**Case Report**

**Helicobacter pylori vacA virulence factor in uncultured Helicobacter heilmannii sensu lato from an infected child**

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**Introduction:** Helicobacter spp. colonizing the human stomach other than *Helicobacter pylori* have been rarely described in children.

**Case Presentation:** We identified an uncultured *Helicobacter heilmannii sensu lato* by histology and PCR in biopsies from a symptomatic child. Surprisingly, the PCR assay demonstrated the presence of the *vacA* gene in this uncultured *H. heilmannii sensu lato* and its sequence related highly to *H. pylori vacA* virulence factor. The analysis of the 16S ribosomal sequence showed higher identity with uncultured *Helicobacter* spp. and the *H. heilmannii* strain, respectively.

**Conclusion:** This is the first report that shows the presence of *vacA* in an uncultured *H. heilmannii sensu lato* extracted from human gastric mucosa.

**Keywords:** 16S rRNA; *Helicobacter heilmannii sensu lato*; *Helicobacter pylori*; phylogenetic tree; uncultured *Helicobacter heilmannii*; *vacA*.

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**Introduction**

*Helicobacter pylori* has generally been considered as the main colonizing bacteria of the human gastric mucosa. Less frequently, colonization by other micro-organisms of the genus *Helicobacter* has been described in the stomach. Increasing studies have been conducted regarding spiral-shaped bacterium-associated gastritis since 1987. The first described bacterium of the non-*H. pylori* type was named *Gastrospirillum hominis*, later referred to as *Helicobacter heilmannii* (Haesebrouck et al., 2009). Subsequently, analysis of the 16S rRNA sequence led to subclassification of *H. heilmannii* type 1 and type 2 (O’Rourke et al., 1992). *Helicobacter suis* is the only representative species of *H. heilmannii* type 1, whilst *H. heilmannii* type 2 is used to describe *Helicobacter felis*, *Helicobacter bizzozeronii*, *Helicobacter salomonis*, *Helicobacter cynogastricus*, *Helicobacter baculiformis* and *H. heilmannii* sp. (De Groote et al., 1999). Some of these fastidious micro-organisms can now be grown in the laboratory, allowing for a more detailed description. Several of the non-*H. pylori* spiral-shaped bacteria extracted from human gastric mucosa are more commonly found in domestic animals, such as cats, dogs, pigs, horses, monkeys and even cheetahs (Solinick et al., 1993; Cantet et al., 1999).

Haesebrouck et al. (2011) proposed the term *H. heilmannii sensu lato* to refer to the whole group of non-*H. pylori* helicobacters detected in the human or animal stomach through histopathology, electron microscopy or crude taxonomic DNA-based data. The term *H. heilmannii sensu stricto* refers to the bacterium on the species level.

The microscopic description of *H. heilmannii* is similar to the spiral morphology displayed by *H. pylori*. Whilst *H. pylori* measures 2.5–4 μm long, *H. heilmannii* measures 7–10 μm long. It has four to six turns per cell and can reach up to 12 flagella per pole (Mention et al., 1999). Due to the mostly non-culturable nature of this bacterium (Sýkora et al., 2004; Iwanczak et al., 2012), a culture-independent identification approach has been accomplished through histological and 16S rRNA sequence analysis.

**Abbreviation:** IRB, Institutional Review Board.

One supplementary figure is available with the online Supplementary Material.
To identify different strains of non-\textit{H. pylori} helicobacters, several groups have used phylogenetic analyses of the 16S rRNA gene and \textit{ureA} and \textit{ureB} subunit sequences (Andersen \textit{et al.}, 1999; O’Rourke \textit{et al.}, 2004; Priestnall \textit{et al.}, 2004).

\textit{H. pylori} infection has been linked to chronic gastritis (Mention \textit{et al.}, 1999), low-grade mucosa-associated lymphoid tissue lymphoma (Monno \textit{et al.}, 2006) and gastro-duodenal ulcers (Dieterich \textit{et al.}, 1998). In addition, two cases of adults with gastric cancer associated with \textit{H. heilmannii} infection have been reported (Morgner \textit{et al.}, 1995; Yang \textit{et al.}, 1995).

