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The use of pulsed-field gel electrophoresis to examine the epidemiology of *Bordetella bronchiseptica* isolated from cats and other species

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SUMMARY

A collection (164) of isolates of *Bordetella bronchiseptica* made predominantly from cats (132) but also from dogs (15), pigs (12) and other species was examined by pulsed field gel electrophoresis following macrorestriction digestion with *Xba*I. Each isolate was analysed twice and the patterns were entirely reproducible. The isolates fell into 17 different strains (> 3 bands different) and within strains there were numerous subtypes. Feline isolates fell into 12 of the 17 strains. In general, cats housed together had similar or identical strains and subtypes of *B. bronchiseptica*. There was no difference in the PFGE patterns of isolates made from carrier cats and those from cats with respiratory disease. Isolates from pigs and dogs were in general similar to the feline isolates and there was no great evidence for species specificity. The PFGE pattern of feline and canine isolates were more related to whether the animals were housed together rather than whether they came from dogs or cats.

INTRODUCTION

Bordetella bronchiseptica is recognized as a pathogen of many mammalian species. It is known to be a primary pathogen in canine kennel cough [1], porcine atrophic rhinitis and pneumonia [2] and may be a primary or secondary pathogen in respiratory disease in various laboratory animal species [3]; it has been increasingly recognised as a primary respiratory pathogen in cats [4–7]. *B. bronchiseptica* has also been implicated in sporadic cases of infection in horses [8], humans [9], and other species [10]. The potential for cross-species transmission has long been pondered but zoonotic spread from pet animals to immunocompromised humans has only recently been described [11].

A recent survey carried out to identify the prevalence of and risk factors for *B. bronchiseptica* infection in cats demonstrated that the prevalence of feline bordetellosis was 11% overall but that infection and respiratory disease rates varied with the cat population studied [12]. During the survey *B. bronchiseptica* was isolated from cats and from in-contact dogs in several households, thus raising the possibility that interspecies transmission could occur under such conditions.

In order to prove that interspecies transmission of *B. bronchiseptica* can occur, stable and discriminatory bacterial typing methods are required. Phenotypic typing methods rely on the expression of characters that may vary according to cultural or experimental conditions and are gradually being superseded by bacterial genomic analysis. The molecular epidemiology of *B. bronchiseptica* has been investigated using a variety of techniques including plasmid analysis [13] insertion sequence polymorphism analysis [14], ribotyping [8, 15] and direct genomic sequence com-

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parisons [16]. Pulsed field gel electrophoresis (PFGE) is a technique that allows separation of much larger DNA fragments than with conventional electrophoresis and is therefore applicable to macrorestriction analysis of bacterial genomes [17]. Recent studies have focused on the analysis of outbreaks of pertussis and have used PFGE to determine the epidemiological relatedness of clinical isolates [18–20]. To date, three PFGE studies have been undertaken on other species of *Bordetella*. PFGE has been used to differentiate human and ovine isolates of *B. parapertussis* [21] where it was also shown that there were strain variations within the isolates from each host species but that similar variants were epidemiologically linked. Another report described the use of PFGE to examine the genetic relatedness of *Bordetella* spp and also showed variation among isolates of the same species [22]. Eight isolates of *B. bronchiseptica* were included which were divided into three types based on restriction fragment patterns obtained by digestion with the rare-cutting endonuclease *Xba*I. The possibility of cross-species transmission was supported by a further study showing epidemiological and genomic relatedness between *B. bronchiseptica* isolates recovered from a human being and rabbits in the same household [23].

The aims of the present study were to examine the value and reproducibility of macrorestriction digestion and PFGE in typing a large collection of feline *B. bronchiseptica* isolates, to compare isolates from infected and carrier cats and to compare feline isolates with those obtained from other mammalian species.

MATERIALS AND METHODS

Bacteria

A total of 164 isolates of *B. bronchiseptica* were examined by PFGE. These comprised isolates from cats (132), dogs (15), pigs (12), snow leopard (1), guinea pig (1), rabbit (1), man (1) and the canine vaccine strain of *B. bronchiseptica* (S55; Schering Plough, Bury St. Edmunds, UK). In addition type strains of *B. pertussis*, *B. parapertussis*, *B. avium*, *B. hinzii* and *B. holmesii* were included. Eleven of the cat isolates were repeat isolations from cats previously tested; repeat isolations were made at intervals varying from 2 weeks to 2 years.

