PCR-based genetic evidence for occurrence of Helicobacter pylori and novel Helicobacter species in the canine gastric mucosa

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Abstract

The canine gastric mucosa is known to be a habitat for various Helicobacter species. So far, five Helicobacter species have been described from the canine gastric mucosa, but histological studies have demonstrated a greater variety. In order to gain more information on diversity of canine gastric mucosa colonising helicobacters, biopsy samples of four pet dogs were examined by DNA-based techniques. PCR with a primer pair binding specifically to the 16S rDNA of the species of the genus Helicobacter and generating a fragment of approximately 400bp indicated the presence of Helicobacter strains in the stomachs of the four dogs. PCR products were cloned into Escherichia coli DH10B™ and PCR-re-amplified 16S rDNA fragments were subjected to amplified ribosomal DNA restriction analysis (ARDRA) employing restriction enzyme HhaI. Restriction profiles indicated the presence of at least two different Helicobacter species in two dogs. Partial sequences of 16S rDNA of six clones were compared with sequences available in the EMBL data bank. Two sequences obtained from different dogs were identical with the corresponding sequences of Helicobacter pylori

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strains. Three sequences showed highest but moderate similarity values to *H. pylori* (96.6–98.0%) and one sequence to *Helicobacter salomonis* (97.3%). In contrast to previous reports our data implicate that the gastric mucosa of dogs may be colonised by strains of *H. pylori* or a very closely related species but they also confirm indications for the presence of so far uncultivated species of *Helicobacter*.

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**Keywords:** Dog mucosa; *Helicobacter* spp.; Partial 16S rDNA sequence; Amplified ribosomal DNA restriction analysis

1. **Introduction**

The discovery of *Helicobacter pylori* in humans (Marshall and Warren, 1984) and its relationship to gastritis, peptic ulcer, and gastric neoplasia has increased interest in gastric bacteria also in cats and dogs. Research has focused on four specific areas: (i) prevalence of gastric bacteria in dogs and cats; (ii) investigation of these domestic animals as a source of zoonotic *Helicobacter* infection of humans (Otto et al., 1994; Thomson et al., 1994); (iii) clinical significance of gastric helicobacters in dogs and cats; and (iv) development of animal models of human disease.

The most common, cultured gastric helicobacters in the stomach of dogs are “*Gastrospirillum hominis*” (Thomson et al., 1994; Hänninen et al., 1995), *Helicobacter felis* (Lee et al., 1992), *Helicobacter bizzozeronii* (Hänninen et al., 1996), *Helicobacter salomonis* (Jalava et al., 1997) and “*Flexispira rappini*” (Eaton et al., 1996). Histological studies have demonstrated that *Helicobacter*-like organisms are present in the gastric mucosas of virtually all adult dogs but only the minority appears to be culturable under standard conditions (Hermanns et al., 1995; Eaton et al., 1996; Happonen et al., 1996).

So far, *H. pylori* has not been detected in biopsies from the stomachs of dogs (Hermanns et al., 1995; Eaton et al., 1996; Fox and Lee, 1997; Yamasaki et al., 1998) and its presence in cats was only reported once (Handt et al., 1994). To gain additional information on diversity of helicobacters in the stomachs of dogs, we have studied biopsy samples of four dogs. One dog (H1) primarily was referred to the 1st Medical Clinic for Small Animals and Ungulates (Veterinary University Vienna) because of chronic cough and respiratory distress. A bronchial foreign body was found and removed endoscopically. No pathology was associated with the gastrointestinal tract on endoscopic and histopathological examination. The other three dogs (H2, H3, H4) underwent endoscopy for clinical signs related to the gastrointestinal tract (e.g. vomiting). In dog H2, endoscopy as well as microscopic examination of biopsies from different regions of the stomach were microscopically normal, suggesting a functional disorder. The final diagnosis in dog H3 was idiopathic acquired megaesophagus; whereas in dog H4 several gastric foreign bodies were found. No histopathological alterations were detected in the latter two dogs on light microscopy. These samples were examined employing a *Helicobacter*-specific PCR directed to the 16S rDNA (Riley et al., 1996), amplified ribosomal DNA restriction analysis (ARDRA) and partial 16S rDNA sequencing.
2. Materials and methods

2.1. Sampling

Four dogs were anaesthetised for flexible endoscopy, and biopsy samples were taken from the cardiac, fundic, and antral regions of their stomachs. Specimens from the three sites of each dog were combined and designated H1/98, H2/98, H3/98 and H4/98.