Due to the close relationship between humans and domestic animals (the most common reservoir for \textit{H. heilmannii sensu lato}), a zoonotic model of infection has been proposed. Scarce information about \textit{Helicobacter} spp. infection is available, so we decided to conduct a report on a paediatric patient infected with uncultured \textit{H. heilmannii sensu lato} and describe some of its genetic characteristics.

**Case Report**

A 13-year-old white Hispanic girl was referred to an upper gastrointestinal endoscopy with a history of 6 months of recurrent abdominal pain, without vomiting, diarrhoea or fever. No history of sleeping disorders, gastrointestinal bleeding or weight loss was recorded. Her anthropometrics parameters were normal (37 kg, 150 cm and body mass index 16.4 kg m$^{-2}$). An unremarkable medical record and family medical history was available. Amongst her epidemiological antecedents, the girl had close proximity to her puppy.

**Ethical statement**

At the time of endoscopy, the girl was enrolled in an Institutional Review Board (IRB) approved research protocol for \textit{H. pylori} eradication (IRB number 12-236), after informed consent from her parents and assent from the patient was obtained, according to international standards and local IRB requirements. The procedure was performed at the Hospital de la Pontificia Universidad Católica de Chile.

**Investigations**

The macroscopic evaluation showed corpus nodular gastropathy, with no erosions, ulcers or other findings. The endoscopy showed gastric mucosa with moderate nodularity in the corpus. The mucosa of the gastric fundus exhibited conserved folds, and both antrum and pylorus were normal (Fig. 1a). During the endoscopy procedure, mucosal biopsies were taken according to protocol. One biopsy was used for the rapid urease test (negative after 24 h incubation at room temperature). A second biopsy was used for histological analysis, which showed a mild superficial chronic gastritis in both the antrum and corpus. A \textit{H. heilmannii}-type of bacilli was unexpectedly revealed in corpus biopsies (Fig. 1b). A third biopsy was retrieved for bacterium culture and homogenized in 1 ml 1× PBS using a TH Tissue Homogenizer (Omni International). Then, 200 μl was seeded on agar plates supplemented with eight different media, commonly used to grow fastidious bacteria (Table 1). No bacterial growth was identified regardless of the media or culture conditions used.

The last biopsy, for total DNA extraction, was used for genetic identification of the bacterium. We first used specific primers for \textit{Helicobacter} spp. that created a positive amplification. Then, we sought to distinguish \textit{H. pylori} from \textit{H. heilmannii} (type 1 and type 2) using specific primers to amplify the 16S rRNA gene from each bacterium (Cantet \textit{et al.}, 1999). Amplification was positive for \textit{H. heilmannii} type 1. We also obtained a positive amplification for control DNA from \textit{H. pylori} when using \textit{H. Heilmannii}-specific primers. This amplification was
secondary to a cross-reaction of one of the primers as revealed later after sequencing analysis. Furthermore, \textit{ureB} gene amplification failed when specific \textit{H. heilmannii} primers for this subunit were used (Fig. S1a, available in the online Supplementary Material). Oligonucleotide primer sequences are listed in Table 2.

In the absence of a PCR-positive control for \textit{H. heilmannii}, we sequenced the PCR product obtained from the amplification of the 16S rRNA gene of this bacterium. The obtained sequence was compared by BLASTN using GenBank. We also compared the sequence obtained from the amplification of the \textit{H. pylori} DNA with the \textit{H. heilmannii} 16S rRNA primers. A 96 % identity was found with other \textit{H. pylori} strains. This was explained by the annealing ability of one of the two primers to the \textit{H. pylori} DNA, resulting in cross-hybridization during the PCR.

