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Factors Affecting Isolation and Identification of *Mycobacterium avium* subsp. *paratuberculosis* from Fecal and Tissue Samples in a Liquid Culture System[▽]

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Culture of *Mycobacterium avium* subsp. *paratuberculosis* is the definitive diagnostic test for Johne's disease, a chronic granulomatous enteropathy of animals. Compared to solid media, the identification of all strains of the organism in liquid media can be more difficult because the appearance of colonies and mycobactin dependence are not observable, and the growth of other organisms needs to be distinguished, commonly by PCR. Factors affecting the isolation rate of S strains and the contamination rate in modified Middlebrook 7H9 broth (Bactec 12B) and 7H10 agar were studied using 11,598 fecal samples and 2,577 tissue samples from sheep from 1,421 farms over 10 years. Minimization of contamination in Bactec cultures required the avoidance of the carryover of fecal particles from the first sedimentation step in the double-incubation centrifugation method, and contamination was reduced significantly by incubating the sample in a solution containing vancomycin, amphotericin B, and nalidixic acid for 3 days compared to 2 days. The growth of irrelevant microorganisms confounded the identification of *M. avium* subsp. *paratuberculosis* in liquid culture by inhibiting IS*900* PCR and in solid medium culture by inhibiting the growth of *M. avium* subsp. *paratuberculosis* or obscuring colonies. The contamination of samples was clustered in certain laboratory submissions and was reduced by including ampicillin in Bactec medium without affecting the odds of isolation of *M. avium* subsp. *paratuberculosis*. The long-term contamination rate for fecal cultures was about 7%, and that for tissue cultures was <0.2%. Liquid medium was more sensitive than solid medium culture for *M. avium* subsp. *paratuberculosis*. The applicability of these findings for C strains is discussed.

Paratuberculosis is a slowly progressive intestinal disease caused by *Mycobacterium avium* subsp. *paratuberculosis.* In its clinical form, there is weight loss, and in some species, diarrhea also occurs prior to death. Most herbivores are susceptible to the infection, and there is no practical treatment for affected animals. Juvenile animals become infected by ingesting the organism through contact with feces from infected adults; transmammary and intrauterine infection also occurs (47). Paratuberculosis is recognized globally, and disease control programs are being developed in many countries (2). To be effective, these programs depend on accurate herd-level diagnosis and the initiation of measures to reduce spread to other farms. Accurate diagnosis at the level of individual animals is also required to enable the removal of cases before they become significant sources of *M. avium* subsp. *paratuberculosis* contamination. Diagnostic tests also enable monitoring of the effectiveness of hygiene measures that aim to break the cycle of transmission on a farm (28). However, the diagnosis of paratuberculosis is problematic.

Clinical signs and humoral immune responses in paratuberculosis tend to be poorly developed until late in the disease process, and tests for cell-mediated immune responses, which may be present earlier in the disease, tend not to be specific or practical. While shedding of *M. avium* subsp. *paratuberculosis* in feces is commonly believed to precede the humoral response, recent analysis of a large data set from a longitudinal study of cows suggested that humoral responses detected by enzyme-linked immunosorbent assay may occur concurrently with or precede fecal shedding (25); such assessments are dependent on the analytical sensitivity of the particular fecal culture method employed. Fecal shedding can be intermittent, and in vitro cultures take several months to develop. Despite these limitations, culture remains the most sensitive means of detection of infection in individuals, and when positive, it is a definitive test (3, 22, 46).

Many methods for the culture of *M. avium* subsp. *paratuberculosis* have been described since the original report (38). These methods are based on the general principles of culture of slowly growing mycobacteria from clinical samples, namely, decontamination to destroy irrelevant, usually rapidly growing microorganisms (both bacteria and fungi); prolonged incubation in appropriate media containing antimicrobial agents to suppress contaminants; and identification of isolates by phenotypic and genotypic means (13, 23). Concentration of *M. avium* subsp. *paratuberculosis* by sedimentation, centrifugation, or filtration of the sample may also be included in the protocol (3, 23, 41). Several decontamination methods have been recommended over the years, and both liquid and solid media are used (3, 6, 16, 17, 23, 34). Antimicrobials such as malachite green, cycloheximide, amphotericin B, vancomycin, nalidixic acid, chloramphenicol, penicillin G, polymyxin, trimethoprim, azlocillin, and ampicillin may be included in decontamination solutions and/or in media (15, 19, 34, 44). Important outcomes

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TABLE 1. Ovine fecal and tissue samples used in this study[a]

Group	Protocol and laboratory	Type of sample (no. of samples/pool)	No. of fecal samples cultured	No. of tissue samples cultured	No. of independent trials	No. of independent farms	No. of independent submissions[d]	Source	Yr
1	Initial protocol; laboratory A	Pooled fecal samples (at most 50)	1,535	0	1	296	343	Expt 3[b]	1998
2	Fixed protocol; laboratory A	Pooled fecal samples (10–50)	378	0	1	51	57	Expt 2[b]	1998
3	Fixed protocol; laboratory A	Pooled fecal samples (at most 50)	4,619	0	1	936	936	Unpublished data	1999–2001
4	Final protocol; ampicillin included in some cultures; laboratory B	Pooled fecal samples (5); individual fecal samples; tissue samples[c]	2,467	772	1	1	15	Field trial; feces collected every 3 mo; tissues collected at necropsy at 2.5 yr of age	2002–2005
5	Final protocol; laboratory B	Pooled fecal samples (5–50); individual fecal samples; tissue samples[c]	2,599	1,805	18	137	172	5 field trials; 6 experimental infection trials; 7 on-farm studies	2002–2008
Total			11,598	2,577	22	1,421	1,523		

