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Evaluation of Modified BACTEC 12B Radiometric Medium and Solid Media for Culture of *Mycobacterium avium* subsp. *paratuberculosis* from Sheep

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Definitive diagnosis of Johne's disease in ruminants depends on confirming the presence of the causative bacterium, *Mycobacterium avium* subsp. *paratuberculosis*, in tissues of the host. This is readily achieved in most ruminant species by culture. However, culture of clinical specimens from sheep in many countries has been unrewarding. Such a culture from sheep was achieved recently in Australia by using a radiometric culture medium. The aims of the present study were to evaluate the culture of *M. avium* subsp. *paratuberculosis* from sheep by using modified BACTEC 12B radiometric medium, to determine the sensitivity of culture in relation to histopathology, and to evaluate a range of solid media. Culture of *M. avium* subsp. *paratuberculosis* from sheep with Johne's disease is a sensitive method of diagnosis: intestinal tissues from all 43 animals with multibacillary disease and all 22 animals with paucibacillary disease were culture positive, while 98% of feces from 53 animals with multibacillary disease and 48% of feces from 31 animals with paucibacillary disease were culture positive. Of sheep without histological evidence of Johne's disease from infected flocks, intestinal tissue from 32% of 41 were culture positive, while feces from 17% of 41 were culture positive. Consequently, culture is recommended as the "gold standard" test for detection of ovine Johne's disease. Of the wide range of solid media that were evaluated, only modified Middlebrook 7H10 and 7H11 agars, which were very similar in composition to modified BACTEC 12B medium, yielded growth of ovine strains of *M. avium* subsp. *paratuberculosis*. The sensitivity of detection of *M. avium* subsp. *paratuberculosis* on solid media was slightly lower than that in modified BACTEC 12B radiometric medium. Both egg yolk and mycobactin J were essential additives for growth of ovine strains of *M. avium* subsp. *paratuberculosis* in both liquid and solid media.

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of Johne's disease or paratuberculosis, a granulomatous enteropathy of economic importance in ruminants throughout the world. Johne's disease is a chronic infection; in the end stages of the disease, large numbers of *M. avium* subsp. *paratuberculosis* organisms may be shed in feces, thereby contaminating the pasture and providing opportunity for transmission of infection to other hosts. Control of Johne's disease is achieved by identification and culling of infected hosts and sometimes entire herds or flocks. Diagnosis is based on serology and pathology, with culture of *M. avium* subsp. *paratuberculosis* from affected tissues being the definitive test. Culture from feces is also used to confirm infection in live animals.

For more than 2 decades a dramatic difference has been noted in most countries in the ease of culture of *M. avium* subsp. *paratuberculosis* from cattle and other ruminants compared to sheep. In Australia, culture from sheep has been largely unsuccessful. Similarly, in the United Kingdom, New Zealand, United States of America, Morocco, South Africa, and Iceland, isolation rates from sheep have been very low, or very lengthy incubations have been required to demonstrate scanty growth (Table 1). The existence of different strains of *M. avium* subsp. *paratuberculosis* in sheep and cattle might explain the above observations (20); it is already known that cattle and sheep tend to be infected with different genotypic variants of *M. avium* subsp. *paratuberculosis* (8). Complicating this interpretation, researchers in India, Iran, Spain, and China have reported reasonable success in culturing *M. avium* subsp. *paratuberculosis* from sheep (Table 1). This suggests that the reported differences in the culturability of strains of *M. avium* subsp. *paratuberculosis* from sheep might also be due to methodological differences between laboratories. Disinfection protocols and type of media are both known to influence in vitro survival and growth of *M. avium* subsp. *paratuberculosis* (35) and other mycobacteria (29). In one Indian study, hexadecylpyridinium chloride disinfection and culture on Herrold's egg yolk medium resulted in a 3.7% isolation rate (33), while in another Indian study, benzalkonium chloride disinfection and glycerol serum agar resulted in a 57% isolation rate (22). In Spain, *M. avium* subsp. *paratuberculosis* from sheep produced minute colonies that required a stereo microscope for visualization on Lowenstein-Jensen medium after up to 40 weeks of incubation (1, 20), whereas growth on Middlebrook 7H11 medium was read easily with the naked eye within 16 weeks (1).

Culture of *M. avium* subsp. *paratuberculosis* offers many advantages over other tests for confirmation of Johne's disease. It is a definitive test, it is cheaper than pathology-based confirmation, and it can be used to advantage in large-scale investigations. Recently we found that it was possible to culture *M. avium* subsp. *paratuberculosis* from tissues and feces of sheep after disinfection in hexadecylpyridinium chloride by using modified Middlebrook 7H9 radiometric medium (38). The aims of the present study were to evaluate this cultural method on a greater number of samples, to determine the sensitivity of culture in relation to the present "gold standard" test (histo-

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TABLE 1. Studies in which culture of *M. avium* subsp. *paratuberculosis* from sheep has been attempted