B. bronchiseptica was isolated from oropharyngeal and/or nasal swabs from cats and other species by plating onto selective charcoal horse blood agar [24] incorporating amphotericin B and cephalexin (Oxoid, Basingstoke). Freeze-dried cultures of reference strains of various *Bordetella* species were obtained (National Collection of Type Cultures, PHLS, Colindale, UK and Laboratorium Microbiologie Ghent Culture Collection, University of Ghent, Belgium) and reconstituted according to suppliers' instructions. All isolates were stored at −70 °C on protect beads until used.

DNA preparation and RE digestion

DNA was prepared, with modification, as described previously [25]. Briefly, one colony of each isolate was taken from a charcoal agar plate, suspended in nutrient broth (3 ml) and incubated at 37 °C overnight. Then 200 μl of the overnight culture was centrifuged for 4 min at 14000 r.p.m. and the pellet resuspended in 100 μl of cold cell suspension buffer (10 mM tris base, 20 mM NaCl, 5 mM EDTA). Four microlitres of lysozyme solution (25 mg/ml) were added to the bacterial suspension followed by 100 μl of 2% LMP agarose (Appligene Oncor, France) and the mixture dispensed into a 12-plug mould (Pharmacia, St. Albans, UK).

For each strain, the plugs were incubated for 1 h at 37 °C in 1 ml lysis buffer (10 mM tris base, 50 mM NaCl, 0·2% sodium deoxycholate, 0·5% sodium lauryl sarcosine, pH 7·2) with lysozyme (1 mg/ml). They were then rinsed in 1·5 ml TE buffer (20 mM tris base, 50 mM EDTA, pH 8·0) for 15 min, then incubated for 16–20 h at 50 °C in a second lysis buffer (100 mM EDTA, 0·2% sodium deoxycholate, 1·0% sodium lauryl sarcosine, pH 8·0). The blocks were washed three times for 45 min in TE buffer and stored at 4 °C in fresh TE buffer, if necessary, prior to restriction endonuclease (RE) digestion with *Xba*I (Boehringer Mannheim Lewes, UK) as described previously [22]. Each of the isolates was subjected to *Xba*I digestion and PFGE twice and the same band pattern was produced on both occasions. Thus the technique produces consistent patterns that are reproducible.

Pulsed field gel electrophoresis and analysis

PFGE was carried out using an LKB Gene Navigator System (Pharmacia) according to manufacturer's instructions. Samples were electrophoresed on 15 × 15 cm 1·2% agarose gel in 0·5 × TBE (4 mM tris

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borate, 2·5 mM EDTA, pH 8·3) gels (Gibco BRC, Paisley, UK) with a digested lambda DNA concatemer molecular size marker (Boehringer Mannheim). The electrophoresis parameters, were based on those of Gueirard and colleagues [23] and comprised an initial pulse time of 10 s for 10 h, stepped up sequentially to 20 s for 12 h, 30 s for 10 h, 60 s for 4 h and 120 s for 4 h. The potential difference across the gel was 10 v/cm and the total run time 40 h. Gels were stained in 0·5 mg/l ethidium bromide (Sigma, Poole, UK) for 30 min, viewed on an ultraviolet transilluminator and photographed using a Polaroid 667 film (Polaroid MA, USA) with an exposure time of 0·5 s at f8. The PFGE patterns were analysed digitally using a Sun Unix system and BioImage Whole Band Analyser Software [26]. Gels were scanned into the system at 150 d.p.i. from photographic images using a Scanmaster 3+ (Howtex, USA). Band patterns were adjusted manually and any small poorly resolved bands were not included in the scanner image. They were then analysed digitally using a deviation table that gave low tolerance for error (2% deviation) at larger band sizes (100–500 kb) and higher tolerance (60% deviation) at the lower band sizes (< 100 kb) where resolution was relatively poor. The percentage similarity value was calculated for each pair of isolates by the computer algorithm, and sorted similarity matrices and dendrograms showing phenetic classification were constructed using the unweighted pair group method with arithmetic averages (UPGMA) algorithm also known as average-linkage clustering [27]. Isolates were classified as identical if they showed no band differences (100% similarity), as subtypes of the same strain if they showed 1–3 band differences (84–99% similarity) and as different strains if they showed 4 or more band differences (83% similarity or lower) [28].

