Effect of egg yolk on the detection of Mycobacterium avium subsp. paratuberculosis using the ESP II liquid culture system

N. Beth Harris, Suelee Robbe-Austerman, Janet B. Payeur

Abstract. Rapid diagnosis of paratuberculosis in infected cattle is important for the successful control of Johne disease within herds. Thus, improving culture methods for Mycobacterium avium subsp. paratuberculosis (M. paratuberculosis) will aid in the identification of asymptomatic animals. Egg yolk is a component of the media used for growing M. paratuberculosis, but its requirement as a supplement has not been reported. Using the ESP II liquid culture system, 2 different sources and 5 concentrations (3.3%, 1.6%, 0.8%, 0.4%, and 0%) of egg yolk were analyzed. Egg yolk source did not affect either recovery rate or time to detection, but both parameters were significantly improved when the 3.3% egg yolk concentrations (final volume) were used over media containing no egg yolk. This study also assessed the recovery of M. paratuberculosis from fecal samples that were cultured multiple times using Herrold egg yolk agar (HEY). Specimens containing greater than 70 cfu/g feces could routinely be identified as positive for M. paratuberculosis after only 1 culture attempt, whereas specimens with fewer bacteria were only intermittently positive, even after 5 replicate cultures. Therefore, this study indicates that the sensitivity of the Trek Diagnostic ESP II liquid culture system for M. paratuberculosis is affected by egg yolk concentration and that single culture attempts using HEY solid media may not identify specimens containing low numbers of bacteria.

Key words: Egg yolk; liquid culture system; Mycobacterium avium subsp. paratuberculosis.

Introduction

Mycobacterium avium subsp. paratuberculosis (M. paratuberculosis) is the etiologic agent of Johne disease, a chronic progressive enteric disease characterized by weight loss and severe diarrhea. This disease causes significant economic losses in the United States and other countries, mainly due to decreased productivity and premature culling.6,10,19,20 Control programs for herds diagnosed with Johne disease are based on early culling of infected animals and improvement of animal husbandry to prevent further spread of infections within the herd. However, these efforts are often less than optimal in controlling this disease due, in part, to the low sensitivity of the current diagnostic procedures used for identifying individual animals infected with M. paratuberculosis. In this regard, fecal culture is more sensitive than serologic tests for identifying those animals with Johne disease.5,6 Unfortunately, isolation of the M. paratuberculosis bacillus from diagnostic specimens remains difficult due to its fastidious nature and slow growth.19,27 Over the last several years, considerable effort has been directed toward determining an optimal culture method for this bacterial pathogen,2,5,19,23,27,28 but efforts continue to be hampered by low recovery rates, especially from fecal specimens of subclinical animals.2,18

Intermittent shedding of M. paratuberculosis in the feces also affects the detection of subclinically infected cattle.4,9 Although this cyclic shedding is generally assumed to be a manifestation of the disease process in the animal,4 others have suggested that shedding may appear to be sporadic due to the limits of sensitivity of the culture method itself.8 Media used for culturing M. paratuberculosis from diagnostic specimens have traditionally included egg yolk emulsion as both a source of supplemental iron and to neutralize the toxic effects of the decontamination method used on fecal and intestinal specimens.12 However, little information is available regarding the optimization of egg yolk concentration in liquid media for M. paratuberculosis. Therefore, a commercially available, automated liquid culture system27 was utilized to analyze this component of M. paratuberculosis culture media. This system was originally adapted from the ESP blood culture system available for the growth and detection of various microorganisms, including mycobacteria, from human clinical specimens.30,31 The underlying principle for detecting the growth of M. paratuberculosis using this method is based on consumption of gasses by viable mycobacteria as part of their metabolic growth cycle. This creates a negative pressure change within the headspace above the broth culture medium in the sealed ESP bottle, which is detected and monitored by a computerized system.
In this study, the capability of solid media to reproducibly recover viable *M. paratuberculosis* from repeated culture attempts of feces from naturally infected animals is described. Overall recovery of *M. paratuberculosis* from these feces was also assessed using the Trek Diagnostic ESP II liquid culture system (ESP). Finally, the effect of egg yolk source and concentration on the recovery of *M. paratuberculosis* from bovine fecal specimens cultured in liquid media was determined.