Facility and country of culture[a]	Sample(s)	Medium(a)	Sensitivity	Time	Reference
Difficulty					
United Kingdom	Lymph nodes	Modified Dubos	50 CFU/g	NA[b]	6
United Kingdom	Gut mucosa	Modified Dubos-actidione-chloramphenicol	75 CFU/ft of intestine	NA	6
New Zealand	Feces	Herrold's	5 of 47 infected sheep; 1 to 15 colonies	6 mo	9
United States	Tissues, feces	Herrold's, Lowenstein-Jensen	3 of 20 infected sheep	6 mo	32
United Kingdom	Tissues	Egg based	Tiny colonies after 7 mo in 3 of 12 tubes	7 mo	15
Australia[c]	Mucosa	Herrold's, Lowenstein-Jensen	1 of 3 infected ewes after 12 wk	5 mo	31
Australia[c]	Mucosa, lymph node, feces	Egg based	4 of 50 infected sheep	5 mo	7
Australia[d]	Mucosa	Herrold's	0 of 1 infected sheep	9 mo	25
Morocco	Feces	Lowenstein-Jensen–mycobactin	2 of 10 infected ewes	16 wk	5
South Africa	Feces, tissues	Herrold's	2% of 59 infected sheep	NA	18
Iceland	Mucosa, lymph nodes	Finlayson's, modified Dubos, Herrold's	9 to 54% of 22 infected samples	8 mo	17
Spain	Feces, tissues	Herrold's, Lowenstein-Jensen	7 and 93% of 41 infected samples, respectively but colonies minute	4 to 8 mo	20
Ease					
India	Feces	Serum agar	57% of 100 infected sheep	NA	22
Iran	Feces, tissues	Finlayson-Taylor, Lowenstein-Jensen	75% (sheep and goats not differentiated)	NA	3
China	NA	Potato broth	60% of 38 infected sheep	NA	14
Spain	Feces, tissues	Lowenstein-Jensen, Middlebrook 7H11-OADC with or without mycobactin J	86% of 25 infected sheep	4 mo	1

[a] Countries are divided with regard to whether difficulty or ease was noted or implied in obtaining results.
[b] NA, not available.
[c] New South Wales.
[d] Victoria.

pathology), and to evaluate a range of solid media for the culture of strains of *M. avium* subsp. *paratuberculosis* that infect sheep in Australia.

MATERIALS AND METHODS

Clinical samples. Feces, mesenteric lymph nodes, and intestinal tissues were collected from sheep known or suspected to have Johne's disease. The status of all animals was evaluated by histological examination of terminal ileum and three other sites of ileum 2 m apart, cecum (one site), proximal colon (one site), and caudal mesenteric lymph node (one site). Feces were collected from the rectum of animals on the farm or during postmortem examination. Feces and tissues for culture were stored at 4°C overnight and then, if required, at −20 or −80°C pending examinations.

Acid-fast stained smears and histopathology. Smears were prepared from feces, scrapings of intestinal mucosa, and cultures; dried in an oven at 65°C: and stained by a Ziehl-Neelsen technique (13). Tissues were fixed in 10% buffered neutral formalin at the time of necropsy, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin and by a Ziehl-Neelsen method (23). Sheep with granulomatous enteritis were defined as having multibacillary Johne's disease if there were numerous acid-fast bacilli (AFB) in at least some high-power fields and as having paucibacillary Johne's disease when there were few or no AFB in any high-power field.

Tissue and fecal samples. (i) Group A. Sheep were selected based on clinical, serological, and/or pathological findings. Individual samples of ileum, or occasionally jejunum, from a group ($n = 36$) representing 34 different farms from New South Wales, Australia, were stored at −80°C for up to 14 months, thawed at 37°C, and then prepared for culture as described below. Twenty-two of these sheep had histological evidence of Johne's disease. Individual fecal samples from a different group ($n = 29$), representing nine different farms from the central tablelands district of New South Wales, were stored at −80°C for up to 11 months ($n = 8$) or for 4 days ($n = 20$) or at −20°C for 3 months ($n = 1$), thawed at 37°C, and then prepared for culture as described below. Twenty-six of these sheep had histological evidence of Johne's disease.

(ii) Group B. Sheep ($n = 30$) were selected based on knowledge of their histological classification. Samples of feces and ileum from each of 10 sheep with multibacillary Johne's disease, representing five different farms from New South Wales, and feces and tissues from each of 20 sheep with paucibacillary Johne's disease, representing the same five farms and another four farms, were cultured. These samples, which had been stored at −80°C for 1 to 4 months, were thawed at 37°C and then prepared for culture as described below.

(iii) Group C. Sheep ($n = 31$) from a farm with a high prevalence of Johne's disease were selected because of low body condition score. The farm was located in the southern tablelands district of New South Wales. Feces were collected and stored at −80°C for several weeks. There were 11 sheep with no intestinal lesions, 6 with paucibacillary Johne's disease, and 14 with multibacillary Johne's disease.

