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Moredun Bordetella Medium, an Improved Selective Medium for Isolation of *Bordetella parapertussis*

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***Bordetella parapertussis*, previously thought to be an obligate human respiratory tract pathogen, has been isolated from sheep. Attempts to assess the prevalence of *B. parapertussis* in conventionally reared sheep by nasal swabbing proved futile with existing selective media because of extensive overgrowth with *Mucor* spp. and other nasal commensals. Moredun Bordetella Medium (MBM), which contains cycloheximide and spectinomycin at final concentrations of 0.5 mg/ml and 100 μg/ml, respectively, was developed as an improved selective medium to isolate *B. parapertussis* from the nasal cavities of conventionally reared sheep. The selective ability of MBM was evaluated with 200 nasal swabs from conventionally reared sheep, and *B. parapertussis* was recovered from 31.5% of the samples. MBM facilitated the simple and effective isolation of *B. parapertussis* from ovine nasal swabs and, in successfully excluding overgrowth with other contaminants, proved superior to other test formulations evaluated and to existing conventional media.**

Bordetella parapertussis is an obligate respiratory tract pathogen known to cause whooping cough in humans (3, 5). It has been isolated also from the respiratory tracts of sheep in New Zealand (2) and more recently from healthy and pneumonic lungs of sheep in Scotland (13). One of the main problems encountered in attempts to isolate *B. parapertussis* from the nasal cavity is overgrowth with unwanted flora during the long incubation period on the highly nutritious media required for its isolation (4). Kurzynski et al. (4) compared modified Bordet-Gengou and modified Regan-Lowe media and reported that although maximum sensitivity was achieved when the media were used in parallel, there was overgrowth with miscellaneous gram-negative rods, gram-positive cocci, and fungi despite the presence of cephalexin and anisomycin. Attempts to overcome this problem have included a two-step isolation procedure, in which suspect *Bordetella* colonies detected on primary isolation using Regan-Lowe medium were subcultured onto a secondary medium containing amphotericin B (14). Modified charcoal agar (CA), successful in isolation of *B. parapertussis* from ovine lungs, tracheal scrapes, and lung lavage samples (13), proved unsuitable for the isolation of the bacterium from the nasal passages of conventionally reared sheep because of persistent overgrowth with fungi and other competitive bacteria (12). A similar problem was reported by Smith and Baskerville (15) when sampling the nasal passages of pigs for the isolation of *Bordetella bronchiseptica.*

This report describes the development of a new selective medium, Moredun Bordetella Medium (MBM), for the improved isolation of *B. parapertussis* from conventionally reared sheep and evaluates its performance with field material.

MATERIALS AND METHODS

Bacterial strains. The bacterial isolates used in this study are described in Table 1. Cultures were preserved by taking a heavy sweep of growth from a blood agar culture and inoculating a Microbank vial (Pro-lab Diagnostics, Bromborough, England) as previously described (13).

Characterization of isolates. Isolates were characterized by using oxidase sticks (BioMérieux UK Ltd., Basingstoke, England) and urea and nitrate discs (both from Difco Ltd., West Molesey, England), and citrate utilization was determined by using Simmons citrate agar (Oxoid Ltd., Basingstoke, England). Fermentation of glucose was determined by Hugh and Leifson's oxidation-fermentation test (7).

Antibiotics and antifungal agents. The following antibiotics were obtained from Sigma (Poole, England): amphotericin B, benzyl penicillin, anisomycin, cycloheximide, and spectinomycin. Antibiograms were obtained by using ATB VET antibiotic susceptibility test strips (BioMérieux).

Conventional media. The following commercial media were prepared according to the manufacturers' instructions: Iso-Sensitest agar (Oxoid), MacConkey agar (Mast Diagnostics Ltd., Bootle, England), and blood agar (Gibco Ltd., Paisley, Scotland) with the addition of 5% (vol/vol) citrated sheep blood. CA and Simmons citrate agar were prepared as previously described (13).

Test media. (i) CA with cycloheximide (CCA). Charcoal agar base (Oxoid) was prepared as per the manufacturer's instructions. After it was cooled to 50 to 55°C, 10% defibrinated horse blood (Oxoid) and *Bordetella* supplement (Oxoid) were added. Amphotericin B, benzyl penicillin, and cycloheximide, at final concentrations of 50 μg/ml, 20 μg/ml, and 0.5 mg/ml, respectively, were added aseptically immediately before pouring.