[a] Farms represent the smallest geographic unit of clustering of samples. There were multiple submissions of samples from some farms.
[b] Details are provided in reference 43.
[c] Ileum and jejunum pooled; intestinal lymph nodes pooled.
[d] A submission is a group of samples collected and consigned from the same farm at the same time.

in the culture of *M. avium* subsp. *paratuberculosis* are the isolation rate and the contamination rate, both of which should be reported in methodological assessments (20, 24, 26, 34). Different protocols recommended for culturing of *M. avium* subsp. *paratuberculosis* have different analytical sensitivities (9, 16, 34). However, there have been few studies to determine the optimum technique, particularly with the distinctive strains of *M. avium* subsp. *paratuberculosis* that commonly cause paratuberculosis in sheep (S strains) (6, 17, 19, 34, 44). S strains have different cultural requirements compared to cattle (C) strains (17, 44).

Evidence to suggest that liquid culture methods for *M. avium* subsp. *paratuberculosis* have greater analytical and diagnostic sensitivity than protocols based on the use of solid media is accumulating (9). Growth can be detected sooner using liquid culture techniques, which is of great practical advantage (4, 6). However, the identification of the organism is more difficult in liquid culture because the appearance of colonies and mycobactin dependence are not observable, and the growth of other organisms needs to be distinguished. The presence of irrelevant microorganisms in a culture is of particular importance, as increased reliance is placed on enrichment culture in liquid medium followed by identification using PCR methods, which removes the need to visualize colonies of the organism (4, 29, 44, 45). Four modern liquid culture methods have been used for *M. avium* subsp. *paratuberculosis* identification: the Bactec 460 radiometric system, the Bactec MGIT 960 fluorescence detection system (12, 14, 15, 32), the Trek ESP pressure detection system (11), and the MB/BacT reflectance detection system (35). In each of these systems, a nondiscriminatory measure of metabolic activity is reported, so it is necessary to verify the cause of microbial growth using an independent and specific method. As the demonstration of growth triggers the need for a secondary test, which may be expensive, it is desirable to reduce the incidence of growth of irrelevant microorganisms (4).

Economical and sensitive diagnoses can be achieved by culturing of fecal samples pooled from groups of animals (30, 43). Pooled fecal culture for sheep is now a routine procedure made available through animal health diagnostic laboratories in Australia. Pooled fecal culture is also used for cattle and goats, and a variation, culturing of pooled samples taken from the environment, also appears to be useful for herd-level detection (8, 10, 18, 21, 36, 40).

The aims of this study were to assess procedural factors that affect the isolation of *M. avium* subsp. *paratuberculosis* in clinical samples, the frequency of growth of irrelevant microorganisms, and the effect that these factors may have on the subsequent identification of *M. avium* subsp. *paratuberculosis*. Experimental design also permitted an assessment of isolation rates of *M. avium* subsp. *paratuberculosis* in liquid and solid media and the value of inclusion of ampicillin in Bactec radiometric medium to discourage the growth of irrelevant microorganisms. The study was based on samples from sheep which contained the S strain, but many of the findings will be applicable to other strains of *M. avium* subsp. *paratuberculosis*.

MATERIALS AND METHODS

Samples. There were five groups of samples for this study (Table 1). The first two groups were pooled fecal samples used in an earlier study (43). The remaining three groups were pooled fecal samples, individual fecal samples, and tissue samples submitted by veterinarians during routine surveillance for ovine paratuberculosis or collected during field-based epidemiological studies or experimental infections. Pooled fecal samples were mixed in a high-speed electric blender with precautions to minimize cross-contamination of samples (43). A negative control fecal sample was homogenized and then cultured at least once each day with the test samples. The negative control fecal sample consisted of a pool of 50 fecal pellets collected from paratuberculosis-free sheep and was stored at −20°C. A positive control fecal sample was included with each batch of cultures. It comprised fecal pellets from infected sheep that were pooled and mixed 1 part in 10 with fecal pellets from negative control sheep. The control positive feces were dispensed in aliquots of 5 ml and stored at −80°C until required.

Fecal samples were submitted chilled (approximately 4°C), sometimes via other laboratories. Upon receipt at the main laboratory, samples were placed at 4°C or at −80°C if they could not be processed within 4 days. Some samples were homogenized upon receipt and then stored at −80°C if culture was to be delayed.

