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Variation in *Bordetella bronchiseptica flaA* does not correlate with typing by macro-restriction analysis by pulsed-field gel electrophoresis

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A genotyping method based on PCR-RFLP analysis of the flagellin gene (*flaA*) was applied to 30 mainly feline isolates of *Bordetella bronchiseptica.* These isolates were separated into three PCR-RFLP groups with the restriction endonucleases *Hae*III, *Msp*I, *Mbo*I and *Rsa*I. *flaA* nucleotide sequences representing each of the three groups differed from each other by 11–13%. One of the groups exhibited far greater *flaA* sequence identity with the cryptic flagellin gene sequence of *B. pertussis* (>97%) than with *flaA* sequences from representatives of the other *B. bronchiseptica* PCR-RFLP groups. Amongst the 30 isolates were at least 10 representing each of the two major genotypes (A and B) identified in a previous study by macro-restriction analysis and pulsed-field gel electrophoresis (PFGE), as well as representatives of other less common genotypes. Each of the major PFGE genotypes contained strains representing more than one flagellin genotype. Indeed, there was no correlation between the two molecular typing methods. PFGE analysis may identify differences due to genomic re-arrangements rather than genuine variations in gene content. If so, relationships inferred on the basis of PFGE or other molecular methods for whole genome comparison should be treated with caution.

Introduction

Bordetella bronchiseptica is a respiratory pathogen of a wide range of domestic and other animals [1] and has also been associated with human infections [2]. It has been recognised as a primary pathogen in canine kennel cough [3] and respiratory disease in cats [4–6]. A number of molecular methods for strain discrimination has been developed to study the epidemiology of *B. bronchiseptica*. These include randomly amplified polymorphic DNA (RAPD) fingerprinting [7], ribotyping [8] and macro-restriction analysis by pulsed-field gel electrophoresis (PFGE) [9, 10]. A study of a collection of 164 (predominantly feline) isolates of *B. bronchiseptica* by PFGE following macro-restriction digestion with the endonuclease *Xba*I concluded that although the isolates fell into 17 different strains, 126 of these isolates were assigned to one of two strains (named A and B) [10]. Isolates were classified as identical if they showed no band differences, as subtypes of the same strain if they showed one-to-three band differences, and as different strains if they showed four or more band differences (corresponding to ⩽83% similarity).

Flagella, consisting of basal body, rod and filament, are the organelles of motility in many bacterial pathogens, including *B. bronchiseptica*. It has been suggested that flagella may also contribute to the adherence of *B. bronchiseptica* to eukaryotic cells [11]. The genus *Bordetella* contains both motile (*B. bronchiseptica* and *B. avium*) and non-motile (*B. pertussis* and *B. parapertussis*) species. However, all four of these species contain DNA sequences homologous to genes encoding the major filament protein, flagellin [12]. The presence of a non-expressed flagellin gene in the *B. pertussis* strain Tomaha I has been confirmed by genome sequencing (data can be obtained from ftp://ftp.sanger.ac.uk/pub). The BvgAS signal transduction system of *B. bronchiseptica* modulates motility as well as a number of well characterised virulence factors, such as filamentous haemagglutinin, pili and adenylate cyclase toxin [13, 14]. The flagellin gene (*flaA*) is known to be negatively regulated by the Bvg regulon such that when the major virulence factors are expressed (Bvg$^+$ phase), the flagellin gene is not and

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vice versa [13]. The bacterial flagellin gene has been used as a variable biomarker to study genetic variation amongst a number of bacterial pathogens [15], including *Burkholderia cepacia* [16] and *Pseudomonas aeruginosa* [17]. The study of variation within individual genes can provide an approach to analysing relationships between bacterial strains that is different from many molecular methods, which rely on whole genome analysis.

This study reports the development of a flagellin gene PCR-restriction fragment length polymorphism (RFLP) technique for *B. bronchiseptica*. This technique, coupled with nucleotide sequencing of *B. bronchiseptica* flagellin genes, was used to assess genetic variation and relationships between strains. The study of single gene variation was compared with whole genome variation as demonstrated by PFGE.

Materials and methods

Bacterial strains

The *B. bronchiseptica* isolates used in this study are listed in Table 1. All the isolates had been typed previously by PFGE after macro-restriction digestion with *Xba*I (Table 1) [10].