(ii) Blood agar base with additives (BA^+). Blood agar base (Gibco) was prepared as per the manufacturer's instructions. After it was cooled to 50 to 55°C, 5% (vol/vol) citrated sheep blood and *Bordetella* supplement (Oxoid) were added. Spectinomycin and cycloheximide, at final concentrations of 100 μg/ml and 0.5 mg/ml, respectively, were added aseptically immediately before pouring.

(iii) MBM. MBM was prepared as described for CCA with the omission of amphotericin B and benzyl penicillin and addition of spectinomycin and cycloheximide at final concentrations of 100 μg/ml and 0.5 mg/ml, respectively.

Stock solutions of cycloheximide were prepared according to the method described in the "Manual of Veterinary Investigation: Laboratory Techniques" (7) and comprised 2.5 g of cycloheximide dissolved in 10 ml of acetone and brought to a final volume of 200 ml by the addition of distilled water. The solution was sterilized at 115°C for 15 min and stored at 4°C for up to 3 months. The solution was added to the melted, cooled medium to give a final concentration of 0.5 mg/ml.

Stock solutions of amphotericin B, benzyl penicillin, and spectinomycin were prepared as aqueous solutions in distilled water to final concentrations of 50, 20, and 100 μg/ml, respectively. They were then filter sterilized with a 0.45-μm-pore-size filter, dispensed, and stored at −20°C for up to 6 months. Stored plates of all media described were kept at 4°C in sealed bags for up to 2 weeks.

Laboratory evaluation of selective agents. The susceptibility of fungal contamination to anisomycin and cycloheximide was tested with a suspension of a *Mucor* sp. in 2 ml of phosphate-buffered saline (PBS), adjusted to McFarland standard no. 4, 100 μl of which was inoculated onto CA. The inoculum was spread evenly over the plate with a sterile plating swab (Difco). Sterile antibiotic assay discs (Whatman International Ltd., Maidstone, England) were impregnated with 10 μl of filter-sterilized solutions of anisomycin (20 μg/ml) or cycloheximide (0.5 mg/ml), and a disc of each agent was placed on the inoculated plate. Plates were incubated at 37°C and examined after 24 and 48 h.

Antibiograms were obtained by using ATB VET strips as per the manufacturer's instructions with an extended incubation of 48 h for *B. parapertussis. B. parapertussis* isolate C (13) was used as a control. The MIC of spectinomycin was determined on Iso-Sensitest agar on the basis of the method described by Stokes and Ridgeway (16). The strains listed in Table 1, with the exception of those of

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TABLE 1. Sources of bacterial isolates

Isolate(s)	Source
A. lwoffii NCTC 5866	NCTC, Colindale[a]
A. faecalis NCTC 415	NCTC, Colindale
B. avium 4091 and 4480	R. Parton, Glasgow University[b]
B. bronchiseptica 452	R. Parton, Glasgow University
B. parapertussis	
Human	
NCTC 10520, NCTC 5952	R. Parton, Glasgow University
Ovine	
C, D1, D2, D3, E1, E2, E3, G1, G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13, H1, H2, H3, H4, H7, H8, H10, J1, K1, K2, K3, K4, K5, 2432, 19752, 9952	Moredun Research Institute
B. pertussis NCTC 18323	R. Parton, Glasgow University
Moraxella sp. strain 995	Moredun Research Institute
P. haemolytica 124/92	Moredun Research Institute

[a] National Collection of Type Cultures, Central Public Health Laboratory, London.

[b] Roger Parton, Department of Microbiology, Glasgow University, Glasgow, United Kingdom.

Bordetella avium, *Bordetella pertussis*, and *Pasteurella haemolytica*, were tested over a range of 0.5 to 140 μg/ml. All plates were incubated at 37°C for up to 48 h.

Field evaluation of cycloheximide. In an initial study to test the ability of cycloheximide to suppress fungal contamination, nasal swabs (Difco Culture Swab Transport System) from 50 conventionally reared sheep were inoculated in parallel onto CA and CCA. The plates were incubated at 37°C for up to 7 days and examined daily for signs of microbial growth.

Laboratory evaluation of Moredun Bordetella Medium. To assess the nutritional properties of MBM, test strains were first grown on blood agar and then subcultured onto CA, BA^+, and MBM. Growth characteristics of test strains on the three media were compared, and the comparative viability of *B. parapertussis* isolate C was assessed by the method of Miles et al. (8).

