Isolation and characterization of *Bordetella parapertussis*-like bacteria from ovine lungs

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Bacteria resembling two *Bordetella* species were isolated from both normal and pneumonic ovine lungs using a selective charcoal agar. Twenty-eight of the 33 isolates showed similarities to stock NCTC *B. parapertussis* strains in their SDS-PAGE gel protein profiles, in their biochemical reactions and in causing browning on tyrosine agar. Five isolates behaved similarly to stock *B. bronchiseptica* strains, in being actively motile, in giving identical positive reactions in three out of four biochemical tests and in causing no colour change in tyrosine agar. Multilocus enzyme electrophoresis separated the isolates into two electrophoretic types distinguishable from those of stock *B. parapertussis* and stock *B. bronchiseptica* strains.

Keywords: *Bordetella parapertussis*, ovine lungs, biochemical tests

INTRODUCTION

Bordetella parapertussis is one of the causative agents of whooping cough in man (Eldering & Kendrick, 1938; Connor, 1986). Its fastidiousness and defined growth requirements (Proom, 1955) make it difficult to isolate without specialized media (Regan & Lowe, 1977) and identification is hampered by the close similarity of members of the genus *Bordetella* with other bacterial genera, in particular *Haemophilus*, *Acinetobacter* and *Alcaligenes* (Johnson & Sneath, 1973). Considered an obligate respiratory tract pathogen for man (Linneman & Perry, 1977; Pittman, 1984) it has recently been isolated from the respiratory tract of healthy and pneumonic lambs in New Zealand (Cullinane *et al.*, 1987). Studies in mice (Chen *et al.*, 1989) and sheep (Chen *et al.*, 1988a, b) have demonstrated its pathogenicity for both species.

This paper describes the first isolation of *B. parapertussis*-like organisms from the lungs of sheep in the United Kingdom and reports a detailed characterization of ovine *Bordetella* isolates, including the use of multilocus enzyme electrophoresis as a tool for their differentiation.

METHODS

Bacterial strains. *B. parapertussis* strains NCTC 5952 and 10520 (both human isolates) and *B. bronchiseptica* strains 11 (pig), 6353 (dog), 452 (dog), 5376 (rabbit) and 10541 (rabbit), previously described by Porter *et al.* (1991), were kindly provided by Dr

Roger Parton (Department of Microbiology, Glasgow University, UK), *B. parapertussis* NCTC 10853 (human), *Alcaligenes faecalis* NCTC 415 and 8582 and *Acinetobacter lwoffii* NCTC 5866 were obtained from the National Collection of Type Cultures, Colindale, UK. *B. bronchiseptica* strains G4 and G30 (both horse isolates) were made available by Mr James Woods (Animal Health Trust, Suffolk, UK).

Cultures were preserved by taking a heavy sweep of growth from a 48 h blood agar culture, inoculating this into a Microbank vial (Pro-Lab) according to the manufacturer's instructions and storing at -70°C .

Media. Modified charcoal agar comprised a charcoal agar base (Oxoid), 10% (v/v) defibrinated horse blood (Oxoid) and 2 ml *Bordetella* supplement (Oxoid). Amphotericin B and benzylpenicillin (both Sigma) were added aseptically immediately before pouring plates, to final concentrations of 160 units ml^{-1} and 0.3 units ml^{-1} respectively. Blood agar (Gibco) was prepared with 7% (v/v) citrated sheep blood. Tyrosine agar consisted of 12.25 g nutrient broth powder no. 2 (Oxoid), 6.0 g bacteriological agar (Gibco) and 0.5 g L-tyrosine (Sigma) dissolved in 500 ml distilled water. Plates were stored at 4°C and discarded if unused after 3 weeks. Simmon's citrate agar (Oxoid) was dispensed as slopes.

Isolation of *B. parapertussis* from ovine lungs. Lungs from 52 slaughter lambs were obtained from a local abattoir. Equal numbers of normal and pneumonic lungs were taken, the presence of pneumonia being determined by meat inspectors. Eleven batches of lungs were collected over a 10 week period and were coded alphabetically (A to K). Three main sampling methods were used.

1. Lung wash (LW). Lungs were perfused via the trachea with 500 ml Hanks' balanced salt solution (HBSS); the washings were collected by straining through gauze. Washes from normal and pneumonic samples were pooled separately. After centi-

Abbreviations: HBSS, Hanks' balanced salt solution; LH, lung homogenate; LW, lung wash; TS, tracheal scraping; MEE, multilocus enzyme electrophoresis; ET, electrophoretic type.

fuging at 180 *g* for 20 min to deposit erythrocytes and cellular debris, the supernatant fraction was decanted and re-centrifuged at 11600 *g* for 30 min, to deposit any bacteria present. This pellet was resuspended in 3 ml HBSS.

