MOLECULAR TYPING AND EPIDEMIOLOGY

Genetic diversity of canine gastric helicobacters, *Helicobacter bizzozeronii* and *H. salomonis* studied by pulsed-field gel electrophoresis

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Genetic diversity of *Helicobacter bizzozeronii* and *H. salomonis*, two recently identified canine gastric *Helicobacter* spp., was studied by pulsed-field gel electrophoresis (PFGE). All 15 Finnish *H. bizzozeronii* strains collected between 1991 and 1996 from pet dogs produced different PFGE patterns with all restriction endonucleases studied (*AscI*, *ApaI*, *SpeI*, *NotI* and *PacI*) suggesting significant genetic diversity. The five independent *H. salomonis* strains produced four different patterns with these enzymes; two strains showed identical patterns with all the enzymes. Three separate isolates from one dog had identical patterns, suggesting long-lasting infection with the same strain. *H. salomonis* strains had several small fragments common for all strains, suggesting relatedness. The PFGE method was shown to be useful for epidemiological studies of canine gastric helicobacter infection. Hybridisation of the DNA digests with digoxigenin-labelled *ureB* or 16S rRNA gene probes generated by PCR indicated conservation in the localisation of these genes in the *H. salomonis* genome, because the probes hybridised with similar size fragments of different strains. In contrast, the probes hybridised with different size fragments of *H. bizzozeronii* strains. Comparison of Southern blots of PFGE patterns digested with *SpeI*, *ApaI* and *AscI* indicated that each species has two 16S rRNA genes and one urease gene. Genome sizes of 11 *H. bizzozeronii* strains estimated from *SpeI* and *NotI* patterns were c. 1.6–1.9 Mb and those of five *H. salomonis* strains estimated from *NotI* and *PacI* patterns were c. 1.7–1.8 Mb.

Introduction

The gastric mucosa of dogs is colonised by three *Helicobacter* spp. – *H. felis* [1], *H. bizzozeronii* [2] and *H. salomonis* [3]. Some dogs are colonised also by an uncharacterised *Helicobacter* sp., provisionally called ‘flexispiras’ [4]. *Helicobacter* infection of canine gastric mucosa is common, almost all dogs being infected with one or more gastric *Helicobacter* spp. [4–6]. The pathogenicity of these organisms is unknown. They are fastidious and their in-vitro culture requires special conditions [2].

H. pylori, a human-adapted *Helicobacter* sp., has been well-characterised because of its significance in the pathogenesis of chronic active gastritis and peptic ulcer. This organism is also strongly associated with human gastric cancer [7]. The genome of *H. pylori* has been characterised and the whole genome

sequence analysis has been published recently [8]. The intra-species genetic diversity has been studied also by various genetic methods such as macro-restriction analysis of the whole genome by pulsed-field gel electrophoresis (PFGE) [9, 10], by multi-locus enzyme electrophoresis (MLEE) [11], ribotyping [12], randomly amplified polymorphic DNA [13] and polymorphism of urease [14, 15] and *vacA* [16] genes. All these methods have revealed significant genetic diversity, probably a necessary characteristic for a pathogen adapted to one mammal species, man. Genetic diversity of other *Helicobacter* spp. is not well known, except for the genome of *H. mustelae*, which was shown to be rather homogeneous by PFGE, when American and European strains were compared [17], whereas the PFGE patterns of *H. hepaticus* showed some diversity [18].

In the present study, genetic diversity of *H. bizzozeronii* and *H. salomonis* strains from Finnish dogs was studied by PFGE with several restriction endonucleases. PFGE patterns allowed the determination of

the genome sizes and the number of *ureB* and 16S rRNA genes.