2.2. Extraction, PCR amplification, cloning, digestion and sequencing of DNA

From each sample, DNA was isolated from 25 mg of gastric tissue and processed using the QIAmp Tissue Kit (Qiagen Inc., CA, USA) according to the manufacturer’s instructions. For detection of helicobacters, purified DNA from specimens was subjected to PCR using “ready-to-go beads” (Amersham Pharmacia Biotech Inc.) and the *Helicobacter*-specific primer pair H276f/H676r (Riley et al., 1996) as described previously (Buczolits et al., 2001). The PCR products were ligated into pGEM-T vector according to the manufacturer’s instructions (Promega, Madison, WI, USA). Competent *Escherichia coli* DH10B™ (Life Technologies, Gibco BRL) cells were transformed with ligated fragments by electroporation (2.5 kV, 200 Ω, 25 μF, 4.6–4.8 ms) using Gene Pulser II and Pulse Controller II (both Biorad). Transformants were incubated over night at 37°C on LB-agar supplemented with ampicillin (125 μg/ml), IPTG (0.5 mM) and X-Gal (50 μg/ml). Positive, white clones selected by blue-white screening and plasmid screening for ampicillin-resistant transformants were considered to contain the 400 bp insert corresponding to the *Helicobacter*-specific amplicon. Presence of this fragment in the plasmid was confirmed by another PCR assay employing primer pair H276f/H676r. DNA was released from whole cells by repeated freeze-thawing cycles and the supernatant obtained after short centrifugation was used as a template for PCR. Amplified ribosomal DNA restriction analysis was done using restriction enzyme *HhaI* as recommended by the manufacturer (Promega). Resulting restriction profiles were analysed on NuSieve Agarose 3:1 as described previously (Buczolits et al., 2002). *H. felis* CCUG 28539T, *H. pylori* NCTC 11637T and Brevundimonas H2/98-FUNDUS were used as controls.

Partial 16S rDNA sequences were purified and sequenced employing primer H276f (Buczolits et al., 2001). Sequence alignment and comparisons were done using either BLAST (Altschul et al., 1997) manually corrected for ambiguous nucleotides or FASTA (Pearson and Lipman, 1988).

3. Results

3.1. Detection of Helicobacter in biopsy samples

After *Helicobacter*-specific PCR, a fragment of approximately 400 bp was detected in all four biopsy samples (results not shown) indicating the presence of *Helicobacter* spp. in all specimens. However, we were not able to cultivate any *Helicobacter* strain, as already reported previously (Buczolits et al., 2001).
3.2. Amplified ribosomal DNA restriction analysis (ARDRA)

The suitability of ARDRA of the *Helicobacter*-specific amplicon for differentiation between *Helicobacter* species was evaluated by theoretical digestion of corresponding sequences of *H. felis* ATCC 49179\(^T\) (Accession no. M57398), *H. salomonis* CCUG 37845\(^T\) (Accession no. U89351), *H. bizzozeronii* CCUG 35545\(^T\) (Accession no. Y09404), “*G. hominis*” isolate 2 (Accession no. L10080), “*F. rappini*” ATCC 43966\(^T\) (Accession no. M88137) and *H. pylori* ATCC 43504\(^T\) (Accession no. M88157) employing the restriction enzyme *HhaI*. These examinations indicated that the 16S rDNA fragment of each species contains one restriction site. In *H. felis* ATCC 49179\(^T\), *H. salomonis* CCUG 37845\(^T\), *H. bizzozeronii* CCUG 35545\(^T\) and “*G. hominis*” isolate 2, *HhaI* cuts after nucleotide 102 of the partial fragment (nucleotide 380, *E. coli* numbering) whereas in “*F. rappini*” ATCC 43966\(^T\) and *H. pylori* ATCC 43504\(^T\) it cuts after nucleotide 276 (nucleotide 579, *E. coli* numbering). Thus, almost identical restriction profiles can be concluded consisting of two fragments of 102 and 274 bp in *H. felis*, *H. salomonis*, *H. bizzozeronii* and “*G. hominis*”, and of 276 and 100 bp in “*F. rappini*” and *H. pylori*, respectively.