(iv) Group D. Sheep ($n = 40$) were selected from two farms based on their reaction in a serological test for Johne's disease. The two farms, located in the central tablelands district of New South Wales, were suspected to have sheep with Johne's disease. Twenty sheep were chosen from each farm on the basis of their having the highest apparent levels of anti-*M. avium* subsp. *paratuberculosis* antibodies as determined by an absorbed enzyme-linked immunosorbent assay. Terminal ileum and feces were collected from each sheep and stored at −80°C for up to 2 weeks before culture. Thirteen sheep, all from one farm, had histological evidence of Johne's disease; 11 had multibacillary disease while 2 had paucibacillary disease.

Preparation of samples and culture methods. The double-incubation method of Whitlock and Rosenberger (36) was used to prepare feces. Briefly, 2 to 5 g of feces was placed in a 15-ml polypropylene tube containing a swab stick. The 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 30 min at room temperature. A 5-ml aliquot of the surface fluid was transferred to a 35-ml polystyrene tube containing 25 ml of 0.9% hexadecylpyridinium chloride (Sigma Chemical Co., St. Louis, Mo.) in half-strength brain heart infusion broth (Oxoid, Basingstoke, England) and allowed to stand at 37°C for 24 h. The tube was centrifuged at 900 × *g* for 30 min. The pellet was collected; resuspended in 1 ml of sterile water with vancomycin (100 μg/ml), nalidixic acid (100 μg/ml), and amphotericin B (50 μg/ml) (all Sigma reagents); and incubated for 48 to 72 h at 37°C.

Intestinal wall specimens, each approximately 5 g, were trimmed of fat and fibrous tissue, cut into small pieces, and homogenized for 30 s in 2 ml of sterile normal saline in a blender. After adding 25 ml of 0.75% hexadecylpyridinium chloride, the homogenates were left standing at room temperature for 48 to 72 hours. The sediment from the base of the tube was collected.

For culture in liquid radiometric medium, 0.1 ml of the prepared fecal or tissue sediment was inoculated into each culture vial. Vials were incubated at 36 to 37°C for 8 weeks. The growth index (GI) was determined weekly by using an

TABLE 2. Composition of Middlebrook media used to culture strains of *M. avium* subsp. *paratuberculosis* from sheep in this study

Ingredient	Amt/liter in:		
	Modified BACTEC 12B[a]	Modified 7H10 agar[b]	Modified 7H11 agar[c]
For base medium			
Casein digest	667 mg[e]	800 mg[d]	800 mg
Ammonium sulfate	333 mg	400 mg	400 mg
Monopotassium phosphate	667 mg	1.2 g	1.2 g
Disodium phosphate	1.7 g	1.2 g	1.2 g
Sodium citrate	67 mg	320 mg	320 mg
Magnesium sulfate	33 mg	20 mg	40 mg
Calcium chloride	0.33 mg	0.4 mg	
Zinc sulfate	0.67 mg	0.8 mg	
Copper sulfate	0.67 mg	0.8 mg	
L-Glutamic acid	333 mg	400 mg	400 mg
Ferric ammonium citrate	27 mg	32 mg	32 mg
Pyridoxine	0.67 mg	0.8 mg	0.8 mg
Biotin	0.33 mg	0.4 mg	0.4 mg
Malachite green		0.2 mg	0.8 mg
Bacto Agar		12 g	12 g
For enrichment			
Oleic acid			40 mg
Albumin fraction V, bovine	3.3 g[e]	4.0 g	4.0 g
Dextrose		1.6 g	1.6 g
Catalase	32,000 U[e]	2.4 mg	3.2 mg
Sodium chloride			680 mg
C_{14} palmitic acid	667 μCi[e]		
As additive			
Egg yolk	167 ml	200 ml	200 ml
Mycobactin J	0.83 mg	1.0 mg	1.0 mg
PANTA PLUS[f]	33.3 ml	40 ml	40 ml

[a] Additives (and 700 μl of water) were added to the enriched BACTEC media at the following rates per 4-ml vial, resulting in a final volume of 6 ml/vial: 1 ml of egg yolk, 5 μg (100 μl of mycobactin J, and 200 μl of PANTA PLUS (4a, 13a).
[b] ADC was used for enrichment.
[c] OADC was used for enrichment.
[d] As added Casitone.
[e] Added by manufacturer to standard Middlebrook 7H9 broth to form BACTEC 12B medium.
[f] Consists of polymyxin B (1,000 U/ml), amphotericin B (100 μg/ml), nalidixic acid (400 μg/ml), trimethoprim (100 μg/ml), azlocillin (100 μg/ml), and polyoxyethylene stearate (4 mg/ml).

automatic ion chamber (BACTEC 460; Johnston Laboratories, Towson, Md.), and samples were collected from these cultures for PCR when the GI was >200.

For culture on solid medium, the prepared fecal sediment (250 μl) or tissue sediment (50 μl) was inoculated onto the surface of the medium. Tubes were incubated at 37°C for 20 weeks. Growth was determined visually at weeks 1, 2, 4, 6, 8, 9, 10, 12, 16, and 20, and colonies were harvested for examination by PCR.

