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 (Ascl, Apal, 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, Apal and Ascl 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 × 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 × SSC (1 × 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 antidigoxigenin-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'CTAT-GACGGGTATCCGCGC3') and H676r (5'ATTCCACC-TACCCTCTCCCA3') 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, SpeI (Fig. 1), AscI and ApaI 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 SpeI digests of H. salomonis are presented in Fig. 2 which shows a number of small fragments <15 kb. NorI 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 NorI, but DNA of most strains of H. bizzozeronii was not digested with this enzyme. SfiI produced c. 10 fragments from H. salomonis DNA; however, H. bizzozeronii strains were not digested with this enzyme (Fig. 3, lanes 9–13). PacI 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 SpeI is shown in Fig. 1. No fragments common to all strains were identified. NorI digested DNA from only 5 of 15 H. bizzozeronii strains (examples in Fig. 3, lanes 5–8).

Fig. 1. Examples of SpeI-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 SpeI 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 (CCUG 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).

Ascl, 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 Ascl 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 Ascl) 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 \textit{H. bizzozeronii} and five \textit{H. salomonis} strains determined from PFGE patterns of \textit{NotI}, \textit{SpeI} or \textit{PacI} digests

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

*\textit{PacI} patterns were identical.

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

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

\textbf{Discussion}

\textit{H. bizzozeronii} and \textit{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 \textit{H. bizzozeronii} strains isolated within the last several years from pet dogs had different PFGE patterns revealing significant genetic diversity. Unassociated \textit{H. salomonis} strains isolated either from pet or experimental dogs also showed diversity in their PFGE patterns, but some common fragments were noted in \textit{SpeI}, \textit{PacI}, \textit{SfiI} and \textit{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 \textit{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 \textit{et al.} [18], \textit{H. muridarum} strains from the USA and Europe showed

\textbf{Fig. 4.} Southern blotting of \textit{AscI} (lanes 1–6) and \textit{SpeI} (lanes 1a–6a) digests of \textit{H. salomonis} probed with digoxigenin-labelled \textit{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, 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 (TATAATTA), 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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References