Decontamination of fecal samples. Several improvements to the decontamination protocol were introduced during the study. Inevitably, there was variation in intervals associated with sample collection and processing. For group 1 samples, the intervals in days between sampling sheep and receipt of samples at the laboratory (sam-rec), between receipt and homogenization (rec-hom), and between homogenization and decontamination (hom-dec) were known for each sample. The effects of these intervals were analyzed, and based on these findings (see Results), the preferred options to minimize the growth of irrelevant microorganisms were described and applied as a final protocol for culture of samples in groups 3, 4, and 5. Fecal samples were prepared using a modified double-incubation and centrifugation method (31, 41) as described previously (44). Briefly, up to 2 g of feces was placed in a 15-ml polypropylene tube containing a swab stick. Two varieties of tube were used: either one with a swab that could be retracted through the lid (catalog number SWP10015G, swab in sterile container, gamma sterile; Bacto Labs, Sydney, Australia) (preferred) or another where the swab could not be retracted (catalog number SWP-100, Kayline disposable swab stick; Bacto Labs, Sydney, Australia). The swab stick was used to break up the feces in 10 to 12 ml of sterile normal saline. After mixing, the tube was allowed to stand for either 15 min or 30 min (preferred) at room temperature. Five milliliters of the surface fluid was transferred by decanting or careful aspiration with a pipette (preferred) to a fresh tube containing 25 ml 0.9% hexadecylpyridinium chloride (HPC) (Sigma Chemical Co., St. Louis, MO) in half-strength brain heart infusion broth (Oxoid, Basingstoke, England), and this was allowed to stand at 37°C for 24 h and then centrifuged at 900 × *g* for 30 min. The pellet was collected and resuspended in 1 ml sterile water with vancomycin (100 μg/ml), amphotericin B (50 μg/ml), and nalidixic acid (100 μg/ml) (VAN) (all Sigma reagents) and incubated for 48 h or 72 h (preferred) at 37°C. Consequently, the total decontamination-to-inoculation interval (dec-inoc) was either 3 days or 4 days (preferred). The sediment was used as the inoculum.

Preparation of intestinal tissues. Intestinal wall or lymph node, approximately 5 g, was trimmed of extraneous tissue, cut into small pieces, and beaten for 30 s in 2 ml of sterile normal saline in a stomacher (BagMixer 100 Minimix; Interscience) set to maximum speed. Contents of the blender bag were transferred into a sterile 5-ml tube; avoiding any remaining pieces of tissue, 2 ml of the liquid homogenate was transferred into 25 ml of 0.75% HPC. The tube was then inverted several times to mix and allowed to stand at room temperature in the dark for 72 h. One hundred microliters of the sediment from the very bottom of the tube was inoculated into Bactec medium.

Culture media, inoculation, and evaluation. One 0.1-ml aliquot of the prepared fecal sediment or tissue homogenate was inoculated into modified Bactec 12B radiometric medium, and the growth index was measured weekly for 12 weeks (44). A growth index of >200 was defined as positive. For samples from groups 1, 2, and 3, a second aliquot of decontaminated fecal sediment was inoculated onto one slope of modified Middlebrook 7H10 agar (7H10) with mycobactin J (MJ) (7H10+MJ) prepared in 35-ml screw-cap polystyrene Macartney tubes (44). These were termed primary cultures. Slopes were examined with the naked eye under strong light every 2 weeks for 20 weeks.

Identification of *M. avium* subsp. *paratuberculosis*. To determine whether the cause of a growth index in Bactec medium was *M. avium* subsp. *paratuberculosis*, 0.1 to 0.2 ml of medium was removed for PCR from each Bactec vial when its growth index was first found to be ≥200 (first sample); again removed when the growth index reached 999, usually in the following week (second sample); and again removed for group 1 samples a week later (third sample). The sample (0.1 ml) was prepared for PCR using differential centrifugation in ethanol, but for groups 1, 2, and 3, if PCR was negative, DNA was purified from the ethanol extract using a commercial kit (Wizard PCR preps; Promega), and the sample was retested by PCR (45). PCR to detect IS*900* and restriction endonuclease analysis (REA) of the PCR product with AlwI to confirm specificity were conducted as described previously (44, 45).

For samples from groups 1, 2, and 3 only, to determine whether the cause of a growth index was *M. avium* subsp. *paratuberculosis* by phenotypic methods, an additional sample was removed from each growth index-positive Bactec bottle when the growth index was ≥200. To determine mycobactin dependency, 0.1 ml was spread onto 7H10+MJ and Middlebrook 7H10 medium without MJ. Herrold's egg yolk medium (HEYM) with and without MJ and Lowenstein-Jensen medium (LJ) slopes were also inoculated. The slopes were examined every 2 weeks for up to 20 weeks. All incubations were performed at 37°C. Colonies consistent with *M. avium* subsp. *paratuberculosis* were removed from primary culture and subculture slopes of 7H10+MJ and prepared for PCR as described previously (44). To assess whether colonies of *M. avium* subsp. *paratuberculosis* might be obscured in mixed cultures, 10 slopes of 7H10+MJ with a moderate to heavy level of growth of organisms not typical of *M. avium* subsp. *paratuberculosis* were also tested by PCR where *M. avium* subsp. *paratuberculosis* had been identified in the parent Bactec culture by PCR. A bacteriological loop was dragged through colonies of the contaminants and across the surface of the slope between them. These colony samples were tested by PCR. Smears were prepared from bacterial colonies, dried in an oven at 65°C, and stained using a Ziehl-Neelsen technique or, alternatively, were prepared from bacterial colonies, heat fixed, and stained using a Gram method (5). Additional tests were conducted to provide preliminary identification of nonmycobacterial isolates (1).