Evaluation of need for additives in BACTEC 12B medium. The standard radiometric medium was based on those described by Collins et al. (10) and Cousins et al. (12) and consisted of enriched Middlebrook 7H9 medium (BACTEC 12B; Becton Dickinson, Sparks, Md.) with 200 μl of PANTA PLUS (Becton Dickinson), 1 ml of egg yolk, 5 μg of mycobactin J (Allied Monitor Inc., Fayette, Mo.) and 0.7 ml of water (Table 2). The requirement for egg, Mycobactin J, and PANTA PLUS in this medium was determined by omitting some or all of these additives; samples were from group A. Two tissue samples and two fecal samples containing high numbers of AFB were used in the first experiment. In a second experiment three fecal samples and three tissue samples containing high numbers of AFB, three fecal samples and three tissue samples containing moderate numbers of AFB, and four fecal samples and four tissue samples containing low numbers of AFB were cultured in BACTEC 12B medium either with all three additives or without any additive.

Evaluation of other media. BACTEC 12B medium consists of Middlebrook 7H9 broth with the addition of bovine serum albumin (0.5%, wt/vol), catalase (192 U per 4 ml), casein hydrolysate (0.1% wt/vol), and radiolabelled palmitic acid (Table 2). Solid media with similar composition were prepared with Middlebrook 7H10 and 7H11 agars (Difco) as the basal media (Table 2). A range of other media were selected either because they have been reported to support the growth of *M. avium* subsp. *paratuberculosis* from sheep or were selected empirically from media that have been used for culture of a diverse range of mycobacterial species.

In a preliminary experiment to evaluate the possibility of growth on solid medium, liquid Noble agar solution (Difco) at 58°C was added to warmed BACTEC 12B medium, after adding PANTA PLUS, egg yolk, and mycobactin J, to result in a final concentration of 2% (wt/vol) agar. After allowing the medium to cool to form a slope, the surface was inoculated with 0.1 ml of sample. Samples were taken from 10 primary BACTEC culture vials with GIs of ≥999; these had been inoculated with tissues from group A.

In a second experiment, inocula prepared from two fecal samples and two tissue samples from animals with multibacillary disease (group A) were cultured on a range of solid media: (i) Middlebrook 7H10 agar base (Difco) with Casitone (0.08%, wt/vol), albumin-dextrose-catalase (ADC) (8%, vol/vol) (Difco), and mycobactin J (1 mg/liter) with or without egg yolk (20%) and/or PANTA PLUS (4%, wt/vol); (ii) Herrold's egg yolk medium containing eight egg yolks/liter and mycobactin J (2 mg/liter) (2) and Herrold's egg yolk medium containing eight egg yolks/liter with or without pyruvate (0.41%, wt/vol) (Difco) and/or mycobactin J (2 mg/liter) or mycobactin P (2 mg/liter) (Allied Monitor Inc.); (iii) Watson Reid medium (26) with or without pyruvate (0.41%, wt/vol) and/or mycobactin J (2 mg/liter) or mycobactin P (2 mg/liter); (iv) charcoal agar (19); (v) American Trudeau Society medium without potato flour (34); (vi) pyruvic acid egg medium (24); (vii) Lowenstein-Jensen agar (21); (viii) Dorset Henley agar (26) with or without mycobactin J (2 mg/liter); (ix) Petragnini agar (28); (x) egg yolk agar (26); (xi) Dorset egg agar (26); (xii) serum agar (26); (xiii) Dubos solid medium (1.5% [wt/vol] Noble agar) (26); and (xiv) Finlayson's medium (16). All media were prepared in 35-ml screw-cap polystyrene Macartney tubes containing 10 ml of medium as a slope. Methylene blue (0.02%, wt/vol) was added to 7H10 media in an attempt to make colonies more easily visible. Malachite green was added to the 7H10 medium to a final concentration equivalent to that in Herrold's egg yolk medium (0.001%, wt/vol) to see whether growth was inhibited.

In a third experiment, media based on Middlebrook 7H10 and 7H11 agars were further evaluated with inocula from the samples in group B. The media were (i) 7H10 agar with Casitone (0.08%, wt/vol), ADC (8%, vol/vol), PANTA PLUS (4%, vol/vol), egg yolk (20%, vol/vol), and mycobactin J (1 mg/ml) and (ii) 7H11 agar with PANTA PLUS (5%, vol/vol), oleic acid albumin-dextrose-catalase (OADC) (8% vol/vol) (Difco), and mycobactin J (1 mg/liter) with or without egg yolk (20%, vol/vol) (Table 2).

The requirement for mycobactin J in 7H10 medium was evaluated in a fourth experiment. Two aliquots of 0.1 ml were removed from each of 14 modified BACTEC 12B medium cultures with a GI of >999 from earlier experiments with samples from group A. One aliquot was inoculated onto 7H10 agar with Casitone (0.08%, wt/vol), ADC (8%, vol/vol), PANTA PLUS (4%, vol/vol), egg yolk (20%, vol/vol), and mycobactin J (1 mg/ml) while the other aliquot was inoculated onto the same medium without mycobactin J. The slopes were incubated at 37°C for 8 weeks.