**Materials and methods**

**Specimens and culture decontamination.** A total of 83 known culture-positive bovine fecal specimens utilized for the 1995–2000 National Veterinary Services Laboratories Johne Disease Proficiency Tests for *M. avium* subsp. *paratuberculosis* were used. Fecal specimens were collected rectally from animals naturally infected with *M. paratuberculosis* into 1-liter containers and frozen at −80°C. Specimens were then thawed once, mixed thoroughly by stirring, and aliquoted into single-use containers containing approximately 5 g each. Samples were then refrozen and maintained at −80°C until used for the study.

At the initiation of this study, all samples were cultured as described below on Herrold egg yolk agar (HEY). Based on these culture results, specimens were divided into 3 separate categories of high–moderate (≥700 colony-forming units [cfu]/g feces) (18 samples), low (70–700 cfu/g feces) (20 samples), and very low (<70 cfu/g feces) (45 samples). Using the culture method and inoculum volume described below, these designations correspond to an average of greater than 26 cfu/tube of HEY (high–moderate), 10–25 cfu/tube (low), and fewer than 10 cfu/tube (very low). All specimens were processed for decontamination using a previously published method. Briefly, 2 g of feces were suspended in 35 ml of distilled water and shaken to disperse visible clumps. After 30 min, a 5-ml aliquot was removed and transferred to a solution containing 0.9% (w/v) hexadecylpyridinium chloride (HPC) in half-strength Brain Heart Infusion broth. After overnight incubation, the specimens were centrifuged, the supernatant discarded, and the pellet resuspended in 1 ml of half-strength BHI containing vancomycin (100 µg/ml), amphotericin B (50 µg/ml), and nalidixic acid (100 µg/ml). Following an additional overnight incubation, 0.5 ml of treated inoculum was aseptically added to an appropriately supplemented liquid culture bottle, as described below.

**Replicate fecal culture procedures.** The 83 fecal samples were processed and cultured on HEY solid media 5 times, each time on different days. For each culture replicate, 2 g of feces were decontaminated as described for the liquid cultures. Fifty microliters of the decontaminated inoculum was placed onto each of 2 tubes of HEY agar containing mycobactin J and 1 tube of HEY without mycobactin. All inoculated HEY tubes were incubated at 37°C in a horizontal position for 1 week with the caps loosened to allow absorption/evaporation of residual moisture on the surface of the medium. Caps were then tightened and the tubes returned to an upright position and incubated at 37°C for a total of 16 weeks. Tubes were examined every 4 weeks using a stereo-microscope at 25X magnification, and any resulting colonies with morphology typical of *M. paratuberculosis* were counted and confirmed as *M. paratuberculosis* by quantitative PCR as described below.

**Preparation of egg yolk supplement.** Eggs purchased from a local grocery store were used to prepare the in-house egg yolk supplement, consisting of 50% concentration (v/v) of egg yolk in phosphate buffered saline. The 50% concentration was selected as a starting point based on the standard operating procedures from laboratories currently using the ESP system for culturing *M. paratuberculosis*. Further 2-fold dilutions of this 50% egg yolk supplement were prepared under sterile conditions with para-JEM broth, to yield final egg yolk supplement concentrations of 25%, 12.5%, and 6.25% (v/v). Similarly, 2-fold dilutions of the egg yolk supplement additive supplied by the company were prepared using para-JEM broth. The concentration of egg yolk present in the commercial supplement is proprietary, and so the assumption was made that the initial concentration was 50%, based on the experience of diagnostic laboratories as mentioned above. Thus, 2-fold dilutions prepared with the commercial additive were assumed to contain the equivalent concentrations of egg yolk as the in-house preparations. Finally, control fecal specimens were cultured with no egg yolk, using unsupplemented para-JEM media as a substitute.

