in clinically affected animals is uncomplicated because of the characteristic clinical presentation of the disease (2). However, diagnosis of paratuberculosis in animals which are infected but not yet showing clinical signs of infection is hampered by the lack of sensitive diagnostic tests (4, 11, 21, 22).

Isolation of *M. paratuberculosis* from feces was first recorded by Twort and Ingram in 1912 (26), and since then many sample-processing procedures and culture media have been described (10, 12, 18, 28-32). These so-called conventional culture methods are relatively insensitive, may take up to 6 months to yield a definitive result, and require large quantities of specialized media.

Recently developed gene probes for the detection of *M. paratuberculosis* in feces require specialized equipment, are expensive, and are reported to be even less sensitive than conventional fecal culture (27, 29).

Radiometric fecal culture (5, 6) is reported to be faster and more sensitive than conventional fecal culture but requires expensive equipment and disposal of radioactive waste materials.

This paper describes the development and evaluation of a sensitive two-stage culture technique for *M. paratuberculosis* that uses elements of a commercial, biphasic, nonradio- metric mycobacterial culture system, Roche MB Check (Becton Dickinson Pty. Ltd., Lane Cove, New South Wales, Australia).

MATERIALS AND METHODS

Roche MB Check system. The MB Check system is composed of three segments that may be individually purchased. They are (i) a base medium bottle containing 20 ml of liquid (modified Middlebrook 7H11 medium), (ii) a paddle holding three solid media (modified Middlebrook 7H11, modified Middlebrook 7H11 plus nitroacetyl aminohydroxy propiophenone, and chocolate agar), and (iii) lyophilized supplement containing growth factors and antibiotics. The components and biphasic culture methodology have been described in detail previously (1, 7, 9). MB Check base medium bottles and supplement sufficient to complete this study were obtained from Roche.

The three media on the paddle supplied with the Roche MB Check system are unsuitable for the growth of *M. paratuberculosis*; therefore, Herrold’s egg yolk medium, with and without 2 mg of mycobactin J per liter (HEYJ and HEY, respectively) (15), was used. Nine milliliters of medium was dispensed into 1-oz (1 oz = 28.350 g) MacCartney bottles and set with a slant.

Comparison of sample preparation methods and culture systems: clinical paratuberculosis. A two-stage culture procedure utilizing elements of the Roche MB Check system was compared with conventional fecal culture. In addition, three methods of sample preparation were compared for use in the two-stage culture method.
(i) **Fecal specimens.** Ten fecal samples that had been stored frozen at −20°C were used for this study. Nine were aliquots from previously culture-positive bovine fecal samples (7 positive on conventional fecal culture and 2 only positive on BACTEC [Becton Dickinson Microbiology Systems, Knoxfield, Victoria, Australia]), and the 10th sample was from a histologically confirmed, bovine, clinical case of paratuberculosis.

(ii) **Conventional culture.** Fecal culture for the diagnosis of paratuberculosis is routinely undertaken in Victoria as follows: 2 g of feces is decontaminated and sedimented for up to 48 h in 40 ml of 0.75% hexadecylcetylpyridinium chloride (HPC). One milliliter of liquid from just above the layer of settled feces is removed and used to inoculate two slants of HEYJ and one slant of HEY. The slants are incubated at 37°C for up to 6 months.

(iii) **Base medium preparation.** MB Check supplement was reconstituted as directed by the manufacturer. The contents of one vial (2 mg) of mycobactin J (Allied Monitor, Fayette, Mo.) were dissolved in 3.7 ml of pure ethanol, and then 1.3 ml of sterile distilled water was added, producing a stock solution of 0.4 mg/ml. One milliliter of reconstituted supplement and 0.1 ml of the stock mycobactin solution were added to 40 MB Check base medium bottles. In addition, 1.0 ml of 50% fresh egg yolk–saline solution was added to 30 of the 40 bottles.

(iv) **Sample preparation.** Figure 1 illustrates the steps involved in sample preparation for the five treatments examined.

Approximately 2 g of fecal specimen was added to each of three 45-ml sterile fecal containers (Hardie Health Care Products, South Oakleigh, Victoria, Australia) containing 40 ml of HPC, which were then shaken and allowed to stand at room temperature for 48 h.

One milliliter of supernatant from just above the settled fecal layer of one container was extracted with a sterile pipette and used to inoculate two HEYJ slants and one HEY slant. The slants were sealed tightly, incubated at 37°C, and examined at 14-day intervals.