Culture definitions. A positive culture was one in which *M. avium* subsp. *paratuberculosis* was grown in Bactec medium and/or in primary or secondary culture on 7H10+MJ medium and identified by PCR with REA. An inconclusive culture was one where PCR yielded a product of the expected size (413 bp) but where REA could not be undertaken due to an insufficient amount of PCR product. A negative culture was one from which *M. avium* subsp. *paratuberculosis* cells were not isolated. A culture was defined to contain irrelevant microorganisms when it had a positive Bactec growth index but when PCR was negative or when colonies other than or in addition to those of *M. avium* subsp. *paratuberculosis* were reported to appear on one or more slopes.

Statistical analysis. For group 1, a generalized linear mixed model with logit link function (GenStat release 10.1.2007; Lawes Agricultural Trust, Rothamsted Experimental Station) was used to determine the effect of four stages within the culture test protocol on the odds of growth of *M. avium* subsp. *paratuberculosis* and separately on the odds of growth of other organisms. The stages assessed were sam-rec, rec-hom, hom-dec, and dec-inoc. As pooled fecal samples were submitted in groups (range, 1 to 11 pooled samples per submission from 343 separate submissions), "submission," defined as a group of samples from one source submitted together, was included as a random factor in the models to allow for possible clustering in microbiological test outcome due to the property of origin or other environmental factors. For group 4, a similar model was used to determine the effect of the inclusion of ampicillin in Bactec 12B medium on the odds of growth of *M. avium* subsp. *paratuberculosis* and separately on the odds of growth of other organisms. Chi-square tests for the difference between proportions were undertaken using Minitab statistical software. Binomial confidence limits for proportions were determined using Prism (GraphPad Software, San Diego, CA). McNemar's test for paired observations was undertaken using software found at www.graphpad.com.

RESULTS

Group 1. (i) Sample processing intervals. A total of 1,535 fecal samples were cultured between May and August 1998 (Table 2).The interval between the collection of samples from sheep on the farm and receipt of those samples at the laboratory ranged from 1 to 7 days (median, 3 days). Processing capacity was sometimes exceeded at the laboratory, necessitating the storage of samples at −80°C upon receipt. Consequently, there were variable receipt-to-homogenization (range, 0 to 54 days; median, 1 day) and homogenization-to-decontamination (range, 0 to 29 days; median, 1 day) intervals. Cultures were set up twice in every 5-day working week by using a protocol which allowed incubation in VAN for either 2 or 3 days and, when combined with the 1-day incubation in HPC, resulted in decontamination to inoculation intervals of either 3 days or 4 days.

(ii) Comparison of primary isolation rates in Bactec medium with solid medium. Of the 1,535 samples, 202 were positive and 11 were inconclusive for *M. avium* subsp. *paratuberculosis.* Four (1.98%) of the positive samples were detected only by growth on primary slopes with 7H10+MJ (Table 2). Primary culture in Bactec medium (198 positive) was more sensitive than primary culture on slopes with 7H10+MJ agar (68 positive) (chi-square value, 120.6; $P < 0.0001$ by McNemar's test). After subculture from Bactec medium to 7H10+MJ, only 75% of the 198 Bactec-positive samples yielded colonies of *M. avium* subsp. *paratuberculosis*.

The peak growth index for 94% of the positive samples occurred within 7 weeks, but for others, it was as late as 12

TABLE 2. Growth index and means of identification of *M. avium* subsp. *paratuberculosis* in 1,535 pooled fecal cultures in group 1

Growth index	Corresponding PCR result	No. of samples with indicated result determined with Bactec medium	No. of positive samples determined with 7H10+MJ solid medium	
			Primary culture	Subculture
Positive	Positive from 1st sample	188	58	143
	Positive from 2nd sample	8	4	5
	Positive from 3rd sample	2	2	1
	Inconclusive	11	0	0
	Negative	342	4[a]	0
Negative	NT	984	0	NT
Total positive		198[a]	68	149

[a] A total of 202 samples were positive. NT, not tested.

weeks. Growth was slower on the primary slopes of 7H10+MJ agar, where 74% of samples had colonies within 14 weeks with a range from 9 to 20 weeks. Colonies were apparent sooner after subculture from Bactec medium to 7H10+MJ medium: 73% within 5 weeks, with a range of 2 to 20 weeks.

(iii) Identification of *M. avium* subsp. *paratuberculosis*. Of the 202 culture-positive samples, 93% were confirmed by PCR with REA examination of the first sample taken from the Bactec medium (Table 2). Additional examinations were required for the remaining 14 positive samples, with 8 being confirmed from the second sample, 2 being confirmed from the third sample, and 4 being confirmed by PCR with REA from colonies from the primary 7H10+MJ agar slopes. Samples were taken from Bactec cultures with a growth index of >200 and were subjected to PCR after a simple ethanol extraction; those that were negative were retested after the purification of DNA using a commercial silica binding column kit. DNA purifications were required for 28% of the samples that were found to be positive. This was considerably higher than the rate of 11% obtained previously (45) and was associated with the fact that many of the positive cultures were mixed cultures, i.e., contained organisms other than *M. avium* subsp. *paratuberculosis* (see below).