**Liquid culture inoculation procedures.** For each concentration of egg yolk tested, 2 g of feces was processed as described. Immediately prior to inoculation, liquid culture bottles containing 12 ml of broth with mycobactin J were supplemented with 1 ml of para-JEM GS (Growth Supplement), 0.5 ml of para-JEM AS (Antibiotic Supplement), and 1 ml of egg yolk supplement. The supplemented para-JEM broth was then inoculated with 0.5 ml of the decontaminated fecal specimen. Therefore, the final concentration of egg yolk present in the liquid media after the addition of the 50% egg yolk supplement and the decontaminated inoculum was 3.3%. Similarly, final egg yolk concentrations for the 25%, 12.5%, and 6.25% supplements were 1.6%, 0.8%, and 0.4%, respectively. Cultures were incubated in the ESP II machines for 56 days or until a positive signal was detected.

**Identification of positive cultures in liquid media and PCR confirmation.** Upon generation of a positive signal, the suspect-positive para-JEM culture bottle was removed from the ESP II machine and shaken vigorously on an orbital shaker for a minimum of 1 minute to release adherent bacteria from the sponges prior to acid-fast staining. Aliquots of 100 µl were then prepared for acid-fast staining, using an automated acid-fast slide stainer and an auramine rhodamine fluorescent stain. If no acid-fast organisms resembling *M. paratuberculosis* were seen, the bottle was returned for further incubation. All acid-fast-positive cultures were confirmed as *M. paratuberculosis* using a previously described quantitative PCR method targeting the IS900 insertion element. For all positive cultures, the time to detection was considered to be the earliest positive signal recorded by the instrument that correlated with both a positive acid-fast stain and positive IS900 PCR result.

**Statistical analysis.** Data were analyzed using JMP version 5.1. To compare HEY agar with liquid media, data were converted to categorical positive/negative and analyzed us-
Table 1. Recovery of *M. paratuberculosis* from known positive fecal specimens subjected to replicate cultures on Herrold egg yolk media.

<table>
<thead>
<tr>
<th>Shedding level*</th>
<th>% positive at first culture attempt†</th>
<th>% positive at second culture attempt</th>
<th>% positive at third culture attempt</th>
<th>% positive at fourth culture attempt</th>
<th>% positive at fifth culture attempt</th>
</tr>
</thead>
<tbody>
<tr>
<td>High–moderate (18)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>92</td>
<td>94</td>
</tr>
<tr>
<td>Low (20)</td>
<td>95</td>
<td>100</td>
<td>100</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>Very low (39)</td>
<td>12.8</td>
<td>12.8</td>
<td>23.1</td>
<td>15.4</td>
<td>20.5</td>
</tr>
</tbody>
</table>

* Semiquantitative estimation of bacterial load present in the fecal specimens. High–moderate ≥ 700 cfu/g, low = 70–700 cfu/g, very low ≤ 70 cfu/g. Numbers in parentheses represent the total number of specimens in each category.
† Percentage of fecal specimens from which viable *M. paratuberculosis* was recovered for each culture attempt. Six of the original 45 samples in the very-low category were removed from analysis due to incomplete data.

Of the remaining 20 culture-positive specimens, only 5 (12.8%) were positive on the first attempt. However, another 3–5 of the remaining specimens were identified as positive with each subsequent culture attempt. These results, together with the results of the 19 culture-negative samples, indicate that fecal specimens in the very-low category contained numbers of *M. paratuberculosis* bacteria that were just at or below the threshold of detection for this culture method.

To determine if the ESP liquid culture method provides an advantage over solid media for the recovery of *M. paratuberculosis*, a comparison of the ESP II system to HEY agar was performed. For this experiment, only ESP cultures that generated a positive signal and were confirmed as *M. paratuberculosis* by PCR were counted, and only the initial replicate on HEY solid media was used for the comparison. As shown in Table 2, no significant differences were observed between the ESP II culture system and HEY media for recovering *M. paratuberculosis* from feces within any of the categories. For the very-low shedding category, ESP liquid media identified 2 of 45 positive samples and HEY solid media identified 5; only 1 sample was positive on both culture methods.