Materials and methods

Bacterial strains

H. bizzozeronii strains were isolated from 15 Finnish dogs between 1991 and 1996, and the 10 *H. salomonis* isolates came from Finnish dogs between 1993 and 1996. Three of the *H. salomonis* isolates were cultured from one dog (dog HS 8, isolates HS 8a, HS 8b, HS 8c) on separate occasions over a period of *c.* 2 years, and one of the isolates originated from a puppy of *c.* 5 months of age when it had been living with the puppies of dog HS 8 (isolate HS 8d). Both *Helicobacter* spp. have already been characterised by phenotypic testing, SDS-PAGE, dot-blot and reassociation DNA–DNA hybridisation and 16S rRNA gene sequencing [2, 3]. The organisms were stored at -70°C , and most of the strains were subcultured 10–20 times after their first isolation.

DNA preparation and digestion for PFGE

The organisms were grown on three-to-six Brucella blood agar plates with antibiotics in a micro-aerobic atmosphere for 2–3 days [2]. Bacterial cells were collected in Brucella broth (Oxoid), pelleted, suspended in PBS and treated with formaldehyde to inactivate endogeneous nucleases [19]. The cells were centrifuged (1800 rpm) for 10 min at 4°C and washed in PBS, and DNA in agar plugs for PFGE was prepared as described previously [20]. Several restriction endonucleases (*AvrI*, *AscII*, *ApaI*, *ClaI*, *EcoRI*, *MluI*, *NotI*, *PacI*, *PvuII*, *SpeI* and *SfiI*) were tested to find the optimal digestion for genetic diversity studies and genome size determination. DNA digests were prepared according to the instructions of the restriction endonuclease manufacturers (Boehringer Mannheim, Mannheim, Germany and New England Biolabs, Beverly, MA, USA).

PFGE

PFGE of digested genomic DNA was performed in agarose 1% gels in $0.5 \times \text{TBE}$ (4.45 mM Tris, 4.45 mM boric acid, 0.1 mM EDTA) with Gene Navigator electrophoresis equipment (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Depending on the fragment sizes, different pulse and electrophoresis run times were used. Genome sizes were estimated from *NotI* digests after five different programmes with a ramped pulse, starting with initial pulses from 2 to 20 s and with final pulses from 40 to 130 s, with running times from 18 to 22 h. For genome size determination from *SpeI* digests, three electrophoresis run programmes were applied: a ramped pulse from 2 to 25 s/18 h; a stepping programme (0.5 s/1 h; 0.7 s/1 h; 0.9 s/1 h; 2 s/3 h; 4 s/4 h and 6 s/4 h); and a short

programme for small fragments of <15 kb, with an initial pulse of 0.5 s and a final pulse of 8 s for 8 h. Different appropriate fragment size markers were used: chromosomal DNA of *Saccharomyces cerevisiae* (range 225–2500 kb), bacteriophage- λ ladders starting from 15 kb (range 15–300 kb) or 48.5 kb (range 50–1000 kb) and a low-range marker (0.1–200 kb). All size markers were from New England Biolabs. After electrophoresis, the gels were stained with ethidium bromide 5%, exposed to UV light and photographed.

Genome sizes

Genome sizes were estimated by measuring the migration distances of individual fragments and referring these to a standard curve of migration distances plotted against log mol.wt of DNA size standards. Genome sizes were obtained by adding the sizes of all the individual fragments.

Southern blotting and DNA hybridisation

DNA from an agarose gel was transferred to a nylon membrane (Micron Separations, Westborough, MA, USA) by Vacu Gene XL, a vacuum blotting apparatus (Pharmacia LKB Biotechnology LKB, Sollentuna, Sweden). After denaturation of DNA under UV light, the membrane was pre-hybridised at 58°C for 2 h and hybridised at 58°C with digoxigenin-labelled *ureB* or 16S rRNA gene probes for 16 h. After hybridisation, the membrane was washed twice for 5 min each in $2 \times \text{SSC}$ ($1 \times \text{SSC}$ contained 0.15 M NaCl, 0.015 M sodium citrate and SDS 0.1%) at room temperature and then twice at 58°C (15 min each). The hybridised fragments were visualised colorimetrically with anti-digoxigenin-alkaline phosphatase, nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate 4-toluidine salt as substrates (Boehringer Mannheim).