RESULTS

PFGE of *Bordetella* spp produced well resolved patterns of bands ranging from 20–500 kb (Fig. 1). The majority of isolates produced between 20–25 bands. The *B. bronchiseptica* type strain (NCTC 452) was included on each gel (i.e. 32 times) and in each case produced an identical pattern.

The type strains of *B. pertussis*, *B. parapertussis*, *B. avium*, *B. hinzii* and *B. holmesii* produced PFGE patterns that differed from each other and from all the *B. bronchiseptica* isolates by more than 4 bands. On UPMGA they each fell into separate branches of the dendrogram.

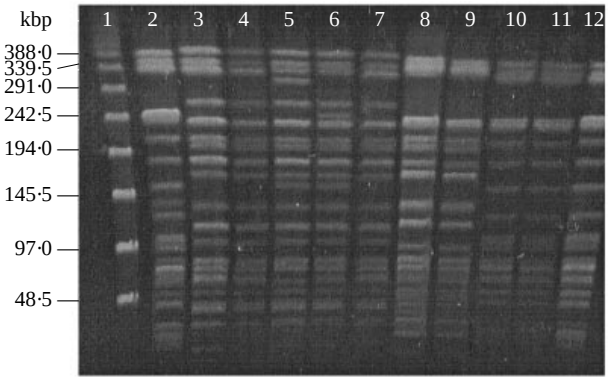

Fig. 1. Pulsed-field gel electrophoresis of *Xba*I-digested genomic DNA from feline isolates of *Bordetella bronchiseptica*. Lane 1: lambda concatemer molecular size marker; Lane 2: type strain of *B. bronchiseptica* (NCTC 452); Lanes 3–7: feline isolates from breeding cattery 2; Lanes 8 and 9: feline isolates from pet household 1; Lanes 10–12: feline isolates from rescue cattery 1.

The 164 *B. bronchiseptica* isolates (Table 1) clustered into a total of 17 strains (differing by > 3 bands) although 126 isolates (77%) fell into 2 strains (A and B). Within 8 of the strains (A, B, C, E, F, K, M, Q) there were varying numbers of subtypes (differing by ⩽3 bands). The 82 isolates in strain A fell into 55 subtypes, and the 44 isolates in strain B into 17 subtypes, while each of the isolates of strains E, K, and Q comprised a separate subtype (Table 1).

The human (NCTC 8750) and guinea-pig (NCTC 10540) *B. bronchiseptica* were the only isolates in strain Q but were of different subtype. The *B. bronchiseptica* type strain, originally isolated from a dog [29] was strain B.

Feline isolates

The feline isolates were represented in 12 of the 17 strains. Eight strains (D, E, G, I, J, L, M and P) contained only feline isolates but four of those strains contained only one isolate (Table 1). Mixed populations were found in some cats. Thus of six cats from which nasal and oropharyngeal isolates were obtained 4 pairs of samples were of identical strain and subtype. However, one cat had isolates of the same strain but different subtypes, and one cat had entirely different strains.

Of the 11 repeat isolations made from cats in breeding colonies, 1, 2, 3 and 4 and research colony 1,

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Table 1. *Similarities and differences between bordetella isolates following* XbaI *digestion and PFGE*

Strain*	Number of isolates	Subtypes*	Number of cat isolates	Number of dog isolates	Number of pig isolates	Other species
A	82	55	68	3	9	Rabbit, vaccine
B	44	17	38	5	0	Snow leopard
C	6	3	4	0	2	0
D	4	0	4	0	0	0
E	6	6	6	0	0	0
F	4	3	0	4	0	0
G	1	0	1	0	0	0
H	1	0	0	1	0	0
I	1	0	1	0	0	0
J	4	0	4	0	0	0
K	2	2	1	0	1	0
L	1	0	1	0	0	0
M	3	2	3	0	0	0
N	1	0	0	1	0	0
O	1	0	0	1	0	0
P	1	0	1	0	0	0
Q	2	2	0	0	0	Human, guinea-pig