The sequence of the 16S rRNA gene from our uncultured \textit{H. heilmannii sensu lato} consisted of 1152 bp. After extensive comparison by BLASTN analysis with GenBank sequences, the sequence showed 95 % sequence identity with an uncultured \textit{Helicobacter} spp. type and similar percentages with several \textit{H. heilmannii} strains. Furthermore, we performed an analysis with a more acute test and selected the 16S rRNA sequence (Bacterial and Archaea)

\textit{Table 1.} Culture medium for growth of fastidious bacteria

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chocolate agar (GC base agar + haemoglobin prepared according to a standard protocol)</td>
<td>Modified from Alikhani \textit{et al.} (2007); Pradhan (2009); Andersen \textit{et al.} (1999)</td>
</tr>
<tr>
<td>BBA agar (blood agar base; Becton Dickinson) supplemented with 7 % defibrinated horse blood</td>
<td>Modified from Pradhan (2009); Anderson \textit{et al.} (1999)</td>
</tr>
<tr>
<td>Brucella agar (Becton Dickinson) supplemented with 7 % defibrinated horse blood</td>
<td>Modified from Andersen \textit{et al.} (1999); Jalava \textit{et al.} (1998)</td>
</tr>
<tr>
<td>Brucella agar supplemented with 5 % FBS (Hyclone)</td>
<td>Modified from Murano \textit{et al.} (1999)</td>
</tr>
<tr>
<td>Brain heart infusion agar (Becton Dickinson) supplemented with 5 % FBS (HyClone)</td>
<td>Modified from Andersen \textit{et al.} (1999); Jalava \textit{et al.} (1998); Alikhani \textit{et al.} (2007)</td>
</tr>
<tr>
<td>Trypticase soy agar supplemented with 7 % defibrinated horse blood</td>
<td>Modified from Alikhani \textit{et al.} (2007)</td>
</tr>
</tbody>
</table>

All plates were incubated under microaerobic conditions (5 % O\textsubscript{2}, 10 % CO\textsubscript{2} and 85 % N\textsubscript{2}) with 95 % humidity at 36 °C for 14 days.

\textit{Table 2.} Primers used for amplification and sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
<th>Position (nt) or size of product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA (genus \textit{Helicobacter})</td>
<td>C97</td>
<td>GCTATGACGGGATGCCATCC</td>
<td>400 bp</td>
<td>Yakoob \textit{et al.} (2012)</td>
</tr>
<tr>
<td></td>
<td>C98</td>
<td>GATATTACCCCTACACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S rRNA (\textit{H. heilmannii} type 1)</td>
<td>FH1</td>
<td>CGCTAAGGATGGTTGCTATGCTATCAT</td>
<td>173–199 nt</td>
<td>Cantet \textit{et al.} (1999)</td>
</tr>
<tr>
<td></td>
<td>FH2</td>
<td>TATTCACCGCAACATGGCTGATTTG</td>
<td>1306–1282 nt</td>
<td></td>
</tr>
<tr>
<td>16S rRNA (\textit{H. heilmannii} type 2)</td>
<td>IS</td>
<td>TTACCTAGGGCTTGACATTGAA</td>
<td>909–929 nt</td>
<td>Cantet \textit{et al.} (1999)</td>
</tr>
<tr>
<td></td>
<td>IR</td>
<td>TTCAATGTCACGGCTACATGAA</td>
<td>929–909 nt</td>
<td></td>
</tr>
<tr>
<td>16S rRNA (\textit{H. pylori})</td>
<td>16S HpF</td>
<td>TTAAGATGTCAGCTATGCT</td>
<td>600 bp</td>
<td>Kumar \textit{et al.} (2008)</td>
</tr>
<tr>
<td></td>
<td>16S HpR</td>
<td>TCCCAAGCTTAAAGGCAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UreB-HhR</td>
<td>CTGTGTTAAATGGTCAAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{vacA} allele m1/m2 (\textit{H. pylori})</td>
<td>VAGF</td>
<td>CAATCTGGTTCAATCAAGCGGAG</td>
<td>567/642 bp</td>
<td>Kumar \textit{et al.} (2008)</td>
</tr>
<tr>
<td></td>
<td>VAGR</td>
<td>CGGTTCAATGAGGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{vacA} allele s1/s2 (\textit{H. pylori})</td>
<td>VAD-S1-F</td>
<td>ATGAAAGATACAAACCAACAC</td>
<td>259/286 bp</td>
<td>Kumar \textit{et al.} (2008)</td>
</tr>
<tr>
<td></td>
<td>VAD-S1-R</td>
<td>CGTTGATGAGGCGCAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{cagA} (\textit{H. pylori})</td>
<td>Cag 2</td>
<td>GGAACCTCTATGGTAATTG</td>
<td>400–600 bp</td>
<td>Kumar \textit{et al.} (2008)</td>
</tr>
<tr>
<td></td>
<td>Cag 4</td>
<td>ATCTTTGAGCTTGCTATCG</td>
<td></td>
<td></td>
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</tbody>
</table>
Fig. 2. (a) Phylogenetic tree of different strains of *Helicobacter* spp., based on sequence similarity of their 16S rRNA gene sequences. The dendrogram was built using the neighbour-joining method with MEGA 6 software. The numbers at nodes correspond to bootstrap values (1000 replicates). (b) Alignment by CLUSTAL W of the amino acid sequence of vacA for the m2 and s2 alleles. Amino acid differences between the query sequence and the sequences from BLASTP are marked by red squares.
option in GenBank. Here, our *H. heilmannii sensu lato* sequence had 90–91% identity with *H. pylori* sequences of strains ATCC 43504, NCTC 11637 and 26695.