2. Lung homogenate (LH). Selected portions (3 × 1 g) from each lung were homogenized with a stomacher in 10 ml HBSS. After centrifugation at 300 *g* for 20 min to deposit tissue, the supernates were withdrawn and centrifuged at 11600 *g* for 30 min. The resultant pellet was resuspended in 2 ml HBSS.

3. Tracheal scraping (TS). TS samples were taken with a sterile scalpel from the lower portion of the trachea. These were pooled, mixed with 3 ml HBSS, then centrifuged at 300 *g* for 30 min to deposit tissue and erythrocytes. The supernate was centrifuged at 11600 *g* for 15 min and the resultant pellet resuspended in 1 ml HBSS.

The final suspensions from the pooled LW, LH and TS samples were inoculated in duplicate 50 µl volumes onto blood agar and modified charcoal agar. Plates were incubated at 37 °C until growth appeared (approximately 3 d), otherwise for 5 d, after which they were discarded if no growth had appeared. Colonies appearing on the selective medium were subcultured onto blood agar then stored at -70 °C and freeze-dried.

Motility test. The motility of all strains was tested by the hanging drop method (Mackie & McCartney, 1989).

Biochemical tests. Isolates were characterized using API strips (BioMérieux), Oxidase sticks (BioMérieux) and urea and nitrate (both Difco). Citrate utilization was determined using Simmon's citrate agar (Oxoid). The oxidase test for the API method was carried out by adding the suspect colony to a filter, then soaking with oxidase reagent (BioMérieux). A purple colour indicated a positive reaction.

SDS PAGE. This was performed by the method of Laemmli (Laemmli, 1970) using 10% (w/v) slab gels. Samples were prepared by suspending the growth taken from a blood agar plate in 1 ml distilled water, washing the cells twice in distilled water (1300 *g* for 5 min) then resuspending the final pellet in 100 µl distilled water. The suspension was boiled in 100 µl 2 × solubilizing buffer. The gels were loaded with 10 µl of each sample, run for approximately 45 min and then stained with Coomassie blue. Strains were compared visually.

Electrophoresis of enzymes. Multilocus enzyme electrophoresis (MEE) analysis as described by Selander *et al.* (1986), was performed on isolates C, D1, E2, G2, H1, J1 and K1; on *B. bronchiseptica* strains G30, 5376, 6353, 13325, 11 and 10541; and on *B. parapertussis* NCTC 10520, 8250 and 5952.

Single colonies from cultures on blood agar were inoculated into tryptose phosphate broth (TPB) and incubated for 24–48 h at 37 °C. Confluent lawns were produced by inoculating 0.2 ml of the TPB cultures onto blood agar and incubating at 37 °C for 24–48 h. The growth from three plates was harvested into 2 ml lysate buffer (10 mM Tris, 1 mM EDTA, 0.5 mM NADP, 0.005% DNase, pH 6.8) and this suspension was homogenized by treatment in a mini beater (BIOSPEC Products, Bartlesville, USA) for 2 min using 0.1 mm diameter zirconium beads. The lysates were clarified by centrifugation (13000 *g* for 1 min) then stored at -70 °C in 40 µl aliquots. Electrophoresis was done in 10.26% (w/v) starch gels and used two buffer systems, buffer A (Tris/citrate, pH 8.0) and buffer F (Tris/malate, pH 8.2).

The electrophoretic profiles of the following enzymes were determined using the buffer A system: L-phenylalanyl-L-leucine peptidase (PLP), isocitrate dehydrogenase (IDH) and indophenol oxidase (IPO). The buffer F system was used for the

following enzymes: catalase (CAT), adenylate kinase (ADK), fumarase (FUM) and β-naphthyl propionyl esterase (β-Est). All standard enzymes were obtained from Sigma. Both the buffer systems and enzymes were chosen after standardization and optimization of the MEE technique.

In MEE the presence of allelic variants is detected by the differences in migration of specific enzymes in starch gels. The farthest-travelling variant is assigned a score of 1, the second-farthest 2 and so on. The scores for each isolate are grouped to give a profile and each profile randomly assigned a number, i.e. its electrophoretic type (ET). By comparing ETs the relatedness of strains can be assessed.