The effect of egg yolk concentration on the number of positive cultures in the ESP II liquid culture system is shown in Table 3. Because egg yolk source was not found to be significant upon the initial analysis, results for egg yolk source were combined and only egg yolk concentration was considered in the final analysis. Because of the occasional missing sample and because each pairwise comparison was calculated independently, the number of pairs in each analysis varied, causing a slightly different percent positive in the 3.3% category for each comparison. The *M. paratuberculosis* was recovered from significantly fewer specimens cultured without egg yolk (*P* < 0.007, Table 3), indicating that egg yolk is a necessary component of the culture media. Interestingly, the comparison of 1.6% egg yolk to 3.3% egg yolk approached statistical significance (*P* < 0.028), suggesting that the higher concentration of egg yolk may be negatively affecting...
Table 2. Identification of bovine feces positive for *M. paratuberculosis* using the ESP II culture system and Herrold egg yolk agar.

<table>
<thead>
<tr>
<th>Shedding level†</th>
<th>HEY‡</th>
<th>ESP§</th>
</tr>
</thead>
<tbody>
<tr>
<td>High–moderate</td>
<td>18/18 (100%)</td>
<td>16/18 (88.9%)</td>
</tr>
<tr>
<td>Low</td>
<td>19/20 (95%)</td>
<td>14/20 (70%)</td>
</tr>
<tr>
<td>Very low</td>
<td>5/45 (11.1%)</td>
<td>2/45 (4.4%)</td>
</tr>
</tbody>
</table>

* Data represent the total number and percentage of bovine fecal specimens that were identified as positive for *M. paratuberculosis* by each method. For HEY media, positive cultures were defined as the observation of bacterial colonies morphologically consistent with *M. paratuberculosis*, followed by PCR confirmation for the presence of IS900. For the ESP II culture system, positive cultures were defined as the generation of a positive signal by the ESP machine, followed by PCR confirmation of *M. paratuberculosis*. All liquid media were inoculated using the commercially available egg yolk supplement at a 3.3% concentration final volume, as recommended by the manufacturer.

† Semiquantitative estimation of initial bacterial load present in the fecal specimens. High–moderate ≥ 700 cfu/g, low = 70–700 cfu/g, very low ≤ 70 cfu/g.

‡ HEY = Herrold’s egg yolk agar; ESP = ESP II culture system.

Table 3. Effect of egg yolk concentration on the number of positive *M. paratuberculosis* cultures identified by the ESP II system.

<table>
<thead>
<tr>
<th>Egg yolk concentration*</th>
<th>Comparison of % culture positive (3.3% egg yolk vs. listed concentration of egg yolk)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3</td>
<td>N/A†</td>
<td>N/A</td>
</tr>
<tr>
<td>1.6</td>
<td>35.4% vs. 42.1%</td>
<td>0.028</td>
</tr>
<tr>
<td>0.8</td>
<td>33.3% vs. 32.6%</td>
<td>0.8</td>
</tr>
<tr>
<td>0.4</td>
<td>33.3% vs. 31.9%</td>
<td>0.72</td>
</tr>
<tr>
<td>0</td>
<td>31.9% vs. 15.9%</td>
<td>0.007‡</td>
</tr>
</tbody>
</table>

* Final concentration of egg yolk present in supplemented paraJEM culture bottles as described in Materials and Methods.

† N/A = not applicable.

‡ Significant results using the Bonferroni correction (P = 0.0125).

the growth characteristics of *M. paratuberculosis* in the ESP II culture system. Because methods that permit more rapid detection of *M. paratuberculosis* are also of value to veterinary diagnostic laboratories, the overall time to detection as a function of egg yolk concentration was analyzed. Similar to the above data, cultures containing 3.3% egg yolk grew significantly faster, averaging 36.5 days to positive versus 46.6 days for cultures grown without egg yolk (Table 4). A significant difference was also noted between specimens cultured with the highest and lowest egg yolk concentration (36.5 vs. 41.9 days to positive, respectively).

**Discussion**

Rapid diagnosis of paratuberculosis in infected animals is valuable for the successful execution of a national Johne disease control program. Therefore, optimization and standardization of bacterial culture methods are necessary to provide a more consistent basis for identification of asymptomatic animals. In this study, we analyzed the repeatability of HEY culture media for recovering *M. paratuberculosis*, evaluated the effectiveness of the ESP II system for recovery of *M. paratuberculosis* from fecal specimens, and determined the effect of egg yolk concentration or source on recovery of this pathogen from liquid media.