PCR-RFLP analysis of B. bronchiseptica flagellin genes

Oligonucleotide primers for amplification of *B. bronchiseptica* flagellin genes were designed from the published *flaA* coding sequence (1176 bp in length) for the strain GPISN [18]. A 1156-bp region of the *flaA* coding sequence (corresponding to a region of GPISN *flaA* from position 2 to 1157) was amplified by PCR with oligonucleotide primers BBFLAF: 5′-TGGCTGCAGTCATCAATA-3′ (N-terminal region, forward primer) and BBFLAR: 5′-ACGTTTTGCGGG ACTTGG-3′ (C-terminal region, reverse primer), obtained from Sigma-Genosys (Cambridge). Strains were screened by first making a suspension of a few colonies in 20 μl of sterile distilled water and boiling for 5 min. This crude DNA (1 μl) was used directly in 50-μl volumes containing 2 units of Dynazyme (Flowgen Instruments, Sittingbourne, Kent), 200 nM of each primer, 1 × Dynazyme buffer and 100 μM nucleotides (dATP, dCTP, dGTP, dTTP). Amplifications were performed in an OmniGene thermal cycler (Hybaid, Ashford, Middlesex) for 30 cycles consisting of 95°C (1 min), 55°C (1 min) and 72°C (2 min). At the end of the amplification 10-μl samples were subjected to electrophoresis on a standard agarose 1.0% w/v gel to confirm the presence of an amplified product. Samples of amplified product (5 μl) were digested with the restriction endonucleases *Hae*III, *Msp*I, *Mbo*I and *Rsa*I with the conditions recommended by the supplier (Helena Biosciences, Sunderland, Tyne and Wear). These digests were then subjected to electrophoresis on MetaPhor agarose 1.5–2% w/v gels (Flowgen Instruments).

Nucleotide sequencing and computer analyses

Both strands of the PCR-amplified products of the flagellin gene were sequenced by the University of Liverpool DNA Sequencing Service with the primers BBFLAF and BBFLAR and other internal primers. Flagellin gene nucleotide sequences were aligned with each other or with other flagellin genes (retrieved from EMBL, GenBank or ftp://ftp.sanger.ac.uk/pub) by the programs GAP, PILEUP, FASTA and PRETTY from the GCG sequence analysis software package (Genetics Computer Group, University of Wisconsin, USA) [19].

Nucleotide sequence accession numbers

The nucleotide sequences obtained from the *flaA* genes of strains SB22, SB521 and SB283 have been assigned GenBank accession numbers AF232939–41.

Results

PCR-RFLP of flagellin genes

PCR amplicons of the flagellin genes were obtained from all the *B. bronchiseptica* isolates tested. Each of the amplicons was subjected to restriction digestion with four different endonucleases. Digestion with *Hae*III, *Msp*I and *Rsa*I each resulted in three different patterns, whereas only two patterns were obtained after digestion with *Mbo*I. The patterns obtained with an individual enzyme were designated A, B or C, depending on whether they were most common (A), second most common (B) or third most common (C). A gel showing examples of digests obtained with *Hae*III is shown in Fig. 1. Table 1 summarises the PCR-RFLP profiles of all the isolates, which fell into three groups. Each of these three groups could be distinguished by using *Hae*III, *Msp*I or *Rsa*I individually.

Of the 11 strains belonging to PFGE subtypes of strain A, five belonged to the most common *flaA* PCR-RFLP group (restriction pattern A with all four enzymes), with the other six belonging to the second most common PCR-RFLP group (restriction pattern B with all four enzymes). The distribution was less equal amongst the 10 strains belonging to PFGE subtypes of strain B, eight of which belonged to the most common *flaA* PCR-RFLP group. Where PFGE subtypes were the same, PCR-RFLP analysis of the flagellin gene supported the relationship (two strains each of subtypes B3 and B7).