The selectivity of MBM was evaluated by inoculation with 100 μl of cocktail suspensions of the following isolates: *B. parapertussis* isolate C and *Acinetobacter lwoffii* NCTC 5866; *B. parapertussis* isolate C and *Alcaligenes faecalis* NCTC 415; *B. parapertussis* isolate C and *Moraxella* sp. strain 995; and *B. parapertussis* isolate C and *P. haemolytica* 124/92. Cocktail suspensions were prepared by suspending a sweep of growth of each organism in 2 ml of PBS, vortexing the suspension, and adjusting it to McFarland standard no. 4. The plates were incubated at 37°C for 48 h.

Field evaluation of MBM and BA^+. Fifty conventionally reared sheep each from three farms and a local abattoir (sites A, B, C, and D, respectively) were selected and sampled by nasal swabbing. In order to avoid bias toward one particular medium, the order of inoculation of BA^+ and MBM plates was alternated. The swab was rotated fully when the well was inoculated and held static when the plate was streaked out. The plates were incubated at 37°C for up to 7 days and observed daily for bacterial growth.

RESULTS

Laboratory evaluation of selective agents. Fungal growth was obtained from an ovine nasal swab and a straw bale at Moredun Research Institute, and both isolates grew luxuriantly on CA (13). The colony morphology was identical to that observed in other experiments and was identified as that of a *Mucor* sp. by the Department of Mycology, University of Glasgow, Glasgow, United Kingdom. The *Mucor* sp. contaminant was resistant to amphotericin B (50 μg/ml) and anisomycin (20 μg/ml) but was shown to be susceptible to cycloheximide (0.5 mg/ml). The antibiogram results showed that only spectinomycin inhibited the growth of most of the competitive commensals but allowed the growth of *B. parapertussis*. The MIC data showed that the optimum concentration of spectinomycin was 100 μg/ml.

B. parapertussis was selectively isolated from all cocktail suspensions with all media containing spectinomycin and cycloheximide. Colony counts were not significantly different from those obtained on media without the selective agents (data not shown).

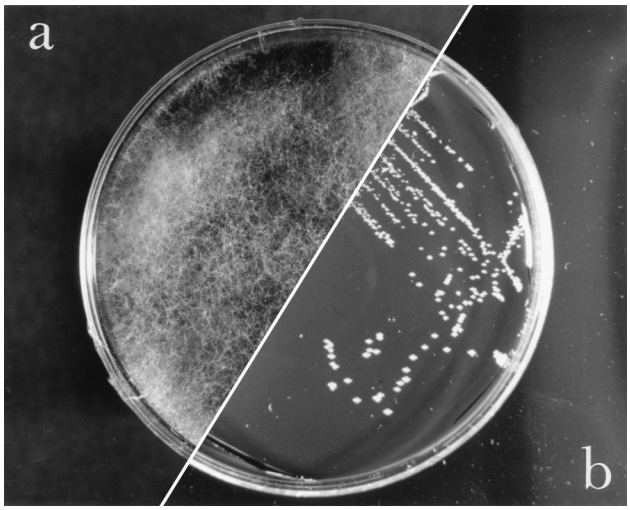

FIG. 1. Clinical specimen plated onto CA (a) and MBM (b) to show the improved selectivity of MBM.

Field evaluation of cycloheximide. Conventional blood agar proved unsuitable for the isolation of *B. parapertussis* because of overgrowth by competitive commensals. Of the 50 samples obtained from conventionally reared sheep in the preliminary study, 24 (48%) were overgrown with *Mucor* spp. (Fig. 1a) and 8 (16%) were overgrown with other fungal species on CA. In contrast, fungal contamination was not observed on medium containing cycloheximide, i.e., MBM (Fig. 1b).

Field evaluation of MBM and BA^+. *B. parapertussis* was isolated from sheep on two of the three farms and from sheep at the abattoir (Table 2). *B. parapertussis* was isolated from 74% of the 6-month-old Suffolks, housed at the time of sampling, on farm A. On farm B, the sheep were at pasture at the time of sampling and *B. parapertussis* was not isolated. It was also observed that a high proportion of swabs from farm B yielded no bacterial growth. On farm C, 4-year-old Greyface ewes, originally housed at farm A but now at pasture, were sampled and only 4% yielded *B. parapertussis*. At the abattoir, mixed-source adult sheep previously transported from market and held overnight in pens were sampled prior to slaughter, and 48% yielded *B. parapertussis.*