A further 2 ml of supernatant from just above the level of the settled feces was removed from the same container with a sterile needle and syringe. One milliliter was added to a base medium bottle containing supplement and mycobactin but without egg yolk (treatment A). The other milliliter was added to a base medium bottle fortified with supplement, mycobactin, and egg yolk (treatment B).

Ten milliliters of supernatant from just above the level of the feces was removed from the second fecal container and centrifuged at 3,000 × g at 15°C for 20 min. The supernatant was discarded, the pellet was resuspended in 5 ml of sterile water and vortexed, and after being centrifuged again the pellet was resuspended in 2 ml of sterile water. One milliliter of the resuspended sample was used to inoculate a base medium bottle containing mycobactin, supplement, and egg yolk (treatment C).

Ten milliliters of supernatant from just above the level of the feces was removed from the third container with a sterile syringe and needle and forced through a 13-mm 0.45-μm-pore-size polycarbonate filter membrane (Millipore Corporation, Bedford, Mass.) (5). The filtrate was discarded, and the filter membrane was rinsed twice with 2 ml of sterile water. The whole filter membrane was introduced into an MB Check base medium bottle containing supplement, mycobactin, and egg yolk (treatment D).
TABLE 1. Comparison of the rate of isolation of M. paratuberculosis from feces by conventional culture with that by two-stage culture method

<table>
<thead>
<tr>
<th>Method of sample prep</th>
<th>Total incubation period (wk)</th>
<th>% Noncontaminated samples showing growth after incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0*</td>
<td>1</td>
</tr>
<tr>
<td>Conventional culture</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>60</td>
</tr>
<tr>
<td>Treatment A</td>
<td>12</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>NA</td>
</tr>
<tr>
<td>Treatment B</td>
<td>12</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>NA</td>
</tr>
<tr>
<td>Treatment C</td>
<td>12</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>NA</td>
</tr>
<tr>
<td>Treatment D</td>
<td>12</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Period of liquid medium incubation (in weeks) prior to transfer to solid medium.

a NA, data not available.

b Liquid medium contained supplement and mycobactin but no egg yolk. Sample was sedimented.

c Liquid medium contained supplement, mycobactin, and egg yolk. Sample was centrifuged.

d Liquid medium contained supplement, mycobactin, and egg yolk. Sample was centrifuged.

e Liquid medium contained supplement, mycobactin, and egg yolk. Sample was centrifuged.

All base medium bottles were incubated at 37°C for 8 weeks. Once each week, 1 ml of medium was removed with a needle and syringe and used to inoculate two HEYJ slants and one HEYJ slant. The slants were incubated at 37°C and examined at 14-day intervals.

Growth on slants was microscopically examined by using Ziehl-Neelsen stain, and then the samples were subcultured onto one HEYJ slant and one HEYJ slant to confirm mycobactin dependence.

(v) Data analysis. Contaminated or overgrown slants were excluded from the analysis. The time taken to produce visible growth on the conventional control slants and two-stage treatment slants was recorded.

Comparison of conventional culture with two-stage culture for the detection of subclinical paratuberculosis. (i) Specimens. As part of a pilot paratuberculosis control program, all cattle older than 2 years in 23 dairy herds are tested annually with the CSL Johne's absorbed EIA (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) and conventional fecal culture. Once processed, the serum and fecal samples (5 g of feces in 40 ml of 0.75% HPC) are stored at -20°C. One hundred of these fecal samples from 16 herds were obtained from the collection for this study. Samples were selected if the cattle were clinically normal at the time of sample collection yet the serum collected at the same herd visit returned a positive result when tested with the CSL Johne's absorbed EIA.

The results of conventional fecal culture were not available at the commencement of the present experiment. Each culture method was conducted independently of the other.

(ii) Conventional fecal culture. Conventional fecal culture was undertaken as described above.

(iii) Specimen processing for two-stage culture. Fecal-HPC samples were removed from the freezer, thawed at room temperature, thoroughly mixed, and processed by using the centrifuge method described above. One milliliter of each resuspended sample was inoculated into a base medium bottle fortified with 1 ml of reconstituted commercial supplement, 1 ml of 50% egg yolk suspension, and 0.1 ml of mycobactin J stock solution. After incubation for 5 weeks at 37°C, 1 ml of the medium was removed aseptically and used to inoculate two HEYJ slants and one HEYJ slant, which were incubated at 37°C and examined at 14-day intervals for 18 weeks for evidence of growth.