Flagellin gene sequences

Representative isolates from each of the three *flaA* PCR-RFLP groups (SB22, SB283 and SB521) were

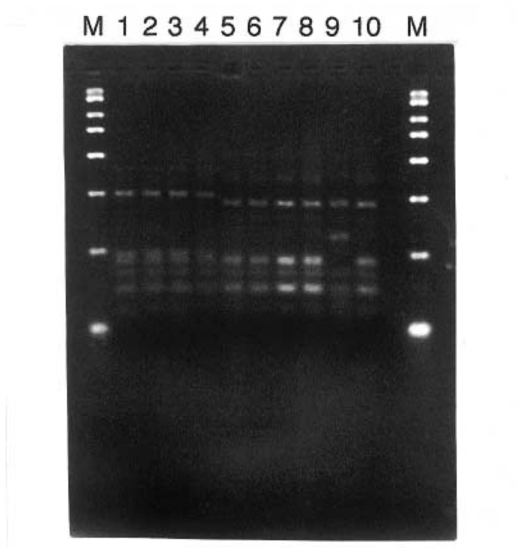

Fig. 1. Flagellin gene PCR products digested with *Hae*III. The gel shows a number of amplicons derived from different isolates of *B. bronchiseptica*, and subsequently digested with the endonuclease *Hae*III. Lane **M**, PCR size marker (fragment sizes 2000, 1500, 1000, 750, 500, 300, 150 and 50 bp; R&D Systems, Abingdon, Oxfordshire). Lanes **1**–**4**, amplicons with PCR-RFLP pattern A; **5**–**8** and **10**, amplicons with PCR-RFLP pattern B; **9**, an amplicon with PCR-RFLP pattern C.

used to obtain flagellin gene sequences. These sequences were aligned with the previously published sequence from *B. bronchiseptica* strain GPISN [18] and the *B. pertussis* strain Tomaha I cryptic flagellin gene sequence (Fig. 2). The percentage identities for each pair of flagellin gene sequences were calculated over a region of the *flaA* gene corresponding to 1051 bp of the strain GPISN sequence (Table 2). Flagellin gene nucleotide sequences representing each of the three groups differed from each other by 11–13%. By far the greatest variation was observed in the central region of the *flaA* gene (Fig. 2). The *flaA* sequence obtained from strain SB283 was 99.5% identical to the reported sequence for strain GPISN (100% identical in predicted protein amino-acid sequence). Interestingly, both nucleotide and predicted amino acid sequence comparisons indicated that *B. bronchiseptica* SB283 and GPISN flagellin sequences are more closely related to the *B. pertussis* Tomaha I flagellin sequence than to the flagellin sequences of the other *B. bronchiseptica* isolates (Table 2).

Discussion

Isolates of *B. bronchiseptica* were separated into three groups by PCR-RFLP analysis of flagellin genes. Each of the groups gave different restriction digest patterns with at least three endonucleases, which means that the PCR-RFLP groups identified in this study can be distinguished by single enzyme digests with *Hae*III, *Msp*I or *Rsa*I. As the strains used in this study were resolved into 10 groups by macro-restriction analysis with *Xba*I followed by PFGE, it is apparent that the

Table 1. Description of strains used in this study

Isolate no.	Source	PFGE subtype*	Flagellin gene RFLP patterns			
			*Hae*III	*Mbo*I	*Msp*I	*Rsa*I
SB22	Cat	A1	B	B	B	B
SB55	Cat	A6	A	A	A	A
SB48	Cat	A10	A	A	A	A
SB80	Cat	A11	B	B	B	B
SB101	Cat	A12	B	B	B	B
SB110	Cat	A13	B	B	B	B
SB116	Cat	A19	B	B	B	B
SB200	Cat	A22	A	A	A	A
SB193	Cat	A24	A	A	A	A
SB197	Cat	A26	A	A	A	A
SB236	Cat	A27	B	B	B	B
SB283	Cat	B1	A	A	A	A
SB297	Cat	B2	A	A	A	A
SB299	Cat	B3	A	A	A	A
SB794	Cat	B3	A	A	A	A
SB452	Cat	B5	C	A	C	C
SB455	Cat	B6	B	B	B	B
SB461	Cat	B7	A	A	A	A
SB503	Cat	B7	A	A	A	A
SB866	Cat	B11	A	A	A	A
SB868	Cat	B12	A	A	A	A
SB18	Cat	C1	B	B	B	B
SB83	Cat	E1	B	B	B	B
SB534	Cat	E2	B	B	B	B
SB998	Dog	H1	A	A	A	A
SB909	Cat	I1	A	A	A	A
SB939	Cat	J1	A	A	A	A
SB521	Cat	K1	C	A	C	C
SB925	Cat	L1	A	A	A	A
SB660	Dog	N1	A	A	A	A

*Determined in a previous study [10].