* Strains differ by four or more bands. Subtypes within the strain differ by three or fewer bands. *B. holmesii* (NCTC 12912, as human), *B. parapertussis* (NCTC 5952, human), *B. avium* (NCTC 12033, turkey), *B. hinzii* LMG 13501, chicken) and *B. pertussis* (NCTC 10739, human) differed by more than four bands from each other and all *B. bronchiseptica* isolates by more than four bands.

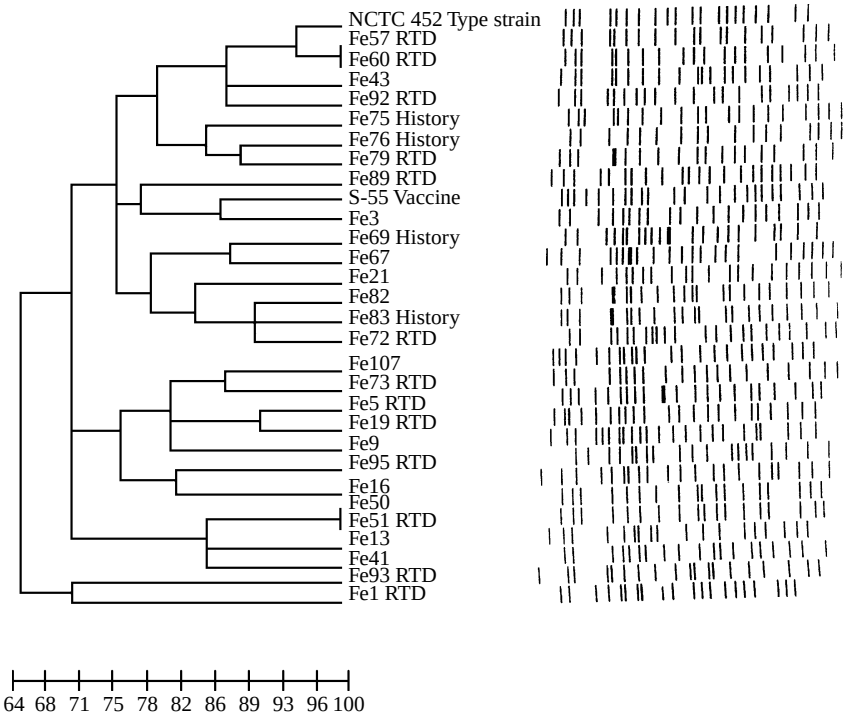

Fig. 2. Dendrogram obtained using average linkage cluster analysis of similarity values obtained from the PFGE patterns represented on the right of the figure. Isolates of *B. bronchiseptica* obtained from a representative selection of cats with and without clinical signs of respiratory tract disease (RTD), or a history of RTD, cluster together.

6 were of the same strain as the original isolation with 5 belonging to different strains and none were of the same subtype.

To examine the possibility that *B. bronchiseptica* isolates obtained from cats with respiratory tract disease were different from those from cats with no

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Table 2. *Molecular epidemiology of* B. bronchiseptica

	Number of isolates tested	Animals	Strain/Subtype (Number of isolates)
Breeding colony			
1*	9	Cats	A1, A2(2), A3, A4, C1(4)
1*	4	Cats	A12(4)
1*	2	Cats	A42, E6
2*	11	Cats	A5, A6, A7, A8, A9, A10(2), D1(4)
2*	11	Cats	A21(4), A22(2), A23, A24(2), A25, A26
3*	2	Cats	A11, E1
3*	13	Cats	A10, A12(2), A13, A14, A15, A16, A17, A18, A19(2), A20(2)
4*	3	Cats	A30, A31, B8
4*	3	Cats	A32(2), A44
5	3	Cats	J1(3)
6	3	Cats	A40(2), J1
Research colony			
1*	6	Cats	A34(4), A35, B11
1*	3	Cats	A37(2), L1
2	3	Cats	M1(2), M2
3	3	2 Cats	A28, A29
		1 Dog†	N1
House Pet			
1	2	Cats	A27(2)
2	2	Cats	A36(2)
3	2	Dogs	A41(2)
Rescue			
1	18	Cats	B1(4), B2(9), B3(5)
2	4	Cats	B5, B6, B7(2)
3	9	Cats	B9(4), B10(5)
4	2	Cat	B3
		Dog	B3
5	4	Cats	B1, B12, B13(2)
6	7	1 Cat	A38
		6 Dogs	A39, F1, F2(2), F3, H1
7	2	Cats	G1, P2