Overall, 84% of the 202 culture-positive samples had colonies of *M. avium* subsp. *paratuberculosis* in either primary culture on 7H10+MJ agar or after subculture on 7H10+MJ agar. However, only 69% of samples that were positive upon primary culture on 7H10+MJ medium yielded colonies of *M. avium* subsp. *paratuberculosis* on this medium after subculture from Bactec medium; the reasons for this were not determined.

(iv) Growth of irrelevant microorganisms. Overall, colonies of irrelevant microorganisms were observed from 16% of the primary cultures on 7H10+MJ agar from the 1,535 samples and from 76% of the 551 subcultures from Bactec medium. A similar proportion (70 to 72%) of each of the five subculture media displayed such colonies. Based on the presence of irrelevant colonies on any of the solid-medium slopes, 49% of the 202 samples that were positive for *M. avium* subsp. *paratuberculosis* yielded mixed cultures with other organisms. Only one slope was overgrown by contaminants and unreadable. Contaminants grew more quickly on 7H10+MJ agar after subculture from Bactec medium (most within 3 weeks) compared to their growth upon primary isolation on this medium (most within 8 weeks). The contaminants comprised gram-negative rods with either brown slimy colonies causing brown discoloration of the media or creamy yellow colonies, gram-negative coccobacilli with white mucoid colonies causing blue discoloration of the media, and gram-positive diptheroids with yellow or orange shiny raised colonies. None of these organisms could be identified using routine medical microbiological keys, suggesting that they were environmental organisms.

The effect of some factors on the isolation of irrelevant microorganisms was assessed by logistic regression in a mixed model that accounted for the clustering of samples. The time taken for samples to reach the laboratory after collection, the interval between receipt and commencement of the culture protocol, the interval between homogenization and decontamination of the sample, and the incubation time in the antibiotic mixture VAN were investigated. Only the decontamination time in VAN was significant. The odds of growth of irrelevant microorganisms were 0.36 times lower (95% confidence interval, 0.26 to 0.51) for samples incubated in VAN for the longer time. Overall, 14.7% of the 812 cultures incubated in VAN for 3 days yielded growth of irrelevant microorganisms, compared to 30.3% of 723 cultures incubated in VAN for 2 days ($P < 0.0001$).

(v) Effect of irrelevant microorganisms on identification of *M. avium* subsp. *paratuberculosis*. To test whether contaminants obscured colonies or inhibited the growth of *M. avium* subsp. *paratuberculosis* upon subculture from Bactec medium, the surfaces of 10 7H10+MJ subculture slopes that did not appear to contain *M. avium* subsp. *paratuberculosis* colonies were sampled with a bacteriology loop and tested by PCR. Six had yielded *M. avium* subsp. *paratuberculosis* colonies on primary 7H10 medium. All 10 slopes were PCR positive, suggesting that colonies of *M. avium* subsp. *paratuberculosis* had been obscured or that their growth had been inhibited.

Of the 60 samples from Bactec medium that required DNA purification on a silica column to yield a positive result by PCR, 72% contained contaminants detected by subculture in solid medium, compared to only 29% of the 152 PCR-positive samples that did not require DNA purification ($P < 0.001$). None of the sampling or processing time factors (sam-rec, rec-hom, hom-dec, and dec-inoc) affected the odds of isolation of *M. avium* subsp. *paratuberculosis*.

Group 2. There was a dramatic difference in the proportions of cultures yielding irrelevant microorganisms between group 2 (1.6%) and group 1 (22%) ($P < 0.0001$). Methodological differences between the two studies were investigated in order to explain this observation (Table 3). Less suspended debris was transferred after the first sedimentation step in saline in group 2 than in group 1. This was due to the longer sedimentation time, removal of the swab stick prior to the removal of the lid of the tube, and careful removal of a variable volume of supernatant with a pipette in group 2.

Based on data from groups 1 and 2, the culture protocol was refined to reduce disturbances of the fecal sediment and to ensure adequate incubation time in VAN as follows: the use of retractable swab tubes, 30 min of sedimentation, careful aspi-

TABLE 3. Comparison of contamination rates and methods for fecal culture

Group	Contamination rate (%) (total no. of samples)[a]	Sampling-to-receipt interval (days)	Settling time for 1st sedimentation step (min)	Tube used to suspend feces for 1st sedimentation step	Mixing for 1st sedimentation step	Decanting supernatant for 1st sedimentation step	Time of decontamination in VAN (days)
1	22 (1,535)	1–7 (85% within 4 days)	15	Fixed swab stick	Gentle inversion	Decanted 5 ml	2 or 3
2	1.6 (386)	0–5 (85% within 3 days)	30	Retractable swab stick	Shaken	Decanted a variable amt of supernatant to avoid sediment	3
3	9.3 (4,619)	Not recorded	30	Retractable swab stick	Shaken	Aspirated up to 5 ml without disturbing sediment	3

[a] Measured as growth-index-positive, PCR-negative cultures.

ration of the supernatant with a sterile disposable pipette, and incubation for 3 days in VAN. Primary culture on 7H10+MJ medium was discontinued due to the low rate of isolation of *M. avium* subsp. *paratuberculosis*. The new protocol was applied to group 3.