Despite the limited discrimination power we decided to employ *HhaI* for ARDRA since we could not identify a single restriction enzyme which was better suited for differentiation between these species. Amplified sequences of all positive clones of samples H1/98 (n = 2), H2/98 (n = 3) and H3/98 (n = 7), and 10 out of 180 positive clones of sample H4/98, were subjected to ARDRA employing *HhaI* to assess *Helicobacter* diversity in our samples. The majority of restriction profiles, including those of *H. felis* CCUG 28539\(^T\) and *H. pylori* NCTC 11637\(^T\), consisted of two fragments which were approximately 100 and 270 bp in size (Fig. 1). Predicted small differences between restriction profiles of *H. pylori* NCTC 11637\(^T\) and *H. felis* CCUG 28539\(^T\) were clearly visible at least for the small fragment (Fig. 1). Among the restriction profiles obtained from cloned sequences of sample H1/98 (n = 2), H2/98 (n = 3) and H3/98 (n = 7), and 10 out of 180 positive clones of sample H4/98, we subjected to ARDRA employing *HhaI* to assess *Helicobacter* diversity in our samples. The majority of restriction profiles, including those of *H. felis* CCUG 28539\(^T\) and *H. pylori* NCTC 11637\(^T\), consisted of two fragments which were approximately 100 and 270 bp in size (Fig. 1). Predicted small differences between restriction profiles of *H. pylori* NCTC 11637\(^T\) and *H. felis* CCUG 28539\(^T\) were clearly visible at least for the small fragment (Fig. 1). The restriction profiles of the two cloned partial 16S rDNA sequences of sample H1/98 were indistinguishable and appeared to be similar to the profile of *H. pylori* NCTC 11637\(^T\) (Fig. 1a). Among the restriction profiles obtained from cloned sequences of sample H2/98, two types were recognised. Clone 1 (H2/98) showed a profile which was most similar to the profile of *H. felis* CCUG 28539\(^T\), whereas profiles of the sequences of clones 2 and 3 showed a unique restriction profile but were indistinguishable from each other (Fig. 1a). It consisted of a heavy band at approximately 100 bp, which, based on visual evaluation, was assumed to represent two different fragments of similar sizes, and one band at approximately 170 bp. Theoretical digestion of partial 16S rDNA of six clones with *HhaI* based on available sequences (see below) confirmed visual evaluation of restriction profiles including the assumed three fragments in clone 3 (H2/98) consisting of 102, 174 and 100 bp. All restriction profiles obtained from sample H3/98 were similar to that of *H. felis* CCUG 28539\(^T\), but one (clone 5) resembled to the profile of *H. pylori* NCTC 11637\(^T\) (Fig. 1b). Restriction profile of cloned sequences of sample H4/98 were indistinguishable from the profile of *H. pylori* NCTC 11637\(^T\) (Fig. 1c).

3.3. Partial 16S rDNA sequence analysis

From the four samples H1/98, H2/98, H3/98 and H4/98 one clone of each distinct restriction profile was selected for sequence analysis. The sequences of the six cloned partial
Fig. 1. ARDRA analyses of HhaI-digested Helicobacter-specific 16S rDNA fragments which are obtained employing the primer pair H276f/H676r. DNA was amplified and cloned from biopsy specimens H1/98, H2/98, H3/98 and H4/98. For reference, restriction profiles of corresponding 16S rDNA of *H. felis* CCUG 28539^T*, *H. pylori* NCTC 11637^T* and *Brevundimonas* H2/98-FUNDUS (Buczolits et al., 2001) are shown. Arrows indicate clones which were subjected to sequence analysis. (a) Lanes 1 and 10, 100 bp marker; lanes 2 and 3, clones 1 and 2 (H1/98); lanes 4–6, clones 1–3 (H2/98); lane 7, *Brevundimonas* H2/98-FUNDUS; lane 8, *H. felis* CCUG 28539^T*; lane 9, *H. pylori* NCTC 11637^T*. (b) Lanes 1 and 12, 100 bp marker; lanes 2–8, clones 1–7 (H3/98); lane 9, *Brevundimonas* H2/98-FUNDUS; lane 10, *H. felis* CCUG 28539^T*; lane 11, *H. pylori* NCTC 11637^T*. (c) Lanes 1 and 15, 100 bp marker; lanes 2–11, clones 1–10 (H4/98); lane 12, *Brevundimonas* H2/98-FUNDUS; lane 13, *H. felis* CCUG 28539^T*; lane 14, *H. pylori* NCTC 11637^T*. 
Table 1
Sequence similarities of six cloned partial 16S rDNAs to selected *Helicobacter* species