RESULTS

Isolation of *Bordetella*-like organisms from ovine lungs

Thirty-three isolates of organisms resembling *Bordetella* species were obtained from 52 ovine lungs. Fifteen isolates were recovered from normal and 18 isolates from pneumonic lungs. Table 1 shows the distribution of these isolates according to lung condition and the sampling methods used. Neither the presence of pneumonic lesions nor the sample type appeared to be important.

Biochemical characterization

All isolates were obtained on a selective charcoal agar designed specifically for the isolation of *Bordetella* spp. from clinical samples and containing benzylpenicillin, amphotericin B and cephalixin (*Bordetella* supplement). Resistance to these antibiotics is in itself useful as a characteristic feature of *Bordetella* species.

The cell morphology of the isolates, namely coccobacilli, and their negative reaction by Gram's stain were typical of *Bordetella* species. The colonies appeared as 'mercury-like' droplets on the charcoal agar. Table 2 summarizes the

Table 1. Distribution of *B. parapertussis*-like isolates from normal and pneumonic lungs

Batch*	No. of isolates from†:					
	Normal lungs			Pneumonic lungs		
	LW	LH	TS	LW	LH	TS
C	1					
D		1	1	1		
E	3					
G	2	3	2	2	2	2
H				3	2	2
J						1
K		1	1	1	1	1
Total	6	5	4	7	5	6

* No *B. parapertussis* isolates were obtained from batches A, B, F or I.

† LW, lung wash; LH, lung homogenate; TS, tracheal scrapes.

Table 2. Differentiation of ovine isolates of *B. parapertussis*-like bacteria and NCTC strains of *B. parapertussis*, *B. bronchiseptica*, *Al. faecalis* and *Ac. lwoffii*

All tests were performed in duplicate. A blank space indicates not tested. +, positive; -, negative; URE, urease; OXI, oxidase; CIT, citrate utilization; NIT, nitrate reduction.

Strain/ isolate	API 20E				Biochemical disc test				Browning of tyrosine agar	Motility*
	URE	OXI	CIT	NIT	URE	OXI	CIT	NIT		
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	+	+	+	-	+	+	-	+	-	+
<i>Al. faecalis</i>										
415	-	+	+	-		+			-	+
8582	-	+	+	+		+			-	+
<i>Ac. lwoffii</i>										
5866	-	-	-	-	-	-	-	-	-	-
<i>B. parapertussis</i>										
10853	-	-	-	-					+	-
10520	-	-	-	-	+	-		-	+	-
5952	+	-	-	-		-			+	-
<i>B. bronchiseptica</i>										
G30	+	+	+	+	+	+		+	-	+
001	+	+	+	+		+			-	+
11	+	+	+	+		+			-	+
6353	+	+	+	+	+	+	+	+	-	+
452	+	+	+	-		+			-	+
5376	+	+	+	+		+			-	+
10541	+	+	+	+		+			-	+

* Motility tested by hanging drop.

Table 3. Growth of three ovine isolates of *B. parapertussis* (G2, K1, J1), *B. parapertussis* (*B. pp.*) NCTC 10853 and *B. bronchiseptica* (*B. bron*) G30 in phosphate-buffered saline (PBS) and tryptose phosphate broth (TPB) during incubation for 48 h at 37 °C

Test fluid	No. of colonies from a 20 µl sample									
	0 h incubation					48 h incubation				
	<i>B. pp.</i> 10853	G2	K1	J1	<i>B. bron</i> G30	<i>B. pp.</i> 10853	G2	K1	J1	<i>B. bron</i> G30
PBS	13	19	9	22	43	0	0	0	0	++
TPB	12	13	20	12	36	++	0	++	++	++

++ , Confluent growth.

biochemical tests recorded with the isolates and with type strains of *B. parapertussis*, *B. bronchiseptica*, *Alcaligenes faecalis* and *Acinetobacter lwoffii*. The latter two species are both similar to *Bordetella* spp.

All isolates except those from batch K caused browning of tyrosine agar after 24 h incubation at 37 °C. The three *B. parapertussis* stock strains also caused browning but the *B. bronchiseptica*, *Al. faecalis* and *Ac. lwoffii* strains did not. All isolates except those of batch K were non-motile, as were the three *B. parapertussis* stock strains and *Ac. lwoffii*. The batch K isolates, *B. bronchiseptica* and *Al. faecalis* strains were all actively motile.

The discriminatory biochemical tests for *B. parapertussis*, namely urease, oxidase, citrate utilization and nitrate reduction, were initially performed by API and then by diagnostic discs or for citrate utilization, on Simmon's citrate medium. The results are shown in Table 2.