Preparation of probes from PCR products of 16S rRNA and *ureB* gene fragments

Helicobacter genus-specific primers, H276f (5'-CTATGACGGGTATCCGGC3') and H676r (5'-ATTCCACC-TACCTCTCCCA3') were used for amplification of a 374-bp fragment from the 16S rDNA gene of *H. bizzozeronii* CGUG 35545 [21], and a nested PCR was applied to amplify first a 1-kb *ureB* fragment and then an 800-bp fragment from DNA of *H. bizzozeronii* CGUG 35545. The primers were from *H. pylori* and '*H. heilmannii*' urease genes, and the PCR amplification programme was as described by Solnick *et al.* [22]. The reaction products were electrophoresed in an ID agarose 2% gel (FMC BioProducts, Rockland, USA) and visualised after ethidium bromide staining under UV light. Both PCR products were labelled by random priming with digoxigenin-dUTP according to the manufacturer's instructions for the DNA labelling and detection kit (Boehringer Mannheim).

Results

Restriction endonucleases

Among restriction endonucleases tested, *Spe*I (Fig. 1), *Asc*I and *Apa*I produced *c.* 20 fragments of *c.* 10–500 kb for *H. bizzozeronii*. The DNA of *H. salomonis* was digested with these enzymes, and many small fragments were produced. Examples of the patterns of *Spe*I digests of *H. salomonis* are presented in Fig. 2 which shows a number of small fragments <15 kb. *Not*I produced three to nine fragments from the DNA of each species with sizes from 40 to 1000 kb (Fig. 3, lanes 1–8). DNA of all *H. salomonis* strains was cut with *Not*I, but DNA of most strains of *H. bizzozeronii* was not digested with this enzyme. *Sfi*I produced *c.* 10 fragments from *H. salomonis* DNA; however, *H. bizzozeronii* strains were not digested with this enzyme (Fig. 3, lanes 9–13). *Pac*I digested DNA from *H. salomonis* and *H. bizzozeronii* strains but much of the DNA was only partially digested, especially from *H. bizzozeronii*. Other endonucleases tested produced a fragment pattern dominated by a great number of small fragments, so these enzymes were not used for further studies. The patterns produced were stable for digests done from the same plugs over a period of several months or from different plugs prepared after several subcultures.

Genetic heterogeneity

All *H. bizzozeronii* strains produced different restriction patterns with all endonucleases. An example of DNA digested with *Spe*I is shown in Fig. 1. No

fragments common to all strains were identified. *Not*I digested DNA from only 5 of 15 *H. bizzozeronii* strains (examples in Fig. 3, lanes 5–8).

Fig. 1. Examples of *Spe*I-digested DNA from *H. bizzozeronii*, showing small and middle sized fragments (<15 kb–150 kb). A stepping programme was used: 0.5 s/1 h; 0.7 s/1 h; 0.9 s/1 h; 2 s/3 h; 4 s/4 h and 6 s/4 h. Lanes 1 and 2 strain CGUG 35545 (HB 1 isolates from 1994 and 1996); 3 and 4, two isolates of HB 6 from 1995 and 1996; 5, strain CGUG 35046 (HB 2); 6, strain HB 16; 7, strain HB 17; 8, strain HB 12; 9, strain HB 3; 10, strain HB 10; 11, strain HB 8; 12, strain HB 14; 13, strain HB 13; 14, strain HB 9; mw, molecular size marker λ concatamer (15 kb). Molecular sizes are indicated on the left.

Fig. 2. PFGE patterns of seven *H. salomonis* isolates digested with *Spe*I and electrophoresed with the same stepping programme as in Fig. 1. Lane 1, HS 6; lane 2, HS 2; lane 3, HS 5 (pattern identical to lane 1); 4, HS 4 (CCUG 37845); 5, HS 8d (CCUG 37848); 6, HS 8b; 7, HS 1. Molecular size marker λ concatamer (15 kb) and the sizes are indicated on the right.