<table>
<thead>
<tr>
<th>Biopsy specimens</th>
<th>Clone</th>
<th>Sequence similarity to the highest scoring <em>Helicobacter</em> species</th>
<th>Sequence similarity with typical gastric <em>Helicobacter</em> species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1/98</td>
<td><em>H. pylori</em> ATCC 43504(^T) Y09404 (%)</td>
<td><em>H. bizzozeronii</em> CCUG 35545(^T) M57398 (%)</td>
</tr>
<tr>
<td></td>
<td>H2/98</td>
<td><em>H. pylori</em> ATCC 43504(^T) (96.6%)</td>
<td><em>H. felis</em> ATCC 49179(^T) M57398 (%)</td>
</tr>
<tr>
<td></td>
<td>H2/98</td>
<td><em>H. pylori</em> ATCC 43504(^T) (98.1%)</td>
<td><em>H. salomonis</em> CCUG 37845(^T) U89351 (%)</td>
</tr>
<tr>
<td></td>
<td>H3/98</td>
<td><em>H. pylori</em> ATCC 43504(^T) (98.0%)</td>
<td>“F. rappini” ATCC 43966(^T) M88137 (%)</td>
</tr>
<tr>
<td></td>
<td>H3/98</td>
<td><em>H. salomonis</em> CCUG 37845(^T) (97.6%)</td>
<td>“G. hominis” isolate 2 L.10080 (%)</td>
</tr>
<tr>
<td></td>
<td>H4/98</td>
<td><em>H. pylori</em> ATCC 43504(^T) (100%)</td>
<td>“F. rappini” ATCC 43966(^T) M88137 (%)</td>
</tr>
</tbody>
</table>

\(^a\) Results are shown for the highest scoring *Helicobacter* species and typical inhabitants of the canine gastric mucosa.
16S rDNA fragments consisted of stretches of 324–347 nucleotides. Sequence comparisons demonstrated highest similarities of clone 2 (H1/98), clones 1 and 3 (H2/98), clone 5 (H3/98) and clone 4 (H4/98) to *H. pylori* ATCC 43504<sup>T</sup> (Table 1). Clone 2 (H1/98) and clone 4 (H4/98) displayed 100% sequence similarity to *H. pylori* ATCC 43504<sup>T</sup>. Their sequence similarities to other *H. pylori* strains were in the narrow range of 99.2–100%. Other nearest *Helicobacter* species, including *H. bilis*, *H. canadensis*, *H. fennelliae*, *H. ganmani*, *H. mesocricketorum*, *H. pametensis*, *H. pullorum* and *H. winghamensis* shared only 96.9–98.1% sequence similarities. Gastric canine *Helicobacter* species revealed only sequence similarity values in the range between 95.0 and 96.5% (Table 1). Thus, these degrees of similarities imply that the stomachs of these two dogs harboured *H. pylori* or at least a very closely related species. Clone 1 (H2/98), clone 3 (H2/98) and clone 5 (H3/98) showed moderate similarities to *H. pylori* ATCC 43504<sup>T</sup> (96.6, 98.1 and 98.0%, respectively), which is in the range found between *H. pylori* and other established *Helicobacter* species (see above).

Among each other, the three cloned sequences shared 95.3–97.2% similarities, indicating that each originated from strains which might be considered to represent novel *Helicobacter* species. Clone 6 (H3/98) displayed highest sequence similarities to *H. salomonis* CCUG 37845<sup>T</sup>, *H. felis* ATCC 49179<sup>T</sup> and *H. bizzozeronii* CCUG 35545<sup>T</sup> (97.6, 97.5 and 97.3%, respectively).

### 4. Discussion

Since it is well known that different regions of 16S rDNA often display different degrees of conservation, similarity values based on partial 16S rDNA sequences do not necessarily reflect similarity values obtained from almost complete sequences. To evaluate the validity of comparison of partial sequences we determined the partial sequence similarity values of *H. pylori* ATCC 43504<sup>T</sup> (Accession no. M88157) and *H. felis* ATCC 49179<sup>T</sup> (Accession no. M57398) to some canine *Helicobacter* species and compared them to corresponding, almost complete sequences (Table 2). These comparisons indicated that similarity values based on partial 16S rDNA sequences were generally higher (0.2–2.4%) than those of corresponding, almost complete sequences. Only in the comparison pair *H. felis* ATCC 49179<sup>T</sup>/"G. hominis" isolate 2 sequence similarity of complete sequences was slightly higher (0.6%) than that obtained from partial sequences. Since deviation between similarity values obtained from partial sequence and almost complete 16S rDNA sequences were low (≤0.6%) when the sequence similarity values were high we conclude, that, in the range >98% sequence similarities, partial sequences usually reflect very well similarities of complete sequences.