Preparation of BACTEC samples and colonies for PCR. The preparation of BACTEC samples and colonies was conducted as previously described (38). Briefly, the rubber stopper-lid of the radiometric culture vial was disinfected with 70% ethanol, the contents were mixed by inverting the tube, and 200 μl of medium was removed and transferred to a microcentrifuge tube. Absolute ethanol (500 μl) was added, and the tube was left to stand for 2 min and then was vortexed for 5 s and centrifuged at $8 \times g$ for 10 min at 22°C. The supernatant was transferred to a clean tube and then centrifuged at $18,000 \times g$ for 5 min. The resulting bacterial pellet was washed twice in 200 μl of sterile phosphate-buffered saline, resuspended in 50 μl of sterile distilled water, and lysed at 100°C for 20 min. A 5-μl aliquot of the lysate was added to each PCR mixture. The lysate was then stored at −20°C. If PCR results were negative, lysates (45 μl) were thawed and DNA was purified from the entire lysate by binding to silica in a column using 6 M guanidine thiocyanate according to the manufacturer's instructions (Wizard PCR Preps DNA purification system; Promega Corporation, Madison, Wis.) and 5 μl of purified DNA solution was added to a second PCR mixture. For solid media, a crude suspension of DNA was obtained by suspending a colony in distilled water, washing the cells three times in water, suspending the cells in 100 μl of water, and boiling the washed cells for 20 min.

IS*900* PCR. A reaction volume of 50 μl containing 5 μl of the DNA sample; 250 ng of each of the *M. avium* subsp. *paratuberculosis* IS*900* primers, P90 (5′GAA GGG TGT TCG GGG CCG TCG CTT AGG) and P91 (5′GGC GTT GAG GTC GAT CGC CCA CGT GAC) (27); 200 μM concentrations of each of the nucleotides dATP, dTTP, dGTP, and dCTP; 66.8 mM Tris-HCl; 16.6 mM $(NH_4)_2SO_4$; 2.5 mM $MgCl_2$; 1.65 mg of bovine serum albumin per ml; 10 mM β-mercaptoethanol; and 2 U of *Taq* polymerase, in buffer (10 mM Tris-HCl, 0.1 mM EDTA [pH 8.8]) was used. Amplification was undertaken in 200-μl tubes in a 96-place thermal cycler (Corbett Research, Sydney, Australia) under the following conditions: one cycle of denaturation at 94°C for 2 min followed by 37 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 15 s, and extension at 72°C for 1 min. Products of approximately 400 bp were predicted and evaluated by electrophoresis at 94 V for 45 min in 2% agarose gels stained with ethidium bromide. The specificity of the reaction for IS*900* was confirmed during optimization experiments by Southern hybridization with an internal probe and then routinely by restriction endonuclease analysis of the PCR product with *Alw*I.

TABLE 3. Culture of ovine tissues and feces in modified BACTEC 12B radiometric medium[a]

Histological grade	Tissue samples		Fecal samples	
	n	No. positive	*n*	No. positive
No lesions	14	5	3	0
Paucibacillary	4	4	7	3
Multibacillary	18	18	19	19
Total	36	27	29	22

[a] Group A. Sheep were selected from numerous farms based on clinical, pathological, and serological criteria. Tissues and feces were not from the same sheep. Data are the number of sheep with a positive radiometric GI and IS*900* PCR result.

RESULTS

Evaluation of modified BACTEC 12B radiometric medium. Samples of tissues and feces (group A) from sheep from numerous farms were evaluated to ensure that culturability was not restricted to unrepresentative strains of *M. avium* subsp. *paratuberculosis*. A GI of >999 was recorded from 27 of 36 (75%) tissue cultures after 2 to 6 weeks of incubation and from 22 of 29 (76%) fecal cultures after 3 to 6 weeks of incubation (Table 3). Culture from feces of sheep with paucibacillary disease appeared to be less efficient than that from sheep with multibacillary disease, and histopathology appeared not to detect all infected individuals from affected farms (Table 3). These findings were confirmed in experiments with samples from groups B, C, and D, which comprised sheep selected for culture based on histological, clinical, and serological criteria, respectively.

From the sheep with multibacillary disease in Group B, all 14 tissues produced GIs of ≥999 within 1 to 4 weeks, while 13 of 14 feces produced GIs of ≥999 within 3 to 5 weeks (Table 4). From the 16 sheep with paucibacillary disease in group B, all tissues produced GIs of ≥999 within 1 to 6 weeks, while 9 (56%) feces produced GIs of ≥999 within 5 to 7 weeks after inoculation. The remaining feces were GI-negative after 8 weeks. All of the sheep with positive fecal cultures also had positive tissue cultures.

Similar results were obtained with the fecal samples from group C; all 14 samples from sheep with multibacillary disease produced GIs of >999 within 3 to 7 weeks while 2 (33%) samples from sheep with paucibacillary disease and those from 3 (27%) sheep that lacked histological evidence of Johne's disease produced GIs of >999 within 4 to 8 weeks (Table 5).