It is generally recognized that subclinical animals do not consistently shed the *M. paratuberculosis* microorganism in their feces. One hypothesis for this observation is that the interaction between the host immune system and this pathogen may contribute to the cyclic pattern of shedding observed early in infection.

Table 4. Effect of egg yolk concentration on the time to detection of *M. paratuberculosis* using the ESP II culture system.

<table>
<thead>
<tr>
<th>Egg yolk concentration*</th>
<th>Average days to positive†</th>
<th>95% confidence interval (days)‡</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3</td>
<td>36.5</td>
<td>33.1–44.0</td>
<td>N/A‖</td>
</tr>
<tr>
<td>1.6</td>
<td>35.4</td>
<td>32.1–38.7</td>
<td>0.53</td>
</tr>
<tr>
<td>0.8</td>
<td>34.8</td>
<td>31.1–38.5</td>
<td>0.36</td>
</tr>
<tr>
<td>0.4</td>
<td>41.9</td>
<td>38.1–45.6</td>
<td>0.005§</td>
</tr>
<tr>
<td>0</td>
<td>46.6</td>
<td>40.1–53.2</td>
<td>0.003§</td>
</tr>
</tbody>
</table>

* Final concentration of egg yolk present in supplemented paraJEM culture bottles as described in Materials and Methods.

† Average time in days for the ESP II culture system to signal a positive culture.

‡ Based on a multiple variable mixed regression model. Calculated confidence interval for the average days to positive, as described in the Materials and Methods.

‖ N/A = not applicable.

§ Significant results.
unclear. Also, the interpretation of various methods reported for culturing *M. paratuberculosis* may be influenced by differences between protocols. For example, some comparative culture studies have classified specimens based on the severity of histopathologic lesions and the number of mycobacteria observed in acid-fast-stained tissue sections, whereas others have utilized a more direct approach and categorized animals as high, moderate, and low shedders based on bacterial counts from cultured specimens. Even among studies that directly quantitate bacterial counts, cutoff points vary for the various shedding status levels. Thus, comparison of results from different laboratories and culture methods need to be interpreted with caution. Nonetheless, it stands to reason that, by limiting the initial volume used to inoculate culture media, sensitivity may be reduced because fewer viable bacteria are being introduced onto the growth medium.

The function of egg yolk in primary cultures of *M. paratuberculosis* is largely unknown, but it most likely acts to neutralize the chemical action of the HPC used in the decontamination step. Residual HPC present in the inoculum may remain active within the media and partially inhibit the growth of mycobacteria. To this regard, 2 recent publications have indicated that the HPC used for decontaminating fecal specimens affected the viability of *M. paratuberculosis* present in the specimen, resulting in up to a 2 log-fold loss of bacteria. Finally, the presence of antibiotics in the decontamination step, the function of which is to help eliminate bacterial and fungal overgrowth, may in fact be either bactericidal or bacteriostatic for *M. paratuberculosis*. A recent study has demonstrated that vancomycin at 10 μg/ml may inhibit the growth of some strains of *M. paratuberculosis*. Synergistic bactericidal effects between the decontaminant and residual antibiotics cannot be ruled out either, as this phenomenon was reported for cetylpyridinium chloride and ethambutol antibiotics for *M. avium, Mycobacterium bovis BCG, Mycobacterium fortuitum* and *Mycobacterium phlei*.1