Fig. 3. PFGE patterns of *NotI*-digested (lanes 1–8) or *SfiI*-digested (lanes 9–13) DNA from *H. salomonis* (HS) and *H. bizzozeronii* (HB). Lane 1, HS 6; 2, HS 5; 3, HS 4 (CGUG 37845); 4, HS 3; 5, HB 8; 6, HB 14; 7, HB 10; 8, HB 4; 9, HS 8d (CCUG 37848); 10, HS 8b; 11, HS 6; 12, HS 2; 13, HS 4 CCUG 37845); molecular size marker yeast chromosome PFG marker (kb).

AscI, *ApaI*, *SpeI* (Fig. 2), *NotI* (Fig. 3, lanes 1–4), *SfiI* (Fig. 3, lanes 9–13) and *PacI* produced patterns useful for study of the genetic diversity of *H. salomonis*. These endonucleases digested all the isolates. In this group, three isolates from one dog (dog HS 8, isolates 8a, 8b and 8c) and from one puppy (isolate HS 8d) had identical PFGE patterns with all the enzymes. The *SpeI* patterns for the isolates HS 8d and HS 8b are shown in Fig. 2 (lanes 5 and 6) and the *NotI* patterns for the same isolates are shown in Fig. 3 (lanes 9 and 10). The *H. salomonis* strain from one dog (HS 1) had a PFGE pattern identical to the isolates from dog HS 8 and the puppy HS 8d with *SpeI* (Fig. 2, lane 7) and *PacI*; however, when *NotI* or *AscI* was used for digestion, the patterns differed. Two other strains (HS 5 and HS 6) with no known association had identical patterns with all the endonucleases (*SpeI*, *AscI*, *NotI*, *PacI*) (Fig. 2, lanes 1 and 3).

Genome size

H. bizzozeronii. Genome sizes for 11 *H. bizzozeronii* strains were estimated from PFGE patterns (Table 1). The sizes for five strains were estimated from *NotI* and *SpeI* digests and, for six strains, the estimation was performed with *SpeI* digest patterns only, because *NotI* did not digest the DNA of these strains. The fragments produced by other endonucleases either had too many small fragments (<15 kb (e.g., *ApaI* and *AscI*) or DNA cutting was unreliable due to partial digestion. Genome

size estimation was performed with three to five different gels electrophoresed under different run programmes to determine the sizes of small, middle-size and large fragments. Genome sizes from *NotI* digests of five *H. bizzozeronii* strains varied from 1630 SD 22 to 1937 SD 21 kb, while those from *SpeI* digests of 11 strains varied from 1558 SD 32 to 1945 SD 35 kb (Table 1). Similar sizes were estimated from *NotI* and *SpeI* digests. Fragments from *NotI* digests were fewer (2–4) than from *SpeI* digests (14–25).

H. salomonis. Genome sizes of five *H. salomonis* strains were estimated from *NotI* digests (Fig. 2) after five electrophoresis runs (Table 1). *SpeI* (Fig. 2), *ApaI* and *AscI* digests were not used for genome size estimation because they produced too many small fragments (<15 kb). *PacI* digests were used for estimation of the sizes of the genomes of three strains. The genome sizes from *NotI* digests varied from 1675 SD 35 to 1805 SD 25 kb and from *PacI* digests from 1769 SD 19 to 1810 SD 28 kb (Table 1). Genome sizes of individual strains were similar from *NotI* and *PacI* digests (Table 1).

ureB and 16S rRNA genes

The *ureB* gene probe was hybridised with *AscI* and *SpeI* digests. The number of fragments which hybridised with *ureB* was one or two, suggesting one urease gene for both *Helicobacter* spp. In *H. bizzozeronii* digests, the *ureB* probe always hybridised with differ-