From the 40 sheep in group D, 53% of tissues and 40% of feces produced GIs of >999 within 3 to 6 and 1 to 7 weeks, respectively (Table 6). Tissues from all 13 sheep with multibacillary and paucibacillary disease were culture positive, while feces from 12 of these sheep were culture positive. In addition, tissues from eight (30%) sheep that lacked histological lesions consistent with Johne's disease were culture positive while feces from four (15%) of these sheep were also culture positive. None of the 20 sheep on one farm had histological lesions consistent with Johne's disease, but *M. avium* subsp. *paratuberculosis* was isolated from tissues and feces of three of these sheep. All of the sheep with positive fecal cultures also had positive tissue cultures.

All cultures that developed a GI in the above-mentioned studies were IS*900* PCR positive. When the results of these experiments are combined (Table 7), culture of intestinal tissues was successful for all sheep with Johne's disease regardless of histological grade. Culture of feces was successful for 98.3% of 58 sheep with multibacillary disease, compared to 48.4% of 31 sheep with paucibacillary disease (chi-square = 32.2; $P < 0.00001$). For animals without histological evidence of Johne's disease that were selected from flocks known to be infected, intestinal tissues from 31.7% of 41 were culture positive while feces from 17.1% of 41 were culture positive. These data indicate that culture of intestinal tissues and/or feces is a more sensitive diagnostic method than histopathology for detection of *M. avium* subsp. *paratuberculosis* infection in sheep.

Requirement for mycobactin J, egg yolk, and PANTA PLUS in BACTEC 12B medium. In the first experiment, for which all three additives were included, all tissue and fecal cultures produced GIs of ≥999 within 4 to 5 weeks, and the GIs remained high in later weeks. Similar results were obtained without PANTA PLUS. In the absence of mycobactin J, egg yolk, or any additive, neither fecal culture developed a GI while both tissue cultures developed a low GI, which peaked at week 1 to 2 and then declined. In the second experiment, a GI of ≥999 occurred in 8 of the 10 fecal cultures by week 6 if all additives were present, but growth did not occur in any fecal culture in the absence of the additives. A GI of ≥999 was present in all 10 tissue cultures by week 7 in the presence of the three additives, while no growth was recorded for 7 of these cultures in the absence of the additives. The other three cultures had very low GIs (12 to 74) at week 3, which then declined. Samples with poor growth included those that contained high or medium numbers of AFB. In all cases GI was associated with positive PCR results. It was concluded that both egg yolk and mycobactin J were essential for isolation of *M. avium* subsp. *paratuberculosis* from feces and dramatically improved the growth from tissues.

TABLE 4. Culture of ovine tissues and feces in modified BACTEC 12B radiometric medium and on modified Middlebrook agar slopes[a]

Histological grade	*n*	Modified BACTEC 12B		Modified Middlebrook agar					
				No. of tissue samples:			No. of fecal samples:		
		No. of positive tissue samples	No. of positive fecal samples	Positive on 7H10	Positive on 7H11	Contaminated[b]	Positive on 7H10	Positive on 7H11	Contaminated[b]
Paucibacillary	16	16	9	15	16	1	8	5	1
Multibacillary	14	14	13	14	12		12	11	0
Total	30	30	22	29	28	1	20	16	1

[a] Group B. The sheep came from nine farms and were selected for culture based on histological findings alone. Tissues and feces were matched from the same sheep. Data are the number of sheep with a positive radiometric GI and IS*900* PCR result or the number of sheep with growth on solid medium and a positive IS*900* PCR result.

[b] Cultures on 7H11 that were overgrown by contaminants.

TABLE 5. Culture of ovine feces in modified BACTEC 12B radiometric medium[a]

Histological grade	n	No. of positive fecal samples
No lesions	11	3
Paucibacillary	6	2
Multibacillary	14	14
Total	31	19

[a] Group C. Sheep were selected based on clinical signs and came from a single farm. Data are the number of sheep with a positive radiometric GI and IS*900* PCR result.

TABLE 7. Combined results of all trials with modified BACTEC 12B radiometric medium[a]

Histological grade	Tissue samples		Fecal samples	
	n	No. (%) positive	n	No. (%) positive
No lesions	41	13 (31.7)	41	7 (17.1)
Paucibacillary	22	22 (100)	31	15 (48.4)
Multibacillary	43	43 (100)	58	57 (98.3)

[a] Tissues and feces are not necessarily matched from the same sheep. Data are the number and percentage of sheep with a positive radiometric GI and IS*900* PCR result.

Evaluation of solid media. In the first experiment modified BACTEC 12B medium was converted to a solid medium slope by the addition of agar. Two weeks after inoculation a GI of ≥999 was recorded in all 10 vials that had been inoculated with medium from primary BACTEC 12B cultures, and after 4 weeks all 10 slopes were covered with numerous small (diameter, <1 mm), white, circular, raised, convex, shiny colonies. Colony diameters were ≤1 mm at 6 weeks and did not change in appearance thereafter. A colony from each vial was confirmed to be *M. avium* subsp. *paratuberculosis* by IS*900* PCR. This experiment confirmed the potential for growth on solid medium and revealed colony morphology, but the high cost of BACTEC medium would preclude its use in this manner.