(iv) Data analysis. The M. paratuberculosis detection rate for each culture method was calculated after samples which were contaminated or overgrown in either culture method were excluded. The contamination rates for each method were calculated. Confidence intervals of 95% were calculated for both the detection and contamination estimates by using standard methods for the approximation of the exact binomial limits (8). The detection and contamination rates were compared by using McNemar's $\chi^2$ test ($\chi^2_M$) with continuity correction (23). An estimation of the level of agreement between the methods was determined by calculating the kappa statistic (14).

The time required for visible growth of colonies morphologically identical to M. paratuberculosis for each sample and method was recorded. A cusum graph (20) of the percentage of positive samples detected in each 14-day period for each culture method was plotted to determine whether there was a difference between methods in the rate of detection of positive samples.

RESULTS

Comparison of sample preparation methods and culture systems: clinical paratuberculosis. After 20 weeks of incubation, growth was evident for six of the conventionally cultured samples.

Table 1 shows the time required to show growth on the HEYJ slants for treatments A, B, C, and D. Treatment A (liquid medium containing no egg yolk) produced no growth for samples incubated in the liquid for longer than 1 week. After 26 weeks of total incubation (incubation in both solid and liquid media), treatments B, C, and D produced more positive samples than conventional culture. Colonies were detected earliest on the HEYJ slants inoculated from the double-centrifuged (treatment C) samples. After 4 weeks of incubation in the liquid medium and 8 weeks on solid medium, 8 of 10 of the treatment C samples exhibited growth and the others were overgrown by contaminants. Overall contamination rates were very low. Only 1 base medium bottle (in treatment D) of 40 became contaminated during the initial sample preparation. Six others, some from each treatment, became contaminated after repeated sampling from the bottles.

Comparison of conventional with two-stage culture for the
TABLE 2. Comparison of detection of *M. paratuberculosis* by conventional culture with that by two-stage system*

<table>
<thead>
<tr>
<th>Culture method</th>
<th>No. of samples</th>
<th>Days to detection (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Conventional culture</td>
<td>15</td>
<td>81</td>
</tr>
<tr>
<td>Two-stage (liquid then solid) system</td>
<td>25</td>
<td>64</td>
</tr>
</tbody>
</table>

* Comparison was done with fecal samples from 100 cattle positive by CSL Johne's absorbed EIA.

**Detection of subclinical paratuberculosis.** Fifteen samples became contaminated when either culture method was used and were excluded, leaving 85 samples for test comparison. Table 2 shows that conventional culture detected *M. paratuberculosis* in 16.5% ± 8.0% of the fecal samples, while the two-stage method detected *M. paratuberculosis* in 29.4% ± 9.9% of the fecal samples. These values are significantly different (χ² = 4.35; *P* ≤ 0.05), and the kappa value (0.251) indicates that the tests are detecting animals from different culture-positive populations. *M. paratuberculosis* was detected in eight (9.4%) samples by both culture methods.

The contamination rates for the conventional fecal culture and the two-stage system were 4% ± 3.9% and 11% ± 6.3%, respectively. A χ² value for these figures could not be calculated because of a 0 value in the two-by-two table. However, the 95% confidence intervals overlap, suggesting that these contamination rates may not be significantly different.

The average times to detection of visible colonies on solid media were 116 days for the conventional method and 123 days of total (liquid and solid medium) incubation for the two-stage method. The cusum plot indicated that there was little difference between the methods in the time taken to detect growth of colonies.

**DISCUSSION**

The Roche MB Check system has been used extensively for the cultivation of mycobacteria, particularly *Mycobacterium tuberculosis* from human-derived specimens (1, 7, 9, 19). In this study, it has been shown that a two-stage culture method performed with elements of the MB Check system is a rapid and reliable method for the isolation of *M. paratuberculosis* from the feces of cattle clinically affected with paratuberculosis.

Three sample-processing methods were compared in this study, and the most successful method involved double centrifugation and then 4 or 5 weeks of incubation in liquid medium followed by incubation on solid medium (treatment C). One probable reason for the success of this method is that the centrifugation steps increase the chances of detecting animals shedding small numbers of organisms (12). In addition, the incorporation of antibiotics in the Roche supplement may have reduced the amount of contamination normally associated with centrifugation techniques (12). However, even if centrifugation is not employed and traditional sedimentation is combined with two-stage incubation (method B), there is a significant increase in the test's recovery rate of *M. paratuberculosis* over that of conventional culture. Filter concentration of *M. paratuberculosis*, although relatively successful, did not result in as high a recovery rate as the centrifugation method and was deemed technically unsuitable for application to large numbers of samples.