Given the existence of a wide variety of strains with a high percentage of identity, a phylogenetic tree for sequence similarity was reconstructed to determine the closest origin of this uncultured *H. heilmannii sensu lato*. Using MEGA 6 software, a dendrogram was obtained using the neighbour-joining method with 1000 bootstrap replicates (Fig. 2a). The phylogenetic tree showed an arrangement in three main clusters. The first cluster was constituted by the genetic proximity amongst *H. felis*, *H. salomonis*, *H. bizzozeronii*, *H. cynogastricus*, *H. baculiformis*, *H. heilmannii*, *Candidatus H. heilmannii*, Helicobacter sp. and *Helicobacter* sp. uncultured bacteria type isolated from biopsies of cheetahs. Interestingly, in this cluster we found our uncultured *H. heilmannii sensu lato* between the *Helicobacter* sp. uncultured bacteria type strain and *H. heilmannii*. Despite this approach, the tree distances between our uncultured *H. heilmannii sensu lato* sequence and the cheetah’s are still large. The second cluster consisted in *H. suis* and different strains of *H. heilmannii* from human, mandrill monkey, rhesus macaque and crab-eating macaque. Finally, the third cluster was an outgroup that had a subcluster with the *H. pylori* strain and other *Helicobacter* spp. most distant with respect to uncultured *H. heilmannii sensu lato*, such as *Helicobacter tursiopsae*, *Helicobacter cetorum*, *Helicobacter pollorum*, *Helicobacter canis*, *Helicobacter hepaticus* and *Helicobacter bilis*, amongst others.

Next, we evaluated the presence of *H. pylori* virulence factors *cagA* and *vacA* (Kumar et al., 2008) (Fig. S1b). As expected, the *cagA* virulence gene was not detected in this bacterium. Surprisingly, we found the *vacA* gene to be present, displaying the s2m2 combination of alleles. The nucleotide sequence analysis indicated 98% identity between the query sequence and *H. pylori* sequences from different strains deposited in GenBank for the m2 allele. Meanwhile, only 87% of identity for the s2 allele was obtained.

We then compared the predicted amino acid sequence of the m and s *vacA* alleles from our query sample with GenBank through BLASTP. Both alleles displayed a highly conserved region between the analysed sequences. For the m2 allele, the sample was similar to the *H. pylori* vacuolating cytotoxin from two *H. pylori* strains. For the strain with GenBank accession number BAS05841.1, 89% identity and 18% coverage were found. We also identified 62% identity and 29% coverage between our query sample to the strain with GenBank accession number BAS05841.1. The alignment by CLUSTAL W demonstrated that our query sample had 18 different amino acids in the alignment region in comparison with both *H. pylori* strains analysed (Fig. 2b). For the s2 allele, we found 77% identity and 60% coverage with the *H. pylori* vacuolating cytotoxin from 10 *H. pylori* strains with a 13 amino acid difference between our sample and the analysed strains (Fig. 2b).