Based on these considerations our examinations provide evidence that two dogs harboured *H. pylori* or a very closely related species, as indicated from identical partial 16S rDNA sequences. The other four sequences analysed in this study did not indicate affiliation of corresponding organisms to any established *Helicobacter* species, thus suggesting that each of these organisms represents a novel species of *Helicobacter*. At least for clone 3 (H2/98) this assumption is supported from the unusual second restriction site for *HhaI* in its partial 16S rDNA sequence. Our results from sequencing also indicate that, apparently, none of the, so far, cultured typical colonisers of the canine stomach such as *H. felis*, *H. salomonis*,...
Table 2
16S rDNA sequence similarities between *H. pylori* ATCC 43504\(^T\), *H. felis* ATCC 49179\(^T\) and some canine *Helicobacter* species based on almost complete sequences\(^a\)

<table>
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<tbody>
<tr>
<td><em>H. pylori</em> ATCC 43504(^T) (Accession no. M88157)</td>
<td>100% (100%)</td>
<td>95.1% (96.5%)</td>
<td>95.6% (96.2%)</td>
<td>95.3% (96.5%)</td>
<td>95.2% (96.2%)</td>
<td>93.2% (95.0%)</td>
</tr>
<tr>
<td><em>H. felis</em> ATCC 49179(^T) (Accession no. M57398)</td>
<td>95.8% (96.2%)</td>
<td>99.5% (99.7%)</td>
<td>100% (100%)</td>
<td>99.4% (100%)</td>
<td>98.8% (98.2%)</td>
<td>92.9% (95.3%)</td>
</tr>
</tbody>
</table>

\(^a\) Values in brackets indicate similarity values which were obtained from corresponding partial sequences.
H. bizzozeronii, “G. hominis” or “F. rappini” predominated in the gastric mucosa of the four dogs in this study. This observation is in accordance with results from Eaton et al. (1996) and Cattoli et al. (1999). These authors demonstrated that in the majority of dogs Helicobacter-like organisms are present but only from the minority of dogs (15–20%) they were able to isolate Helicobacter strains which were identified as H. felis, H. bizzozeronii and H. bilis. Thus, we assume that the majority of canine Helicobacter-like organisms are unculturable when conventional, Helicobacter-specific cultivation strategies are employed. This assumption might also explain why we were unable to isolate any Helicobacter strain from these four dogs as reported recently (Buczolits et al., 2001).

ARDRA analyses of the partial 16S rDNA applying HhaI for assessment of Helicobacter diversity were limited by the fact that restriction profiles of H. pylori and H. felis are almost indistinguishable. The similarities in restriction profiles were not due to identical restriction sites, but only due to restriction fragments of almost identical size. Validity of this specific approach is also limited, since on the one hand H. pylori and “F. rappini” and on the other hand H. felis, H. bizzozeronii, H. salomonis and “G. hominis” display in their partial 16S rDNA sequences identical restriction sites for HhaI. However, this result is not surprising since the species H. felis, H. bizzozeronii and H. salomonis are highly related to each other, as demonstrated by identical 23S rDNA-based PCR-RFLP profiles (Jalava et al., 1999) and high 16S rDNA sequence similarities (99.2–99.5%) and “G. hominis” isolate 2 is the next phylogenetic relative of this group, as indicated by sequence similarities of 98.8–98.9%.

Thus, the probability for differentiation of these species using the ARDRA approach is low since the sequence similarities among these species are very high. Nevertheless, the ARDRA profile of clone 6 (H3/98) clearly showed the same restriction profile like H. felis CCUG 28539T (Fig. 1b), and H. salomonis CCUG 37845T shared the highest sequence similarities with this cloned sequence (Table 1). On the other hand, close relatedness of the sequence of clone 1 (H2/98) to H. felis CCUG 28539T, as implicated from the restriction profiles (Fig. 1a), was not confirmed from sequence comparisons (Table 1), which indicated a separate position of the corresponding organism among Helicobacter species. These observations demonstrate that identical restriction profiles of the Helicobacter-specific amplicon obtained after digestion with HhaI do not necessarily indicate a very close relationship.