Group 3. Between April 1999 and April 2001, a total of 4,619 fecal cultures were undertaken with the improved protocol. Of these cultures, 3,755 did not develop a positive growth index, 420 (9.1%) developed a growth index and were positive by PCR, and the remaining 428 (9.3%) were growth index positive but PCR negative, consistent with growth of irrelevant organisms (Table 3). Of the latter, 92% grew contaminants when subcultured on solid media, compared to 7.4% of the PCR-positive cultures ($P < 0.001$). Colonies of *M. avium* subsp. *paratuberculosis* were obtained after subculture from 90% of the PCR-positive Bactec cultures (in five cultures as mixed growth with irrelevant organisms). Only 6% of the 420 positive samples that were confirmed by PCR on Bactec medium required more than one PCR examination, and only 2.6% required DNA purification (Table 4). However, all 444 of the growth index-positive samples that were reported to be PCR negative had received multiple PCR tests and DNA purification. As the subculture of Bactec medium to solid medium did not significantly increase the rate of isolation of *M. avium* subsp. *paratuberculosis*, it was discontinued in subsequent studies. As DNA purification over silica was required for only 2.6% of 420 samples to provide a positive PCR result but needed to be undertaken for all growth positive-PCR negative samples, it was also discontinued.

Group 4. Fecal samples collected quarterly over a 2-year period from 840 Merino sheep were cultured in pools of five samples using the final protocol at laboratory B. Individual samples from positive pools were then cultured, while intestinal tissues were cultured at necropsy. There were a total of 217 positive fecal cultures and 34 positive tissue cultures (Table 5). A wide range of contamination rates were noted between submissions, possibly associated with changes in nutrition including hand feeding of grain in some seasons. Ampicillin was added to media to reduce the contamination rate following a pilot study (15). This led to a significant reduction in the rate of isolation of irrelevant microorganisms, from 26% of 1,190 fecal cultures in media without ampicillin to 8% of 1,277 cultures when ampicillin was included ($P < 0.001$). The odds of growth of irrelevant microorganisms was 0.30-fold lower (95% confidence limits, 0.11 to 0.89) in the presence of ampicillin after allowing for clustering in the data due to submission. There was no significant difference in the odds of isolation of *M. avium* subsp. *paratuberculosis* between media with ampicil-

TABLE 4. Growth index and means of identification of *M. avium* subsp. *paratuberculosis* in 4,619 pooled fecal cultures in group 3

Growth index	Corresponding PCR result	No. of samples with indicated result determined with Bactec medium	No. of positive samples determined with subculture with 7H10+MJ solid medium
Positive	Positive from 1st sample	401 (8)[a]	364
	Positive from 2nd sample	19 (3)[a]	14
	Inconclusive	0	0
	Negative	444	0
Negative	NT[b]	3,755	NT
Total positive		420	378

[a] Numbers in parentheses are numbers of samples requiring DNA purification to yield a positive result.
[b] NT, not tested.

TABLE 5. Growth and identification of *M. avium* subsp. *paratuberculosis* in fecal and tissue cultures in groups 4 and 5

Result	No. of samples with indicated result				
	Group 4			Group 5	
	Feces with:		Tissues with no ampicillin	Feces with no ampicillin	Tissues with no ampicillin
	No ampicillin	Ampicillin			
Growth index positive (PCR result)					
Positive from 1st sample	70	147	34	944	427
Positive from 2nd sample	11	1	0	38	41
Inconclusive	0	0	0	0	0
Negative	308	99	1	184	4
Growth index negative[a]	812	1,030	737	1,471	1,374
Total	1,190	1,277	772	2,599	1,805

[a] Samples that were growth index negative were not tested by PCR.

lin and medium without ampicillin after allowing for clustering due to submission.

Group 5. Fecal samples collected antemortem and intestinal tissues collected at necropsy were cultured at laboratory B. There were 944 positive fecal cultures and 427 positive tissue cultures (Table 5).The mean rate of isolation of irrelevant microorganisms from feces was 7.1% of 2,599 fecal samples (95% confidence limits, 6.1 to 8.1%). However, there was substantial clustering within trials and submissions. For example, the lowest rate for trials with more than 100 samples was 1.7%, and the highest was 11.0%.

Requirement for multiple PCR tests in groups 4 and 5. Only 4.1% of 1,211 culture-positive fecal samples required more than one broth sample to be examined by PCR in order to obtain a positive outcome, but this was required for 8.7% of 505 positive tissue cultures. In the case of tissues, this was generally because of a desire to obtain additional PCR products to complete REA; only 3.2% failed to react in the first PCR.

Tissue cultures from groups 4 and 5. The rate of isolation of irrelevant microorganisms from tissue cultures was substantially lower than that from feces. Only 1 of 772 tissue cultures in group 4 and 4 of 1,805 tissue cultures in group 5 had evidence of growth of other microbes. The overall rate of isolation of irrelevant microorganisms from tissue cultures was <0.2% (95% confidence limits, 0.063 to 0.45%).

Mycobactin dependence, HEYM, and LJ medium. In groups 1, 2, and 3, the lack of growth on 7H10 medium without MJ confirmed mycobactin dependence for each sample where *M. avium* subsp. *paratuberculosis* cells grew on 7H10+MJ. None of these samples yielded colonies of *M. avium* subsp. *paratuberculosis* that were visible on HEYM with MJ, HEYM without MJ, or LJ medium.

Confirmation of IS*900* PCR results using REA. No samples were positive by PCR but negative by REA.