**Discussion**

There are several reports on *H. heilmannii* infection in children. However, very few share a genetic description as described in this paper. In general, the studies described between 0.2 and 0.9% prevalence of *H. heilmannii* infection. The main reported symptom is abdominal pain. The histological assay displayed mild chronic gastritis. A few cases reported duodenal ulcer, but some were normal. In some cases, the urea breath test or rapid urease test were positive. All studies reported negative culture for *H. heilmannii*. The treatment for the infection was the same as used for *H. pylori* infection. The combination of a proton pump inhibitor plus two antibiotics (amoxicillin, clarithromycin or metronidazole) was shown to be effective for the eradication. Almost all the reported cases were associated with the patient living with pets – often dogs and, less frequently, cats (Qualia et al., 2007; Šykora et al., 2004; Boyanova et al., 2003; Iwanczak et al., 2006, 2012; Kato et al., 2005; van Loon et al., 2003).

In this research, the analysis of the 16S rRNA gene sequence demonstrated that the bacterium found in the stomach of the child belonged to an uncultured *Helicobacter* sp. By comparing the results obtained from phylogenetic analysis of the 16S rRNA gene sequence with those obtained by O’Rourke et al. (2004), the sequence of our uncultured *H. heilmannii sensu lato* was grouped with Cluster 2 of O’Rourke et al. (2004). Cluster 2 includes human isolate 2 (HU2), isolates from cheetah, bobcat, tiger, wild dog and cat, the putative ‘*H. heilmannii*’ isolate cultured from a human, and the recognized species *H. salomonis*, *H. felis* and *H. bizzozeronii*. From Cluster 2, we obtained a phylogeny based on the partial ureA and ureB gene sequences. The sequence was regrouped in four new clusters. Unfortunately, we could not amplify the ureA and ureB genes from our bacterium. However, given the proximity of the 16S rRNA gene sequence with the sequences of isolates from cheetahs, it may be possible that our bacterium could group in the so-called Cluster B (which corresponds to new putative species) or Cluster D (with *H. felis* strains). To reach a species definition for our uncultured *H. heilmannii sensu lato* would require more evidence. However, despite the similarity amongst symptoms and pathological findings between our uncultured *H. heilmannii sensu lato* and *H. pylori*, we can confirm that they are different types of bacterium because *H. pylori* is in an outgroup more distant than our bacterium in the phylogenetic tree. In conclusion, the uncultured *H. heilmannii sensu lato* found in the stomach of the girl shares some pathological, morphological and genetic features with *H. heilmannii*.

Interestingly, the bacterium presents the *vacA* virulence gene with s2m2 alleles like those described in *H. pylori*. *VacA* in the host cells causes ‘vacuolation’ – a process
characterized by the accumulation of large vesicles that possess the hallmarks of both late endosomes and early lysosomes (Papini et al., 1994). In addition, it disrupts mitochondrial functions, stimulates apoptosis and blocks T-cell proliferation (Cover & Blanke, 2005). It has been shown that H. pylori with \textit{s1m1} and \textit{s1m2} \textit{vacA} cause more severe chronic inflammation when compared with the \textit{s2m2} allele. The \textit{s2} type encodes a \textit{VacA} protein with an additional N-terminal hydrophilic amino acid segment that prevents vacuolation (McClain et al., 2001).

To date, a homologue of the \textit{vacA} gene has been found only in \textit{H. suis} isolated from the gastric mucosa of a pig (Vermooten et al., 2011). However, the \textit{H. suis vacA} homologue exhibits no \textit{vacA} signal sequence, indicating that it might encode a dysfunctional cytotoxin. Therefore, it is possible that \textit{vacA} gives a comparative advantage to the uncultured \textit{H. heilmannii sensu lato}, allowing it to persist in the gastric mucosa of the child.

Considering these findings, it is reasonable to propose that \textit{H. heilmannii sensu lato} has spread out of its niche, colonizing other hosts (including humans) in order to avoid difficulties that result from both the natural barrier and the immune system of its specific original host. Effective methods for identifying new micro-organisms of the genus \textit{Helicobacter} are needed as well as relevant clinical and epidemiological information.

**Acknowledgements**

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**References**