Table 1. Genome sizes of 11 *H. bizzozeronii* and five *H. salomonis* strains determined from PFGE patterns of *NotI*, *SpeI* or *PacI* digests

Strain no.	Genome size, kb, and (number of fragments)		
	<i>NotI</i>	<i>SpeI</i>	<i>PacI</i>
<i>H. bizzozeronii</i>			
CCUG 35545 (HB 1)	Not digested	1766 SD 24 (18)	...
CCUG 35046 (HB 2)	1937 SD 21 (3)	1945 SD 35 (16)	...
HB 6	1745 SD 13 (2)	1770 SD 23 (15)	...
HB 5	1630 SD 22 (3)	1688 SD 34 (17)	...
HB 10	1797 SD 18 (3)	1795 SD 22 (25)	...
HB 14	1780 SD 15 (4)	Not done	...
HB 17	Not digested	1706 SD 16 (18)	...
HB 16	Not digested	1595 SD 21 (18)	...
HB 9	Not digested	1570 SD 80 (14)	...
HB 13	Not digested	1558 SD 32 (14)	...
HB 3	Not digested	1804 SD 27 (18)	...
<i>H. salomonis</i>			
CCUG 37848 (HS 8d)	1805 SD 25 (4)	...	1796 SD 16 (12)*
CCUG 37845 (HS 4)	1679 SD 25 (6)	...	Not done
HS 6	1752 SD 30 (3)	...	1810 SD 28 (11)
HS 1	1799 SD 27 (6)	...	1769 SD 19 (12)*
HS 3	1675 SD 35 (4)	...	Not done

**PacI* patterns were identical.

ent fragments of different strains (results not shown). In contrast, the probe always hybridised with the same fragments of *H. salomonis* *AscI* or *SpeI* DNA digests (Fig. 4).

The 16S rRNA probe hybridised with two to four fragments of *AscI* and *ApaI* digests of both *H. bizzozeronii* and *H. salomonis*, suggesting that these species have two copies of 16S rRNA genes. The probe always hybridised with the same fragments of *H. salomonis* strains, but with different fragments of *H. bizzozeronii* strains (Fig. 4).

Discussion

H. bizzozeronii and *H. salomonis* are recently described canine gastric helicobacters [2, 3] and their genetic diversity has not been studied previously. The present study showed that these two species may differ in the level of diversity, although the number of strains

studied was limited because strains have been isolated only in Finland. All *H. bizzozeronii* strains isolated within the last several years from pet dogs had different PFGE patterns revealing significant genetic diversity. Unassociated *H. salomonis* strains isolated either from pet or experimental dogs also showed diversity in their PFGE patterns, but some common fragments were noted in *SpeI*, *PacI*, *SfiI* and *NotI* digests. All three isolates from one dog and an isolate from a related puppy were identical, suggesting that the method produces stable patterns useful for studies of the epidemiology of canine gastric helicobacter infections. The dog was infected with same strain for the whole study period, >2 years.

The genome of *H. pylori* has shown significant diversity by all methods used and it has been regarded as one of the most heterogeneous species known [10]. The genomes of other *Helicobacter* spp. are not well described. In studies by Saunders *et al.* [18], *H. muridarum* strains from the USA and Europe showed

Fig. 4. Southern blotting of *AscI* (lanes 1–6) and *SpeI* (lanes 1a–6a) digests of *H. salomonis* probed with digoxigenin-labelled *ureB* gene probe. The molecular sizes of hybridised fragments are shown by an arrow.

different PFGE patterns, suggesting genetic diversity. In contrast, in the PFGE analysis of a limited number of strains, the genetic diversity of *H. mustelae* was low [17]. It has been suggested that the significant genetic diversity of *H. pylori* strains reflects a long evolutionary history as a human pathogen [10, 13, 18]. Recently, the only recognised host for *H. bizzozeronii* and *H. salomonis* has been dogs [2, 3]. In dogs, mixed infections have been noted with *H. bizzozeronii* and *H. felis* [4, 23], unlike *H. pylori* colonisation in human gastric mucosa [10]. Mixed colonisation of the same niche by two species may provide conditions for genetic exchange between species.