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       351                                                    401                                              450
SB283  .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Bb     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Bp     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
SB22   .......... .......... .......... .......... .......... .......... .......... .......... ccgc...ca. .tc.......
SB521  .......... ..ga.gga.. ..g....... .......... .......... .......... .........c .......... ccgc...ca. .tc.......
CON    CCTGAAGTCC AACGCCACCG ACATGACCCT GTCGATCCAG GTCGGCGCCA AGGACAACGA AACGATCGAT ATCAAGATCG ATCGCAACTC GAACTGGAAC

       451                                                    501                                              550
SB283  .......... .......... .......... .......... .......... .......... ......t... .......... .......... ..........
Bb     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Bp     .......... .......... ......c..t .......... .......... .......... .......... .......... .......... ..........
SB22   a.t..c.... ...ct..tg. ga....c... ac.ca...t. .......a.. c....aa..g gc...c.... .......... ......c..t .ctg...g..
SB521  .aa..c.... .gc....... g......a.. .......... .......a.. c....aa..g gc...c.... .......... ......c... ac.g.at...
CON    CTGTATGACG CCGTGGGCAC CGTCCCGGGC GGCACGGTCA ACGGCGAGGC TCGCACCGTC AACGCGCTGG GCTTTGACGT GCTGTCGGCC GTCACGACCA

       551                                                    601                                              650
SB283  .......... c......... .......... .......... ......t... .......... .........t .......... .......... ..........
Bb     .......... c......... .......... .......... ......t... .......... .........t .......... .......... ..........
Bp     .......... g......... .......... .......... .......... .....t.... .......... .......... .......... ..........
SB22   ..g.t...g. a..t.....t t.g.....t. .......... t..t...atc xxx..t.gcc at..t.g... cacg.gt... gag...cg.. ..........
SB521  .ggc..acgt g..ag..a.c t.g....... ....t..c.. ca........ xxx...aagc a.a....... c.c.ac.a.. gt....aa.c a.........
CON    CCATCGCTTC -GACACCGTG ACCTTCGACG CCGCCGTGGC GGCCGCCGAA CAGGCCGCTG GCGCCGCCGC AGGCGACGGC AGCGTCGTCT CGTACGGCGA

       651                                                    701                                              750
SB283  .a........ .......... .......... xxx....... .......... .......g.. .......... .......... .......... ..........
Bb     .a........ .......... .......... xxx....... .......... .......g.. .......... .......... .......... ..........
Bp     .......... .......... .......... xxx......c .........c .......... .......... .......... .......... ..........
SB22   c..a...... .......... .c..g...c. gg.t...gcg .g....gac. .cg.ag.... t......... .......... .t........ ...t......
SB521  .......... ..c..g.... .a........ ta.t...gg. .g..t.gac. xxx..g.... .........t .......... .......... .a........
CON    TGCCGCCAAC CCGCAATACG CGGTCGTGGT --CCGACAAT GCCGGCACGA TGACCTCCTA CGCCCTGACC TTCGACAAGG ACGGCAAGGC CGCCCTGGGC

       751                                                    801                                              850
SB283  .....g.... .......... c........t ..g....... ........a. .......... .......... .......... .......... ..........
Bb     .....g.... .......... c........t ..g....... ........a. .......... .......... .......... .......... ..........
Bp     .......... .......... t.....a..c ..t....... .......c.t .......... ..ct...... a......... .......... ...aa....a
SB22   ...g...... .c..g..t.. t.....a..c ..tctc...t .gacgac..t t.t..c.aa. a.c.a.acg. xxxxxx.... .......... ...aa.....
SB521  ..ag....c. .c..g..... ga.......a ..c.cc..tt .gacgacg.. ..t.ac.aat ...t.gacg. g..xxx.... .......... ..........
CON    GACCAACTGG GTGCCGTCGC -TCGCAGGC- GC-GAAGCCG CCGTCGGTGC CAACGACGTC GCTGCCGGCG CCAACGTCAC CGTGTCCGGC GGCGCCGCCG
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Fig. 2. Flagellin gene nucleotide sequence alignment. The *flaA* sequences were edited to correspond to a 1051-bp region of *B. bronchiseptica* strain GPISN and aligned by the programs PILEUP and PRETTY. The figure shows only the central, most variable region of the flagellin gene (commencing at position 351 and ending at position 850 of the 1051-bp region). The sequences include those from *B. bronchiseptica* GPISN (Bb) and *B. pertussis* Tomaha I (Bp). A consensus sequence (CON) is also shown. In the other sequences, only nucleotides differing from the consensus are shown. Gaps in individual sequences are represented by a cross (x). Positions for which there is no consensus are indicated by a dash (–).