* Indicates that the same colony was sampled repeatedly but not necessarily the same animals.
† The dog and cats were housed separately.

disease, UPGMA cluster analysis was performed. From Fig. 2 it can be seen that there was no correlation between an isolate that was made from a symptomatic cat and the strain found.

Isolates from other species

The 15 canine isolates of *B. bronchiseptica* were distributed among six strains (A, B, F, H, N, O), and of these only strains A and B contained isolates from other species. Only two isolates were of identical strain and subtype. The canine bordetellosis live vaccine (S-55; originally obtained from a pig) was in strain A but was of different subtype to the three other canine isolates in strain A. Nine (75%) of the 12 porcine isolates were in strain A and of these, three were of identical subtype, despite being obtained from pigs in different parts of the country.

Molecular epidemiology

The cats sampled in this study came from two major types of housing. Some were maintained in relatively stable households (breeding colonies, research colonies and house pets) and others were cats in rescue homes (Table 2). The cats in stable households were predominantly colonized by strain A *B. bronchiseptica* (66/82:80%) whereas those from rescue cats were of

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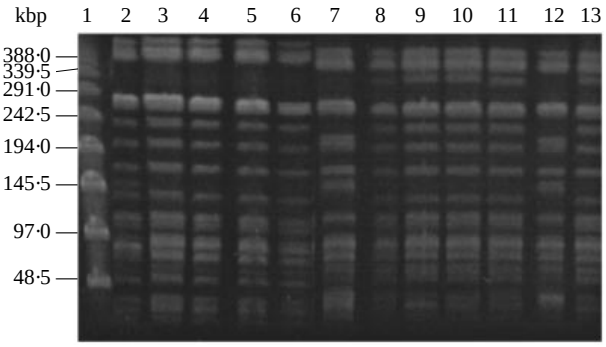

Fig 3. Banding patterns obtained from dogs and cats kept on the same premises had similar banding patterns. Lane 1: lambda concatemer molecular size marker; Lane 2: type strain of *B. bronchiseptica* (NCTC 452); Lanes 3, 5 and 6: feline isolates from rescue colony 3; Lane 4: canine isolate from rescue colony 3; Lane 7: feline isolate from rescue colony 6; Lanes 8–13: canine isolates from rescue colony 6. The feline isolate in lane 7 can be sen to be most clearly related to the canine isolate in lane 12.

strain B (36/39:92%). In general, isolates made from cats within the same household were of the same strain and often the same or closely related subtype (Fig. 1, Table 2).

In households where there were cats and dogs in contact the *B. bronchiseptica* isolates tended to be of the same strain and of closely related subtype (Fig. 3). Although no samples were obtained from pigs in contact with cats or dogs the majority of pigs (75%) had isolates of *B. bronchiseptica* very similar to those of cats and dogs and none was in a species-specific strain (Table 1).

The canine bordetellosis vaccine strain was originally obtained from a pig and was typed as strain A to which most of the porcine isolates belonged. Interestingly the majority of the feline isolates (50%) were strain A but only three (25%) of the canine isolates were in this strain.

DISCUSSION

In this study we have investigated the usefulness of macrorestriction PFGE in typing *B. bronchiseptica* isolates from cats and other species. Using *Xba*I the 164 isolate fell into 17 distinct strains that differed by 4 or more bands on PFGE [25, 28]. Each isolate was tested twice and the type strain of *B. bronchiseptica* on 32 occasions and the band patterns were completely reproducible.