To our knowledge, this is the first time that evidence is provided for the presence of H. pylori in pet dogs. Eaton et al. (1996) reported the presence of gastrospirilla in the stomach of dogs based on histological investigations and 16S rDNA sequence analyses. They identified these isolates as H. felis-like, H. bilis and a so far unknown species. Since they could not identify any H. pylori or H. pylori-like strain in the 54 dogs examined, they concluded that pet dogs do not represent a source of H. pylori for the human population, at least in central Ohio. However, these authors did not try to identify H. pylori by molecular means, and it can be assumed that the presence of “viable but non-culturable” strains of H. pylori has been overlooked. Although partial 16S rDNA sequencing indicated the presence of H. pylori we also failed to isolate H. pylori from the gastric biopsy specimens of these dogs (Buczolits et al., 2001). This may lead to the conclusion that for unknown reasons, cultivation of H. pylori from the canine gastric mucosa is more complicated and conditions for cultivation have to be adapted. This non-culturability might be explained by the fact that, under unfavourable conditions, H. pylori can change its shape from spiral to coccoid
and that the coccoid forms are viable but non-culturable (Bode et al., 1993; Cellini et al., 1994; Dunn et al., 1997; Mizoguchi et al., 1999; Ren et al., 1999). Stomachs of dogs may provide such unfavourable conditions for *H. pylori* which cause transformation of the spiral to the coccoid shape and coincides with the switch to the viable but non-culturable state.

The results of our study confirm those of other examinations suggesting that the presence of *Helicobacter* and *Helicobacter*-like organisms is almost universal in dogs (Henry et al., 1987; Geyer et al., 1993; Hermanns et al., 1995). At least two dogs (H2/98 and H3/98) could be assumed to harbour two different species of *Helicobacter* in their gastric mucosa. This observation supports the results of Jalava et al. (1998) who showed that several distinct species can colonise the canine gastric mucosa. In contrast to other publications we provide strong evidence for the presence of *H. pylori* in the gastric mucosa of dogs. Despite the fact that they might be viable but non-culturable using conventional cultivation strategies, the zoonotic potential of *H. pylori* should be seriously examined.

Presence of *H. pylori* in two of four dogs might be explained by close contact between humans and their pet animals. *H. pylori* is found in 30–100% of human gastroscopies (Stolte et al., 1994). Transmission from humans would explain their presence in the gastric mucosa of these dogs. Vice versa, risk of transmission of *H. pylori* from pet dogs to other humans cannot be excluded here. However, to substantiate this assumption, epidemiological studies would be required, but unfortunately, these dogs are not available for continuing studies.

The use of the *Helicobacter*-specific primer pair H267f/H676r for detection of helicobacters in any samples as recommended by Riley et al. (1996) is tempting, since this approach is rapid and not cost intensive. Nevertheless, a positive result should be confirmed by other results, e.g. sequence analysis, since false positive results can occur. Recently, we have reported the isolation and characterisation of strain *Brevundimonas* H2/98-FUNDUS from specimen H2/98 (Buczolits et al., 2001) which gave a positive result in PCR amplification with the *Helicobacter*-specific primer pair. This strain displayed growth requirements which are characteristic for *Helicobacter* but unusual for members of the genus *Brevundimonas*. Nevertheless, it could be unambiguously identified as a strain of *Brevundimonas* based on chemotaxonomic characteristics and 16S rDNA sequence analysis. Thus, this strain is the first example which gives false positive results when the *Helicobacter*-specific primer pair is employed alone indicating that its reliability for detection of *Helicobacter* is limited.

5. Conclusions

(1) ARDRA analysis of the *Helicobacter*-specific amplicon using *HhaI* reflects only partial *Helicobacter* biodiversity in a natural samples since identical restriction profiles do not necessarily indicate a very close relationship.

(2) Application of the *Helicobacter*-specific 16S rDNA directed primer pair H267f/H676r for amplification, cloning and sequencing of the resulting amplicon is suitable for detection and assessment of *Helicobacter* biodiversity in natural samples.

(3) *H. pylori* or a very closely related species as well as several unknown *Helicobacter* species can be found in the canine gastric mucosa.
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