Pedicle growth was not observed in any of the base medium bottles; however, particulate material could be seen in the liquid of treatment C bottles after about 4 weeks of incubation. The liquid in the bottles is deeper than has been recommended for optimal growth of *M. paratuberculosis* (30), and repeated weekly sampling resulted in some agitation of the fluid, which also has been shown to reduce the growth of the organism (30). Although growth in the system may not have been optimal, it appears to have been sufficient to allow a considerable increase in the number of CFU inoculated onto the HEYJ slants, resulting in the more rapid appearance of colonies.

Significant transfer of mycobactin from the liquid system to the HEY slants does not appear to have occurred. There is, however, the possibility of mycobactin-dependent organisms growing at the solid-liquid interface at the very bottom of the HEY slants. Routine subculture of any colonies grown should allow the differentiation of such organisms from mycobactin-independent strains.

The results of treatment A compared with those of the other three treatment preparation methods indicate that egg yolk is essential in the liquid medium if the samples have been decontaminated with HPC.

The second part of this study was undertaken to compare the sensitivities of the conventional method and the best of the two-stage culture methods when applied to animals with subclinical *M. paratuberculosis* infection.

In order to determine test sensitivity, the disease status of each animal should be determined with a highly specific test which is unrelated technically to the test under assessment (14). Traditionally this has meant that culture-based tests have been used to identify infected animals to allow the assessment of serological tests. In this case, a highly specific serological test, the CSL Johne's absorbed EIA (17), has been used to identify 100 clinically normal but paratuberculosis-infected cattle. Because the animals were selected this way and the two culture methods were performed independently, an unbiased comparison of the tests may be made.

The two-stage system produced significantly more culture-positives results than the conventional culture. The two-stage culture was undertaken on frozen, stored samples, whereas fresh samples were used for conventional culture. If both methods had been undertaken on freshly collected samples, there may have been an even greater difference in the performance of the tests.

The contamination rate was slightly higher for the two-stage method than for the conventional culture. This may be due partly to the centrifugation process and partly to the longer storage of the samples.

A kappa value of 0.251 suggests that the two culture methods demonstrate organism detection in animals from different populations. It is not clear what characteristics of the organism, animal, herd, or processing method produced the effect observed. However, when the conventional culture and the two-stage method were used in parallel, *M. paratuberculosis* was recovered from 31 (36.5%) of the 85 noncontaminated samples. This represents a considerable
improvement in test sensitivity and requires few additional technical or physical resources.

The sensitivity of either of the culture methods described in the second part of this study is lower than reported previously for fecal culture when evidence of paratuberculosis at slaughter (3), known culture-positive samples (5, 6, 10, 21), or clinically affected cattle (12) were taken as the “gold standard” for test evaluation. None of the earlier results can be compared with those of the present study, which selected clinically normal animals on the basis of serological test status. Many clinically normal animals giving positive CSL Johne’s absorbed EIA results have minimal histological evidence of paratuberculosis at necropsy (5a). Consequently, the animals in this study probably represent cases in an earlier stage of the disease than those included in any other published report, making direct comparison of the results difficult.

The time taken to detect growth on solid media for samples from clinically affected, known culture-positive cattle was much shorter than for samples obtained from subclinically affected cattle. This presumably relates to the number of *M. paratuberculosis* CFU inoculated into the base medium. Lambrech et al. (15) have shown that the growth rate of the organism may be influenced by the inoculum size.

As well as having a major role in the diagnosis of individual infected animals, fecal culture has been used extensively in herd-based test and removal control programs for paratuberculosis. The test has the advantages of both 100% specificity and detection of the animals that are most likely to spread infection. Large-scale processing of samples for this purpose requires relatively uncomplicated procedures, readily available reagents, and the production of minimal quantities of dangerous or waste materials. The two-stage system described here offers the prospect of fulfilling these criteria. In addition, the improved sensitivity of this processing method (or the two methods in parallel) over conventional fecal culture should allow more rapid and successful disease control in herds (16).

The addition of HEY and HEYJ media to the Roche MB Check system paddle would further facilitate large-scale sample processing. Further trials of a true biphasic system using such paddles need to be undertaken to examine the paddle design, inoculation method and frequency, and the possibility of the transfer of mycobactin from the liquid to the solid media.

The processing and culture method outlined above should be equally suitable and useful for the isolation of *M. paratuberculosis* from tissue samples.

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REFERENCES

ican Society for Microbiology, Washington, D.C.