It has been suggested previously that *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* are sufficiently closely related to be regarded as a single species [16, 22, 30]. In addition to the above species we also included *B. hinzii*, *B. holmesii* and *B. avium* in our study. On UPGMA cluster analysis each of the *Bordetella* species differed from each other and each of the *B. bronchiseptica* strains by more than four bands, producing well separated branches on the dendrograms (data not shown).

The 132 *B. bronchiseptica* isolates from cats fell into 12 strains, but the vast majority of isolates (77%) fell into 2 strains (A and B). However, within each of these 2 strains there were large numbers of subtypes. This is in contrast with a study of human and ovine isolates of *B. parapertussis* which showed only 3 distinct patterns each [21]. The diversity of *B. bronchiseptica* isolates demonstrated by PFGE in our study confirms findings obtained using multilocus enzyme electrophoresis (MLEE) [31] and analysis of genomic insertion sequences [14]. Furthermore these results support the hypothesis that *B. bronchiseptica* may have diverged relatively early in evolutionary terms which may be related to its ability to infect many different mammalian host species [31].

The 2 or 3 fragment differences in the banding patterns of related isolates within a strain is probably linked to a single genetic event such as point mutation, insertion or deletion of DNA [28]. Such differences do provide markers for distinguishing subtypes but are presumably insufficiently large to distinguish isolates for epidemiological purposes. The clustering of isolates within household groups supports the observations of other authors that PFGE is valuable in investigating outbreaks of *B. pertussis* where intrafamilial epidemic transmission can be tracked [32, 33].

The *B. bronchiseptica* isolates from cats within the same households tended to be of the same strain and were often within the same subtype. Overall strain B predominated in rescue catteries and strain A in other types of household. Differences seen in isolates from repeat samplings may indicate co-infection or re-infection with different strains. In experimental studies in specific-pathogen-free cats banding patterns appear to remain stable over 19 weeks repeated sampling [34].

There was no difference in PFGE patterns of isolates from cats with and without respiratory disease. Although *B. bronchiseptica* is known to be a cause of respiratory disease in cats, longterm asymptomatic carriage of the organism also commonly occurs [6, 12, 35]. These results suggest that *B. bronchiseptica* is an opportunistic pathogen of cats, and that the likelihood of a particular *B. bronchiseptica* isolate producing disease is probably related to host or

environmental factors such as crowding, stress and hygiene [36]. It may also be related to differential expression of virulence determinants as has been demonstrated for canine or porcine infections where phase variation occurs [37, 38].

Isolates from pigs and dogs were in general similar to the feline isolates and there was no great evidence for species specificity. The clustering of *B. bronchiseptica* isolates from cats and dogs appeared to be based on epidemiological origin rather than on the particular host species. None of the porcine isolates were of a host-species specific strain but 7 of the 15 dog isolates were in canine specific strains. Unfortunately the origins of the porcine isolates were unknown. The relative lack of host specificity amongst *B. bronchiseptica* isolates is supported by MLEE data [31] and by previous PFGE studies [22, 23]. In contrast, analysis of insertion sequence elements found the majority of pig and rabbit isolates similar, but different from most of the canine and feline isolates [14]. Interestingly, using PFGE, human and ovine isolates of *B. parapertussis* appeared to be host specific [21].

The *B. bronchiseptica* (S-55) strain which is used as a live intranasal vaccine to prevent canine kennel cough was originally isolated from porcine lung [39]. It was not closely related to any canine isolate and only one feline isolate was in the same strain and subtype. Thus, in the small number of dogs tested, there was no evidence that the vaccine strain spread or persisted.

Finally, the lack of absolute host-species-based clustering of *B. bronchiseptica* isolates has implications for cross-species transmission in particular between cats and dogs. Isolates from dogs and cats housed together showed identical or very similar PFGE patterns. This lends support to the anecdotal reports of cross-species transmission [40]. The possibility of zoonotic spread of *B. bronchiseptica* to man has been raised [9]. A subsequent study demonstrated transfer of *B. bronchiseptica* from a rabbit to a human [23]. Interestingly the only human isolate (type strain) from our study clustered in a strain separate from all other isolates except for the guinea-pig isolate type strain.

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