There was no difference in isolation rates on BA^+ and MBM. Colony size on initial isolation was variable; "small" colonies were circular, translucent, and <0.5 mm in diameter, and "large" colonies were whitish-grey, circular, convex, and 0.5 to 1.0 mm in diameter. On subculture onto conventional

TABLE 2. Isolation of *B. parapertussis* from ovine nasal swabs

Site	No. of swabs	No. positive on:			Total no. positive
		BA^+ alone	MBM alone	Both BA^+ and MBM	
A	50	3	2	32	37
B	50	0	0	0	0
C	50	1	0	1	2
D	50	1	3	20	24
Total	200	5	5	53	63

blood agar, both colony types reverted to the typical morphology of *B. parapertussis* as described by Pittman (10). Slight pleomorphism was evident by Gram staining on initial isolation, but following subculture, the morphology was more characteristic of *B. parapertussis*. Confirmatory tests gave the following results: oxidase positive, urease positive, citrate positive, no oxidation or fermentation of glucose, growth on MacConkey agar, and no growth anaerobically.

DISCUSSION

A novel selective medium, MBM, containing cycloheximide and spectinomycin, was used successfully in isolating *B. parapertussis* from ovine nasal swabs. Existing selective media for the isolation of *Bordetella* spp. have been shown to be problematic as regards suppression of fungi and other commensals (4, 14), particularly in a veterinary context in which overgrowth with fungal contamination from ovine nasal swabs is a persistent occurrence (12). Attempts to isolate *B. parapertussis* from sheep at Moredun Research Institute by using existing selective media were aborted because of overgrowth with *Mucor* spp. and other commensals, such as *Neisseria* spp., *Moraxella* spp., and *Proteus* spp. The extent of overgrowth was due in part to the highly nutritious charcoal agar base and the suboptimal choice of antibiotics, exacerbated by a 7-day incubation period. These problems justified the development of a new, improved selective medium.

Cycloheximide proved to be a highly effective antifungal agent, and its efficacy in CCA was successfully demonstrated in the laboratory and with field material. Conventional blood agar and CA (13) were used in parallel in this pilot study, but their use was discontinued because of the extent of overgrowth of both fungi and other commensals.

Inhibition of most of the other nasal commensals was achieved by the addition of spectinomycin. Variation in colony morphology was observed with both small- and large-type colonies appearing on the same plate. However, both colony types reverted to the characteristic *B. parapertussis* colony morphology on subculture. A possible explanation may be differences in cell sizes during in vivo growth and survival. Morita (9) noted the miniaturization of cells in vivo when bacteria were in a starvation survival menstruum. Porter (11) reported similar findings with *B. bronchiseptica* when the bacterium was grown in PBS or lake water for extended periods at different temperatures. On microscopic examination, slight pleomorphism which may be attributed to the effect of cephalexin was observed in cell morphology, as previously reported by Sutcliffe and Abbott (17).

Although BA^+ and MBM were comparable in their selective abilities, the dark background of MBM facilitated the recognition of *B. parapertussis*-like colonies and therefore MBM was the preferred medium.

Other *Bordetella* species which were tested during this study were *B. bronchiseptica*, *B. avium*, and *B. pertussis*. However, no data are available for the two new recently described species *B. hinzii* (18) and *B. holmesii* (19), as these species were described after the survey was completed. Since both *B. bronchiseptica* and *B. avium* grew on MBM but *B. pertussis* did not, further work would be required to determine whether MBM could be adapted to other *Bordetella* species.

Differences in the recovery of *B. parapertussis* from the three farms may be attributed to the animal husbandry methods employed at each farm. Recovery was at high levels (74%) when animals were housed (farm A) but was at low levels ($\leq$4%) when animals were at pasture (farms B and C). These results, when considered with the 48% recovery from the abattoir, might suggest that prior yarding and transportation could result in cross-infection between closely confined sheep and that similar opportunities for cross-infection exist during many routine husbandry practices. Another factor which may influence the prevalence of *B. parapertussis* is seasonal variation. A higher level of recovery of *B. parapertussis* from conventionally reared sheep was observed from October to February (12). Seasonal variation with regard to bacterial populations has already been reported for sheep and calves (1, 6).

To conclude, MBM was developed to improve the isolation of *B. parapertussis* from the nasal cavities of conventionally reared sheep. The new medium was evaluated by using field material and proved superior in terms of isolation to existing selective media. The significance of this report lies in the ability of MBM to isolate *B. parapertussis* selectively from conventional sheep, which may have wider epidemiological implications in assessing the prevalence of this human pathogen in sheep.

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