Probing with a house-keeping gene (16S rDNA) and a *Helicobacter* species-specific urease gene revealed significant differences between *H. bizzozeronii* and *H. salomonis* in the localisation of these genes. Each gene was located in a different fragment in each *H. bizzozeronii* strain, whereas localisation in *H. salomonis* strains was homogeneous. Variability in gene order, i.e., macro-diversity, is typical of the *H. pylori* genome and, in this characteristic, the *H. bizzozeronii* genome may resemble the genome of *H. pylori* [10]. The macro-diversity of *H. salomonis* is perhaps less than that of *H. pylori*. More studies on gene order are needed. However, recently, genome mapping of *H. bizzozeronii* and *H. salomonis* has been restricted by the fact that except for 16S rRNA genes [3], sequences of other genes of these species are unknown. As in *H. pylori* [9], one urease gene was found in both organisms. Both organisms are urease positive [3]. The two 16S ribosomal genes seen in *H. bizzozeronii* and *H. salomonis* equals the number seen in *H. pylori* [8, 10]. The number of 23S ribosomal genes was not determined, but bacteria usually have the same number for both genes [24]. The enterobacteria *Escherichia coli* and salmonellae usually have seven 16S–23S ribosomal genes [24]. A high number of copies is associated with the short generation time of fast-growing organisms, and a low number with the long generation time of slow-growing organisms – a characteristic typical of *H. bizzozeronii* [2], *H. salomonis* [3] and *H. pylori* [25].

The genome sizes of *H. bizzozeronii* and *H. salomonis* were in the range *c.* 1.6–1.9 Mb and 1.7–1.8 Mb, respectively. Similar sizes have been reported for the *H. pylori* genome, 1.6–1.7 [9, 10], while the genome size of *H. hepaticus* was estimated to be 1.3 Mb [18]. The genetically related species *Campylobacter jejuni* and *C. coli* also have a genome of size of 1.7 Mb [26], whereas *E. coli* and *Salmonella* spp. have genome sizes of 4.6 Mb [24]. Genome size estimation of a larger number of species of phylogenetically related *Helicobacter* and *Campylobacter* spp. [1] will make it possible to judge whether small genome size is typical of this group of organisms. Small genome size is characteristic of bacterial species that are

metabolically inactive [10, 24], such as *H. bizzozeronii* and *H. salomonis* [2, 3].

NotI and *NruI* are the enzymes most commonly used to study micro- and macro-heterogeneity of *H. pylori* [9, 10]. Only *NotI* was useful for *H. bizzozeronii* and *H. salomonis*, and even *NotI* failed to cleave the DNA of one-third of *H. bizzozeronii* strains. The other rare-cutters tested, *PacI* and *SfiI*, either did not cut DNA or produced partial digestion, especially with *H. bizzozeronii*. These results, as well as results obtained with a frequently cutting enzyme *HaeIII* which also does not cleave the DNA of many *H. bizzozeronii* strains (unpublished observations), suggest that methylation sites are common in the DNA of *H. bizzozeronii*. Because the G + C mol% content of *H. bizzozeronii* and *H. salomonis* is *c.* 42–43 (unpublished observations), *NotI*, *SfiI* and *AscI* as eightbase-cutters with GC-rich sequences were more applicable than enzymes which recognise AT-rich sequences for digestion of *H. bizzozeronii* and *H. salomonis* DNA. However, *PacI* (TTAATTAA), which cleaves DNA rich in AT sequences, was useful for digestion of *H. salomonis* DNA.

In conclusion, macro-restriction analysis revealed significant genetic heterogeneity for *H. bizzozeronii* compared with homogeneity for *H. salomonis*. Both have a small genome and two 16S ribosomal genes, characteristics typical of organisms that are metabolically inactive and have a slow growth rate.

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