Of the solid media evaluated in the second experiment, growth was detected only on Middlebrook 7H10-based media. Visible colonies developed for all samples on all media with a Middlebrook 7H10 agar base with Casitone, ADC, and mycobactin J, provided that egg yolk was included. The inclusion of PANTA PLUS did not appear to influence growth. Colonies appeared at 4 weeks and did not change in appearance after 6 weeks. At 6 weeks, colonies which were well separated had diameters of ≤1 mm and were white, circular, shiny, raised, and convex, but over most of the inoculated surface there were innumerable tiny colonies. Isolation of *M. avium* subsp. *paratuberculosis* was confirmed in each case by IS*900* PCR using a colony. Addition of methylene blue to the 7H10-based medium resulted in the colonies becoming deep blue by 6 weeks. Addition of malachite green to 7H10-based medium to a final concentration equivalent to that in Herrold's egg yolk medium did not appear to inhibit growth.

Using the larger number of samples in the third experiment, modified Middlebrook 7H10 agar was compared with modified Middlebrook 7H11 agar. As with 7H10-based media, egg yolk was required for growth on 7H11-based media. Similar results were obtained for the two media with egg yolk, although slightly fewer positive cultures were obtained with 7H11 (Table 4). On modified 7H10 medium all but one of the negative fecal cultures were from animals with paucibacillary disease. Radiometric culture appeared to be slightly more sensitive than culture on solid media (Table 4). Four fecal samples that were culture negative on solid media were positive in radiometric culture, although one fecal sample that was negative in radiometric culture was culture positive on both solid media. The colonies on modified 7H11 medium were identical in appearance and growth rate to those on modified 7H10 medium. For tissue cultures, colonies were first apparent at 6 weeks for samples from animals with multibacillary disease (innumerable colonies) and 6 to 8 weeks for those from animals with paucibacillary disease (25 to 200 colonies). For fecal cultures, colonies were first apparent at 6 to 8 weeks for samples from animals with multibacillary disease (innumerable colonies) and 8 to 10 weeks for samples from animals with paucibacillary disease (1 to 25 colonies).

TABLE 6. Culture of ovine tissues and feces in modified BACTEC 12B radiometric medium[a]

Histological grade	n	No. of positive tissue samples	No. of positive fecal samples
No lesions	27	8	4
Paucibacillary	2	2	1
Multibacillary	11	11	11
Total	40	21	16

[a] Group D. Twenty sheep were selected based on serology from each of two farms. Tissues and feces were matched from the same sheep. Data are the number of sheep with a positive radiometric GI and IS*900* PCR result.

Mycobactin J was an essential additive in 7H10 medium. Of the 14 subcultures made from modified BACTEC 12B medium onto modified 7H10 medium with mycobactin J, growth of *M. avium* subsp. *paratuberculosis* was recorded from 10, while 2 subcultures were overgrown by contaminants and 2 subcultures had no visible growth at week 8. In contrast, on modified 7H10 medium without mycobactin J, no growth was recorded from 11 subcultures, while 3 subcultures were overgrown by contaminants at week 8.

DISCUSSION

The culture of *M. avium* subsp. *paratuberculosis* from sheep generally has been an unrewarding task (Table 1), despite the fact that the organism can be cultured readily from other ruminants with Johne's disease. The reasons for the difference in culturability are probably related to the existence of different strains of *M. avium* subsp. *paratuberculosis* in the different ruminant hosts, a feature of the biology of the organism which has been confirmed by genomic analysis (4, 8). In Australia, where *M. avium* subsp. *paratuberculosis* has been frequently isolated from cattle but almost never cultured from sheep, distinct strains of *M. avium* subsp. *paratuberculosis* are present in cattle and sheep populations (11, 37). However, the work reported here indicates that culture of *M. avium* subsp. *paratuberculosis* from sheep is possible if appropriate media are used. Three closely related basal media, namely, Middlebrook 7H9, 7H10, and 7H11, were found to support the growth of ovine strains of *M. avium* subsp. *paratuberculosis* when supplemented appropriately. Middlebrook 7H11 agar has been used with success for the culture of *M. avium* subsp. *paratuberculosis* from sheep in Spain, but egg yolk and mycobactin J were not required (1).

Culture from intestinal tissues was more sensitive than culture from feces, because feces from some sheep with pauciba-

cillary Johne's disease were culture negative. For some sheep that lacked histological evidence of Johne's disease tissues but not feces were also culture positive. Where matched radiometric cultures of feces and tissues from the same sheep were undertaken, no cultures were positive from feces and not tissues. The success of culture from feces is clearly related to the likelihood that sufficient numbers of *M. avium* subsp. *paratuberculosis* are being shed from the intestinal lesions. A similar trend was noted in a Spanish study where the success of culture from tissues was directly related to the number of AFB observed in lesions (30).

Egg yolk and mycobactin J were essential additives for isolation of ovine strains of *M. avium* subsp. *paratuberculosis* in Middlebrook 7H9 broth (as modified BACTEC 12B radiometric medium). The specific components of egg yolk that stimulate growth are not yet known. PANTA PLUS does not appear to affect the growth of *M. avium* subsp. *paratuberculosis* from sheep but might play a useful role in reducing contamination of the cultures.