Table 2. Comparison of nucleotide sequences of flagellin genes

Flagellin sequence of isolate no.	Percent identity* to flagellin sequence of strain			
	GPISN	SB22	SB521	SB283
SB22	87.9 (86.5)			
SB521	87.2 (83.9)	88.7 (86.4)		
SB283	99.5 (100)	87.7 (86.4)	87.1 (83.9)	
B. pertussis Tomaha I	97.3 (97.7)	88.7 (87.3)	86.9 (83.6)	97.1 (97.7)

*Levels of identity were determined over a 1051-bp region of *B. bronchiseptica* GPISN *flaA* sequence using the GAP program. Values in parentheses indicate comparisons between predicted amino-acid sequences for the equivalent region of *flaA*.

flagellin gene PCR-RFLP method is not as discriminatory as PFGE. The isolates used in this study were chosen to include at least 10 representatives of each of the major PFGE types (A and B) identified in a previous study [10]. When the results from PFGE and flagellin gene PCR-RFLP were compared, there was no direct correlation between PFGE types and *flaA* PCR-RFLP groups. Nucleotide sequence analysis confirmed the presence of three flagellin gene groups differing by 11–13% from each other.

Proteins present on the surface of bacterial cells, such as the flagellin protein, are known to be more variable than other bacterial proteins [20]. However, flagellin gene variation reflects the clonality of the organism under study. For example, in *Burkholderia cepacia*, flagellin genes are highly variable and can be readily separated into numerous PCR-RFLP groups [16, 21], whereas in *Bur. pseudomallei* very little flagellin gene variation is observed, especially amongst clinical isolates [22]. These observations correlate with the genetic variation observed in other approaches. In *Bur. cepacia*, genomic variation is so apparent that the species has been subdivided into genomovars [23]. In one study, the genetic relatedness of *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* was examined by macro-restriction digestion of DNA with the rarely cutting endonucleases *Xba*I and resolution by PFGE. When the results of this work were compared with those of previous DNA-DNA hybridisation and multilocus enzyme electrophoresis studies, the authors concluded that these three species are so closely related that they should be considered members of the same species [9]. Indeed, *flaA* nucleotide sequence data suggest that the most common *B. bronchiseptica* flagellin gene group is more closely related to *B. pertussis* Tomaha I than to the other *B. bronchiseptica* flagellin gene groups. This has implications for the whole genome analysis following on from the completion of the genome sequences of *B. pertussis* Tomaha I and *B. bronchiseptica* RB50, the *flaA* sequence of which shares 99.9% identity with the *flaA* gene of GPISN (data not shown). Greater genetic variation may occur between strains currently designated as *B. bronchiseptica* than occurs between the strains of these two species chosen for whole genome sequencing. As has been observed in many other studies of flagellin genes, the variation is apparent mainly in the central region of the gene [15].

It is clear from this study that relationships between *B. bronchiseptica* isolates inferred by macro-restriction analysis do not agree with those calculated on the basis of sequence similarity in the flagellin genes. A recent study comparing the genomic organisation of 14 clinical isolates of *B. pertussis* revealed that whilst the genomic maps demonstrated general similarity in gene order, there were a number of examples of genomic re-arrangements in the form of large chromosomal inversions [24]. If such events were also found in *B. bronchiseptica*, then many of the current molecular methods for strain differentiation would discriminate between strains containing essentially the same genetic information. Whilst such genetic re-arrangements would not invalidate the use of such techniques in epidemiological studies aimed at identifying identical strains, the possibility of such events suggests that relationships inferred from macro-restriction analysis by PFGE and other whole genome approaches should be treated with caution. The study of variations in other genetic loci in *B. bronchiseptica* will help to resolve the question of whether relationships inferred on the basis of flagellin gene sequences are more reliable than those calculated on the basis of restriction site variation detected by PFGE.

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