DISCUSSION

There are two predominant types of strains of *M. avium* subsp. *paratuberculosis*, S and C, and both occur worldwide. The strains that are epidemiologically associated with Johne's disease in sheep in Australasia (S strains) have extraordinary cultural requirements that have limited the study of their geographical distribution compared to the more easily grown C strains that are associated with cattle and other species (7, 17, 44). Unlike the C strains, the S strains do not grow readily on HEYM but can be grown in modified Middelbrook 7H9 broth with added egg yolk and MJ. Middlebrook 7H9 is the base for Bactec 12B medium. Related media such as Middlebrook 7H10 agar or 7H11 agar are also suitable for routine culture provided that egg yolk and MJ are added (44). Bactec 12B medium is now commonly used for the culture of the S strains. While culture is expensive, approximately AU$100 per sample, for the cost-effective diagnosis of paratuberculosis at the flock level, the feces from 50 sheep can be pooled in a single culture (43). The validation experiments for pooled fecal culture (43) involved a protocol where both modified Bactec 12B and 7H10 agar were used for primary culture, and all the samples with growth in Bactec 12B were tested by IS*900* PCR and were subcultured onto modified 7H10 agar. In the event that PCR analysis of Bactec broth with growth was negative, up to two additional samples from Bactec broth were tested by PCR to confirm the presence of *M. avium* subsp. *paratuberculosis*. In the event that PCR was negative, the extract of DNA from the Bactec vial was purified over a silica column and retested. It was unclear whether all of these steps were justified in terms of sensitivity, specificity, and cost. For this reason, the values of various steps in the protocol were assessed in subsequent trials, and the results are reported here.

In an attempt to maximize the diagnostic sensitivity of culture of *M. avium* subsp. *paratuberculosis*, primary isolation using both modified Bactec 12B medium and modified 7H10 agar was undertaken in groups 1 and 2. However, only a few extra positive samples (4 out of 202) were detected by inclusion of 7H10 agar, and only 34% of Bactec-positive samples were positive on primary 7H10 agar in group 1. This study has confirmed unequivocally that the analytical sensitivity of 7H10 agar is lower than that of Bactec 12B medium. This would lead to a lower diagnostic sensitivity if solid medium alone was used for primary culture. While subculturing of growth index-positive Bactec cultures on 7H10 agar provided additional assurance that the Bactec growth index was due to *M. avium* subsp. *paratuberculosis*, no additional samples were found to be positive through subculture from Bactec 12B medium to 7H10 agar. Thus, there is minimal practical benefit in the use of the solid medium. Even in the absence of radiometric technology, a better option than the use of solid media may be culturing in modified Middlebrook 7H9 broth and conducting PCR after a set incubation period of, for example, 10 to 12 weeks; this approach would be costly in terms of molecular confirmation. Those few cultures that were positive by primary 7H10 medium but negative by Bactec culture may represent samples containing low numbers or clumps of *M. avium* subsp. *paratuberculosis* cells. In both cases, there is an unequal likelihood that randomly selected aliquots of an inoculum would contain *M. avium* subsp. *paratuberculosis* cells.

The solid media HEYM with MJ, HEYM without MJ, and LJ did not support the development of visible colonies of *M. avium* subsp. *paratuberculosis* after enrichment culture in Bactec medium from any of the 149 samples from which the organism grew on modified 7H10 agar; this supports the findings reported in a previous study (44).

When a growth signal is detected in the Bactec or any other liquid culture system, additional tests must be performed in order to confirm the presence of *M. avium* subsp. *paratuberculosis*. Thus, the cost of culture is significantly increased by the presence of irrelevant microorganisms that cause a growth signal. Furthermore, the chance of detection of *M. avium* subsp. *paratuberculosis* can be reduced in the presence of other organisms. Contamination was found to interfere with PCR for IS*900*, requiring that DNA samples be further purified on a silica column prior to retesting by PCR. Bactec cultures containing irrelevant bacteria were more than twice as likely to require the purification of DNA in order to obtain a positive result by IS*900* PCR. This may have been due to by-products of the irrelevant bacteria or due to competition from excess DNA in the reaction mixture. Provided that serial PCR tests were performed on purified DNA, it was possible to detect *M. avium* subsp. *paratuberculosis* in the presence of irrelevant microorganisms in this study. Similarly, contaminants obscured or inhibited the growth of *M. avium* subsp. *paratuberculosis* cells on

solid media, even when contaminant growth was subconfluent. *M. avium* subsp. *paratuberculosis* could still be detected on the agar surface by PCR in the absence of visible *M. avium* subsp. *paratuberculosis* colonies. These results are consistent with those of an earlier study using bovine feces in which agar enrichment culture prior to PCR led to the identification of samples containing *M. avium* subsp. *paratuberculosis* cells by PCR where no *M. avium* subsp. *paratuberculosis* colonies were visible on slopes (29).

Contamination of cultures by irrelevant microorganisms was highly clustered within submissions and often within farms, which is suggestive of seasonal and environmental factors such as the type of ration fed to livestock, which may influence the microbial flora in feces.