B. parapertussis is described as urease and citrate positive and oxidase and nitrate negative (Pittman, 1984). Most of the isolates were urease positive by API within 4 h, the exceptions being G8, G10 and all of batch H except H8 and H10, which were urease negative. Two of the three stock *B. parapertussis* strains were also negative. All the *B. bronchiseptica* strains were strongly positive within 4 h. The two *Al. faecalis* and single *Ac. lwoffii* strains were all negative.

The API oxidase test showed all isolates except J1 and those from batch K to be negative, as were the three stock *B. parapertussis* strains and *Ac. lwoffii*. The J1 and batch K isolates were oxidase positive, as were the *B. bronchiseptica* and *Alcaligenes* strains.

The citrate results by API were even more variable, with variation apparent within batches of isolates. Positive citrate reactions were recorded for isolates C, E2, G2, G3, G5 and all batch K. All other isolates were citrate negative. All the *B. parapertussis* stock strains, and *Ac. lwoffii*, were negative, and the *B. bronchiseptica* and *Al. faecalis* strains were positive for citrate utilization.

All isolates, *B. parapertussis* stock strains and *Ac. lwoffii* did not reduce nitrate by API, whereas all *B. bronchiseptica* strains (except NCTC 452) did reduce nitrate. The NCTC

8582 and NCTC 415 strains of *Al. faecalis* were positive and negative respectively.

When diagnostic discs were used, the results for oxidase were identical with those obtained by API, but the urease reactions showed some differences, all isolates giving positive reactions, including those which were negative by API (H1, H2, H3, H4 and H7). Seven isolates (G3, G4, G6, H9, K2, K3 and K5) reduced nitrate when tested by discs, in contrast to the API results.

On Simmon's citrate medium more positive reactions were observed than with the API test, although 16 of the isolates remained negative. Intra-batch variation remained and two isolates (G12, G13) negative by the discs were positive by API.

While all of the isolates from batch K gave positive results for the four biochemical tests by API, only K2 was positive in all four tests by diagnostic discs or slopes. This may reflect differences in the efficiency or suitability of the biochemical methods and not necessarily a difference between isolates.

Two stock organisms. *B. bronchiseptica* strain 6353 and *Ac. lwoffii*, were positive and negative, respectively, for all four tests by both API and discs.

Gel profiles

The isolates and stock strains were compared by SDS-PAGE by running whole-cell preparations on 10% slab gels. Differences in protein profiles were observed between *Bordetella* species and *Alcaligenes* and *Acinetobacter* but no differences could be seen either within the isolates or between the isolates and stock *B. parapertussis* and *B. bronchiseptica* strains (results not shown).

Growth in phosphate-buffered saline (PBS)

As previously reported (Porter *et al.*, 1992), *B. bronchiseptica* can grow and survive for extended periods in PBS but *B. parapertussis* cannot, a characteristic which may be used to differentiate the two species. Table 3 shows the results of an experiment in which the survival and growth of isolates G2, K1 and J1 were compared with

Table 4. MEE of ovine isolates of *B. parapertussis* and NCTC strains of *B. parapertussis* and *B. bronchiseptica*

Enzymes: IDH, isocitrate dehydrogenase; IPO, indophenol oxidase; PLP, L-phenylalanyl-L-leucine peptidase; CAT, catalase; ADK, adenylate kinase; FUM, fumarase; β -Est, β -naphthyl propionyl esterase.

Strain/isolate	Buffer A			Buffer F				Profile	ET
	IDH	IPO	PLP	CAT	ADK	FUM	B-Est		
<i>B. parapertussis</i>									
10520, 8250	1	1	1	0	1	1	2	1110112	1
5952	1	1	0	0	1	1	0	1100110	2
<i>Ovine isolates</i>									
C, D1, E2, G2, H1	2	1	0	0	1	1	2	2100112	3
J1, K1	1	1	0	0	1	1	2	1100112	4
<i>B. bronchiseptica</i>									
G30, 5376, 11, 10541	1	1	1	2	1	1	2	1112112	5
13325	1	1	1	1/2*	1	1	2	111(1/2)112	5/7
6353	1	1	1	2	1	2	1	1112121	6

* Mobility difficult to assign, hence 1/2 designation.