Colonies of *M. avium* subsp. *paratuberculosis* were reliably obtained for the first time in Australia with solid media based on Middlebrook 7H10 and 7H11 agars, after growth on the surface of solidified BACTEC 12B radiometric medium was first demonstrated. All three media were of very similar composition, although the modified BACTEC 12B radiometric medium had lower concentrations of most ingredients, including egg yolk and mycobactin J, than did the two agar media (Table 2). Further experiments to determine optimal concentrations of these ingredients may be worthwhile.

The colonies on modified 7H10 agar developed to a maximum diameter after about 6 weeks, and their size was inhibited by crowding. The colonies were easily visualized, but they were made more obvious by including methylene blue in the medium. Detection of growth on solid medium was slower than that in modified BACTEC 12B medium and also appeared to be less sensitive. Of the animals with paucibacillary Johne's disease, 60% were identified by culture of feces in modified BACTEC 12B, compared to 50% by culture on modified 7H10 or 7H11 agar. Although this difference was not statistically significant, if proven in a larger study, it would tend to favor the use of the more expensive liquid radiometric medium. Liquid radiometric culture also appeared to be more sensitive than culture on solid medium for diagnosis of Johne's disease in cattle (38).

A range of solid media have been used to culture *M. avium* subsp. *paratuberculosis* from sheep in other countries, but none of these supported the growth of *M. avium* subsp. *paratuberculosis* from Australian sheep in this study. Although some of the media have been evaluated in Australia in the past, mostly with negative results, several isolates of *M. avium* subsp. *paratuberculosis* were obtained in New South Wales in the 1980s from Herrold's egg yolk medium cultures of sheep intestinal tissues. Some of these isolates have been shown to be bovine strains by restriction fragment length polymorphism analysis using IS*900* probes (11, 37). This observation and the fact that *M. avium* subsp. *paratuberculosis* can be cultured from sheep on a range of solid media in several countries (Table 1) suggests that there are a number of variants of *M. avium* subsp. *paratuberculosis* in sheep populations and that these have quite different cultural requirements. It is also possible that some isolates obtained from sheep in some studies are not strains of *M. avium* subsp. *paratuberculosis*. However, it would be useful to compare a range of isolates from different countries under standard conditions, because the pattern of culturability on different media might be a useful phenotypic typing method for epidemiological purposes. For example, *M. avium* subsp. *paratuberculosis* has been isolated from sheep in Spain on Herrold's egg yolk medium, Lowenstein-Jensen medium, and Middlebrook 7H11 agar without egg yolk (1, 20, 30). Only the latter medium supports the growth of *M. avium* subsp. *paratuberculosis* from sheep in Australia, and then only when egg yolk is added. Furthermore, Spanish isolates of *M. avium* subsp. *paratuberculosis* from sheep do not require mycobactin J (1) whereas those from sheep in Australia display the conventional dependence on mycobactin J. This suggests fundamental differences between the strains of *M. avium* subsp. *paratuberculosis* present in sheep in Australia and Spain.

The development of a GI in radiometric medium inoculated with samples from sheep with Johne's disease does not guarantee the presence of *M. avium* subsp. *paratuberculosis*. There are now several options for confirmation of *M. avium* subsp. *paratuberculosis* in radiometric culture medium. Detection of IS*900* by PCR on an aliquot taken from the primary culture is a rapid and effective test but is quite costly. Subculture from the primary radiometric culture to modified Middlebrook 7H10 agar with and without mycobactin J to check for the presence of colonies of appropriate morphology containing acid-fast organisms with mycobactin dependence is an alternative and cheaper method. However, a further incubation of at least 4 to 6 weeks is required, during which overgrowth by irrelevant microorganisms may be a problem. Environmental mycobacteria and other contaminants can be detected in radiometric medium by concurrent subculture to a general-purpose medium such as Lowenstein-Jensen agar. In Australia, the strain of *M. avium* subsp. *paratuberculosis* can be determined provisionally by concurrent subculture to Herrold's egg yolk medium, where no growth would be expected from typical ovine strains of *M. avium* subsp. *paratuberculosis*.

The development of a solid medium for culture of *M. avium* subsp. *paratuberculosis* from sheep in Australia has benefits other than those immediately applicable to diagnosis. It will now be possible to produce clonal isolates for antigen production for use in serological tests and vaccines and for in vivo inoculation experiments. It will also be possible to evaluate enumeration methods based on colony counts.

In conclusion, the results of this study confirm that culture of *M. avium* subsp. *paratuberculosis* from intestinal tissues and feces of sheep is a sensitive method of diagnosis of ovine Johne's disease. It is particularly notable that some sheep were detected by culture and not by histopathology of intestinal tissues, the current gold standard diagnostic test in Australia. These results support the preliminary data from an earlier study (38) and suggest that culture of *M. avium* subsp. *paratuberculosis* should be the test against which the diagnostic performance of other laboratory tests for ovine Johne's disease is assessed.

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