Given the need for PCR tests to rule out the presence of *M. avium* subsp. *paratuberculosis* when irrelevant microorganisms are cultured, the inclusion of additional antibiotics such as ampicillin in Bactec medium may be warranted. In this study, the inclusion of ampicillin was shown to significantly reduce the odds of contamination without affecting the odds of isolation of *M. avium* subsp. *paratuberculosis* from ovine feces. These data extend the findings of a pilot study (15). A wide range of antibiotics have been included in culture media for *M. avium* subsp. *paratuberculosis*. However, it cannot be assumed that all strains of *M. avium* subsp. *paratuberculosis* have similar patterns of antimicrobial resistance, and each combination of antibiotics will need to be evaluated carefully with both S and C strains of *M. avium* subsp. *paratuberculosis.* For example, vancomycin is less inhibitory to C strains than it is to S strains (15).

The contamination rate for fecal culture observed using the final protocol over 7 years in this study was about 7%. This is the proportion of fecal cultures that contained organisms other than *M. avium* subsp. *paratuberculosis*. Comparisons of published data on contamination rates for mycobacterial culture are confounded by a lack of consistency in the definition of contamination, for example, mixed culture with the target organism, light growth of irrelevant organisms, or overgrowth of irrelevant organisms, and also by the many variations reported in decontamination methods and culture media. However, contamination rates for culture on solid media in human clinical mycobacteriology were reviewed and ranged from 0.4 to 41% (39). Contamination rates for fecal samples for *M. avium* subsp. *paratuberculosis* cultured on HEYM ranged from 26% for a sedimentation method to 60% for a centrifugation method in one study (20), while in another study, total contamination rendering cultures useless occurred at a rate of 13% to 14% among 2,513 bovine fecal cultures on HEYM and LJ medium (26). Using the Whitlock version of the sedimentation method and HEYM, there was an outgrowth of contaminants in 30% of 463 bovine fecal cultures (29). In another study of 2,989 bovine fecal samples using the Jorgensen method, 7% of approximately 12,000 LJ slopes were contaminated, but only 0.13% of fecal samples were lost due to the contamination of all four replicate slopes (19). Contamination rates of bovine feces in Bactec were 35% to 44%, and those for camelid feces were 10% to 16% after overnight incubation and sedimentation in HPC (4). In a study of 179 bovine fecal samples using cultural methods similar to those reported in the present study, 11% of cultures were contaminated (9). Thus, the reported contamination rates for fecal cultures for *M. avium* subsp. *paratuberculosis* culture are extremely variable, and there may still be a need for improved culture protocols.

Based on assessments of the impact of some variables on the contamination rate in groups 1 and 2, it was possible to improve the method that had been used up to 1999. This method was based on the double-incubation centrifugation method developed at Cornell University first described by Shin (31) and later modified by Whitlock et al. (42). The main factors leading to a high contamination rate when using the double-incubation method were the inclusion of fecal sediment in the culture after the initial sedimentation step (floating particles should also be avoided) and inadequate contact time with the antibiotic solution VAN during the second incubation. The initial sedimentation procedure must be closely monitored, and the type of disposable tube is important. Efforts must be made to minimize disturbances of the first fecal sediment if contamination of cultures is to be avoided. The amount of feces cultured also needs to be standardized, as other researchers have shown previously that it can have an impact on the contamination rate (34). Vancomycin, a component of VAN, is potentially inhibitory to the S strain of *M. avium* subsp. *paratuberculosis* (15), but the impact of too little incubation time in VAN on contamination outweighs this consideration. Logistic regression analysis indicated that there was no additional detrimental effect of VAN on the odds of isolation of *M. avium* subsp. *paratuberculosis* due to an extra day of incubation in this mixture but a considerable penalty in contamination for the shorter period. Apart from these specific points, the handling of samples can be quite flexible, with shipping times and storage of samples for short periods at 4°C or for longer periods at −80°C having no detectable effect on the odds of isolation of *M. avium* subsp. *paratuberculosis* or the odds of contamination. However, common sense would dictate that times of sample transit to the laboratory should be minimized, as samples can be exposed to uncontrolled environmental conditions.

The contamination rate for intestinal tissues and associated lymph nodes in this study was negligible, which is consistent with previously reported results of studies using other culture protocols (33, 37). The decontamination practice for tissues in the present study involved a prolonged incubation in HPC, which was shown not to be deleterious to the S strain of *M. avium* subsp. *paratuberculosis* (27). These types of samples have an inherently lower microbial burden than feces.

In summary, liquid culture of S strains of *M. avium* subsp. *paratuberculosis* from sheep was far more sensitive than culture on optimized solid medium of similar composition and should replace solid medium if the objective is to maximize diagnostic sensitivity. A question arising from this study is the extent to which the findings can be applied to cultures of C strains of *M. avium* subsp. *paratuberculosis.* In brief, the ability of HEYM and other solid media to support their growth is well established, but there is some evidence that culture in Bactec medium is more sensitive (9); in one small study, growth in Bactec medium was confirmed equally well by subculture for mycobactin dependency or by IS*900* PCR (9). The factors that affect the identification of growth of *M. avium* subsp. *paratuberculosis* cells in liquid media by PCR or subculture would not be expected to differ between strains. Precautions must be taken to minimize the rate of contamination by irrelevant microorgan-

isms, as this can reduce the diagnostic sensitivity of culture and increase the complexity and cost of confirming the presence of *M. avium* subsp. *paratuberculosis*.

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