B. parapertussis NCTC 10853 and *B. bronchiseptica* G30 after their inoculation into PBS and tryptose phosphate broth (TPB) and subsequent incubation for 48 h at 37 °C. None of the isolates nor *B. parapertussis* NCTC 10853 survived the 48 h incubation in PBS; however, all isolates (except G2) and strain NCTC 10853 grew in TPB and gave confluent growth from a 20 μ l sample on blood agar. *B. bronchiseptica* G30 grew sufficiently in both PBS and TPB to give confluent growth on blood agar after 48 h incubation.

Multilocus enzyme electrophoresis

MEE was used to differentiate *B. parapertussis* from *B. bronchiseptica* strains and to compare the different batches of isolates (Table 4). Six ETs were recorded. The stock *B. parapertussis* strains lay within ETs 1 and 2. The ovine lung isolates tested all belonged to ET 3 except K1 and J1, which were ET 4. The *B. bronchiseptica* strains were found in ETs 5 and 6.

MEE showed the lung isolates to cluster in a manner similar to the biochemical tests, with K1 and J1 deviating from the others. The lung isolates were closer in ET profile to the stock *B. parapertussis* strains than to the *B. bronchiseptica* strains.

DISCUSSION

Characteristics of the ovine lung isolates

This study is the first in the United Kingdom to report the isolation of *B. parapertussis*-like organisms from ovine lungs, although similar isolations have been made from lambs in New Zealand (Cullinane *et al.*, 1987). This organism is more usually considered to be an obligate respiratory tract pathogen of man (Linneman & Perry, 1977; Pittman, 1984). In all, 33 *Bordetella* isolates were

obtained from the lungs of 52 young sheep. Pneumonic and normal lungs were equally infected, which substantiates the report by Cullinane *et al.* (1987) concerning isolations of *B. parapertussis* from the respiratory tract of New Zealand sheep.

In this study, the three different sampling methods (LW, LH and TS) proved effective, though not always on the same set of lungs. For example, in batch E, only LW yielded isolates of *B. parapertussis*-like organisms, while in batch D, isolates were made from LH and TS but not LW. Thus this study indicates that a combination of LW and TS should be employed for maximum efficiency. In contrast, Cullinane *et al.* (1987) reported lung washings and nasal swabbing to be the most successful methods of isolation.

In general, most of the isolates showed biochemical reactions which were similar to those described for *B. parapertussis* (Pittman, 1984). The main characteristic biochemical property was the ability of the isolates to causing browning of tyrosine agar, a characteristic unique to *B. parapertussis* (Ensminger, 1953). All the isolates except those of batch K possessed this ability, while *B. bronchiseptica*, *Al. faecalis* and *Ac. lwoffii* strains did not. Cullinane *et al.* (1987) reported that their isolates failed to brown peptone agar, possibly because of a lower tyrosine concentration in this medium.

In some cases the API strips produced inconsistent results, both with the isolates and with NCTC strains. These inconsistencies may not be relevant, as API report the percentages of positive reactions for urease, citrate, nitrate and oxidase tests by *Bordetella/Alcaligenes* spp. as 59%, 60%, 63% and 95%, respectively. It is not reported specifically what proportion of *B. parapertussis* strains give positive results in these tests. Inconsistencies were also observed between the API results and those obtained with

diagnostic discs or slopes. The contrary results by the two different methods may indicate their efficiency for particular biochemical reactions.

The SDS-PAGE protein profiles were useful as an indication of speciation and differentiated between genera, but were ineffective in distinguishing between *B. parapertussis* and *B. bronchiseptica*.

The isolates from batch K lungs were consistently different from all other isolates and from the NCTC *B. parapertussis* strains, being motile and not browning tyrosine agar. Thus, although their SDS-PAGE profiles were similar to those of the other isolates, they appeared to be more like *B. bronchiseptica*. The isolates from this group were probably *B. bronchiseptica*, although two inconsistencies were recorded. Porter *et al.* (1991) have previously reported that *B. bronchiseptica* strains can grow and survive in PBS and freshwater samples, and in this study isolates G1, J1, K1 and *B. parapertussis* NCTC 10853 failed to multiply in PBS, while *B. bronchiseptica* grew as previously described. Furthermore, isolates from batch K failed to reduce nitrate, whereas six of seven reference *B. bronchiseptica* strains did reduce nitrate.