Because it is possible any residual HPC or antibiotic carryover from a bacterial inoculum into liquid media would be diluted sufficiently to prevent inhibitory effects, this study endeavored to determine if egg yolk is a required component of broth cultures for growing *M. paratuberculosis* from fecal specimens. Based on the protocol provided by the manufacturer for growing *M. paratuberculosis* in the ESP II system, a 50% egg yolk supplement was selected as the initial concentration for these experiments, corresponding to a final concentration of 3.3% in the growth media. Our results demonstrate that media containing 3.3% (final volume) concentrations of egg yolk resulted in the growth of *M. paratuberculosis* from significantly more cultures than cultures grown without egg yolk. These results are consistent with previous studies utilizing egg yolk in radiometric liquid cultures, where higher egg yolk concentrations, ranging from 6 to 10% (final volume), improved both the recovery rate and the time to detection of *M. paratuberculosis* from both ovine and bovine fecal specimens. In addition, higher concentrations of egg yolk resulted in more rapid identification of positive cultures. However, the overall number of specimens identified as positive by the ESP II system was similar to that of conventional solid media, unlike previous studies, which indicate that liquid culture methods for *M. paratuberculosis* tend to be more sensitive. One possible explanation for these divergent results may be due to the total volume of specimen used to inoculate culture media in the respective studies. Throughout the present study, lower volumes for inoculating both liquid and solid media were used, which may influence overall results, as previously discussed.

The relatively high proportion of negative cultures reported for both solid and liquid media from specimens in the very-low category may be due in part to the lack of viable microorganisms. Although feces used in the present study were aliquoted individually and stored at −80°C with no freeze-thaw cycles after being aliquoted, it is possible that a proportion of the bacteria were rendered nonviable as a result of this storage. To this end, a loss of viability of *M. paratuberculosis* has been reported by others for bovine feces stored at −70°C. In these previously reported studies, the reduction in average colony counts for fecal specimens cultured after freezing was between 54% and 98%, indicating that freezing may reduce viable bacterial organisms to below detectable levels, especially in samples containing low numbers of bacilli. Furthermore, a recent study by Stabel et al. also reported that 29% of fecal samples previously defined as culture-positive and containing 1–7 cfu/g were subsequently culture negative. However, other researchers have indicated that minimal loss of viability was noted from bovine feces as a result of freezing (R. H. Whitlock, personal communication). Therefore, more research is necessary to conclusively address this issue.

Another possibility for the lack of recovery in these specimens may be that the bacteria present in these samples were viable but their generation time was such that insufficient growth prevented detection. The generation time of *M. paratuberculosis* reported in other liquid-culture studies ranged from less than 1 day to greater than 7 days and was inversely proportional to the number of bacteria present in the original inoculum. Furthermore, Kim et al. determined that the average threshold of detection for the ESP II system was approximately 10³ cfu/ml or more, indicating that
at concentrations below this level, bacterial growth may not be sufficient to generate a positive signal by the system.

In conclusion, this study demonstrates that growth of \textit{M. paratuberculosis} in the ESP II system is enhanced by the presence of egg yolk and that concentrations of approximately 3% (final volume) provide the highest recovery rate. The identification of \textit{M. paratuberculosis} from bovine specimens using HEY media could be reliably detected with a single culture attempt for samples with an initial bacterial load of >70 cfu/g feces but was only intermittent for specimens containing fewer bacteria when using this culture protocol. This study also confirms that the ESP II liquid culture system is a rapid, sensitive method for identifying \textit{M. paratuberculosis} from bovine feces, making it a reliable tool for the diagnosis of Johne disease.

\textbf{Acknowledgements}

The authors wish to thank Mr. David Farrell and Ms. Doris Bravo for their excellent technical assistance. We would also like to gratefully acknowledge Dr. Annette O’Connor for her assistance with the statistical analyses. N.B.H. was the recipient of a USDA-APHIS fellowship.

\textbf{Sources and manufacturers}

a. ESP Culture System II\textsuperscript{a} and ESP\textsuperscript{a} para-JEM\textsuperscript{a} liquid culture reagents, TREK Diagnostic Systems Inc., Cleveland, OH.

b. Sigma Chemical Co., St. Louis, MO.

c. Becton-Dickinson Diagnostic Systems, Sparks, MD.

d. Vibrax\textsuperscript{b} VXR shaker, Janke & Kunkel, Staufen, Germany.

e. Aerospray\textsuperscript{c} stainer, Wescor, Inc., Logan, UT.

f. SAS Institute, Cary, NC.

\textbf{References}


17. Smithwick RW, Stratigos CB, David HL: 1975, Use of cetylpyridinium chloride and sodium chloride for the decontamination of sputum specimens that are transported to the laboratory for the isolation of \textit{Mycobacterium tuberculosis}. J Clin Microbiol 1:411–413.