We propose that the majority of the isolates be classified as *B. parapertussis*-like, except for batch K, which appeared to be more like *B. bronchiseptica*, until such time as further evidence is collected and the taxonomy of this group of organisms is clarified. Characterization by immunological methods, either by simple agglutination or antigen detection, was not used in this study as previous reports have indicated a lack of specificity with these techniques due to cross-reacting antigens within *Bordetella* species and between *Bordetella* and other genera (Preston, 1988). Cullinane *et al.* (1987) did not describe in detail the method employed for the characterization of their New Zealand ovine isolates. However, they did draw attention to notable differences between their isolates and *B. parapertussis* NCTC 5952, such as the speed of urease reaction being similar to that of *B. bronchiseptica* (as observed in our study) and the failure of the isolates to cause browning on peptone agar.

MEE analysis

MEE has been applied previously to *Bordetella* species by Musser *et al.* (1986), who measured genetic diversity and relationships within and among three nominal *Bordetella* species by assaying electrophoresis variation in 15 metabolic enzymes. Contrary to our findings, they found that all the *B. parapertussis* isolates lay in a single ET. However, none of the isolates examined by Musser *et al.* (1986) were of ovine origin. With *B. bronchiseptica* strains, these workers reported differences in ET between and within animal source. *B. bronchiseptica* strains formed ten and *B. pertussis* three ETs. *B. parapertussis* and *B. bronchiseptica* were placed in one group (A) with *B. pertussis* in another (B), though one of the *B. pertussis* isolates (18-323) was found to lie in group A (Musser *et al.*, 1986).

Musser *et al.* (1986) stated that relatively limited genetic variation existed in populations of *Bordetella* species and

that this, in conjunction with other genetic evidence, such as DNA homology, was insufficient to warrant single-species status for all three *Bordetella*. In contrast, some avian isolates characterized as *B. avium* were found to be sufficiently genetically divergent to warrant specific status (Musser *et al.*, 1986).

Our findings cannot be directly compared with those of Musser *et al.* (1986) since different enzymes and buffer systems were used, but we did find the technique useful in distinguishing between *B. parapertussis* and *B. bronchiseptica*.

Epidemiological implications

The isolation of *B. parapertussis* from sheep is significant since the organism is a human pathogen which hitherto has been reported purely as an obligate respiratory tract parasite. The organism has now been isolated from sheep in two countries, New Zealand and Scotland, and found not only in lambs with pneumonia but also in healthy lambs. The health risk of exposure to these animals is not known but may have zoonotic implications. The animal pathogen *B. bronchiseptica* can be transmitted to man (Byrd *et al.*, 1981) and a similar situation may pertain with *B. parapertussis*. Another possibility is that man is the source of infection for sheep. The recent demonstration (Porter *et al.*, 1991) that *B. bronchiseptica* strains can grow in PBS and freshwater, when it had been believed that the organism was an obligate respiratory tract pathogen of animals (Goodnow, 1980), suggests that *Bordetella* may be more widespread than stated in recent reviews (Pittman, 1984). The difficulty of isolating *B. pertussis* and *B. parapertussis* from clinical samples could explain why these organisms have not previously been detected or why *B. bronchiseptica* and *B. avium* have not been isolated more readily from the environment. It has been suggested that *B. parapertussis* may be a cause of pneumonia in lambs in its own right (Alley, 1991). Alternatively, *B. parapertussis* may predispose lambs to secondary infection with agents such as pasteurellae. This type of relationship has been described for *B. bronchiseptica* and *Pasteurella multocida* in atrophic rhinitis of pigs (Rutter, 1985).

It has been reported that the toxins produced by *B. parapertussis* are responsible for the clinical symptoms seen in infections of humans with the organism and that, unlike infections involving *B. bronchiseptica*, the presence of live organisms is not required for clinical signs (Bemis *et al.*, 1977). The damage caused by these toxins in lambs may be the factor that allows colonization of the lower respiratory tract by organisms such as *Pasteurella haemolytica*. If this is the case then *B. parapertussis* may, in some cases, have been eliminated from the lungs by the time *P. haemolytica* becomes established. Interestingly, the first batch of lungs (A) were all pneumonic and yielded *P. haemolytica* (serotypes A2 and A6) but not *B. parapertussis* (data not shown).

Ovine isolates of *B. parapertussis* have been used to produce pneumonia in mice (Chen *et al.*, 1989) and lambs (Chen *et al.*, 1988a), and cell-free extracts of cultures of *B.*

parapertussis have also caused pneumonia in mice (Chen *et al.*, 1990). In one study (Jian *et al.*, 1991), the combined administration of *B. parapertussis* and *P. haemolytica* resulted in a more severe bronchopneumonia than produced by either agent administered alone.

The results of our study indicate the need for a review of the epidemiology of *Bordetella* species.

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