**Helicobacter ganmani** Infection Associated with a Spontaneous Outbreak of Inflammatory Bowel-like Disease in an IL-10-Deficient Mouse Colony

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**Summary**

Background: A breeding colony of IL-10 deficient B6.129P2-Il10 tm1Cgn/J mice, kept under conventional conditions, developed an inflammatory bowel-like disease (IBD) with rectal prolapse and blood tinged diarrhoea. No clinical signs of disease were observed at the time of arrival to our animal house. These animals were originally planned to serve as a negative control group in an experimental infection study with *Helicobacter* species to investigate colonization of the murine gut.

Results: A spiral-shaped, Gram-negative bacterium was isolated from the breeding mice colony. In a first group of six animals, tissue specimens from the liver, small and large intestines, faeces and blood, were analysed by culture, PCR-denaturing gradient gel electrophoresis (PCR-DGGE), species-specific PCR assays and DNA-sequencing, histology and serology. *Helicobacter ganmani*, but no other *Helicobacter* species, was isolated from the liver, small bowel, caecum, colon and faeces. We found inflammation in caeca, colon and livers, most pronounced in the caecal areas of culture positive mice with a severe typhilitis with cystic dilatation of glandular structures and irregular crypt architecture. Some animals showed a pronounced colitis with mucosal and sub-mucosal inflammatory infiltrates. Other animals displayed large lymphoid infiltrates in the livers and hepatitis. Tissue samples and sera from 18 additional animals from the same breeding colony were analysed by the same methods, except for culture. *H. ganmani* was identified by PCR in most tissue samples of the 18 additional animals as well. Sero-conversion to *H. ganmani* correlated well with histopathological changes. Conclusions: Our findings emphasize the importance of using *Helicobacter*-free animals to develop murine models of chronic hepatitis and colitis.

**Introduction**

Since the discovery of *Helicobacter pylori* some 25 years ago the genus of *Helicobacter* has expanded dramatically and now more than 25 species of *Helicobacter* and a similar number wait to be classified (Nilsson et al., 2005). These novel species have been isolated from a variety of animals and were shown to cause gastrointestinal disease in various animal models (Solnick et al., 2001). Some bile-tolerant enteric species of *Helicobacter* have been detected in humans with diarrhoea, bacteraemia and chronic inflammation in the liver and bile ducts (Kiehlbauch et al., 1994; Fox et al., 1998; Hsueh et al., 1999; Fox et al., 2000; Nilsson et al., 2000; Nilsson et al., 2001). Interleukin-10 deficient
(IL-10 -/-) mice develop inflammatory bowel disease (IBD) when housed under conventional but not under germ-free conditions, strongly suggesting a role for enteric bacteria in this model of human IBD (Berg et al., 1996; Sellon et al., 1998). IL-10 -/- mice housed under specific pathogen-free (SPF) conditions, without *Helicobacter* spp., develop a less severe IBD and an experimental infection of IL-10 -/- mice by *Helicobacter hepaticus* was shown to be associated with colitis, and proposed as a model to study a “microbial trigger” of IBD (Kullberg et al., 1998). A number of other bile-tolerant enterohepatic *Helicobacter* spp. have been reported to be associated with IBD in naturally and experimentally infected mice with various immune deficiencies (Shomer et al., 1997; Franklin et al., 1998; Franklin et al., 1999). Some recent studies reported detection of specific enteric *Helicobacter* spp. in the human colon, but whether these microbes play a role in any human intestinal diseases is unclear (Oliveira et al., 2004; Streutker et al., 2004; Sturegård et al., 2004). *Helicobacter ganimani* is an enterohepatic and urease negative anaerobe species of *Helicobacter* isolated from intestines and livers of mice (Roberson et al., 2001). Whether *H. ganimani* belongs to the murine indigenous gut microflora or whether this species can cause disease in rodents, other animals and man is not yet understood. We now report on a colony of IL-10 deficient B6.129P2-Il10 tm1Cgn/J mice, with *Helicobacter*-associated typhlocolitis, hepatitis, diarrhoea and rectal prolapses. These animals were originally planned to serve as a negative control group in an experimental infection study to investigate colonization of the murine gut.

**Materials & Methods**

**Animals**

In this outbreak study, six female mice, 6-month-old, B6.129P2-Il10 tm1Cgn/J (The Jackson Laboratory, Bar Harbor, ME, USA), and later on further 18 animals from the same breeding colony at a Lund University animal house, were studied. The animals were housed in 425 x 266 mm polycarbonate cages (Scanbur A/S, Koge, Denmark) with aspen chips as bedding and given the RM1 expanded feed (SDS, London, UK) and tap water ad libitum. Environmental conditions for the animals included >25 conditioned fresh air changes per hour, a temperature range of 20-22 °C, relative humidity between 50 and 65% and a 12 h light/dark cycle with twilight. The animals were tested for viral, bacterial, and parasite infections according to the Federation of European Laboratory Animal Science Associations (FELASA, www.felasa.eu) Working Party Report at the National Veterinary Institute (Uppsala, Sweden). All tests except for *Staphylococcus aureus Pasteurellaceae* and *Helicobacter* spp. were negative. No clinical signs of disease were observed at the time of arrival. The planned animal experiments were approved by the Lund University Animal Ethics Committee (permit no. M77-02). Later, some animals in the breeding colony displayed rectal prolapses and blood-tinged diarrhoea. Faecal samples were collected for culture and PCR analyses one month prior to sacrificing the animals, by carbon dioxide according to local regulations. Blood was drawn by heart puncture, sera separated and frozen for serological analyses. Samples from liver, proximal and distal small bowel, caecum, proximal and distal colon were collected for culture, PCR analyses and histology.

**Culture**

Tissue and faecal samples were collected in a transport medium (tryptone soy broth, 10% horse serum, 20% glycerol), homogenised in the same medium with a sterile glass tissue grinder and filtered gently in two steps; first through a 5.0 µm Minisart® filter followed by a 0.45 µm filter (Sartorius, Goettingen, Germany). 100 µl of the filtrate was plated onto fresh Brucella agar (Becton Dickinson, Bedford, MA, USA) supplemented with 10% (v/v) horseblood, 10% (v/v) inactivated horseserum (GibcoBRL, Paisley, Scotland), 1% (v/v) IsoVitalex (Becton Dickinson) and 1% (v/v) bovine haemin chloride (ICN, Aurora, OH, USA). Faecal cultures
were incubated under anaerobic (10% CO₂, 10% H₂ and 80% N₂) and microaerobic (6% O₂, 7% CO₂, 7% H₂ and 80% N₂) conditions at 37°C, using the Anoxomat culture system (MART®, Lichtenvoorde, The Netherlands). All cultures were observed repeatedly during several weeks for colonies of Helicobacter-like organisms (HLO’s), tested for urease activity, Gram-stained and analyzed by a Helicobacter genus-PCR followed by sequencing (see below).

**DNA-extraction**

DNA was extracted from tissue specimens (15-20 mg), from agar cultures using the QIAamp DNA Tissue Kit (QIAGEN, Hilden, Germany) and from faecal samples with the QIAamp DNA Stool Kit (QIAGEN) according to the manufacturer’s instructions. Extracted DNA was aliquoted and stored at -20°C for further analysis.

**PCR assays**

PCR amplifications were carried out using a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Foster City, CA, USA). Helicobacter genus- as well as species-specific primers are listed in Table 1. A nested Helicobacter genus-specific PCR assay included primers C97 and C05 in the first step and primers 16S1F-GC and 16S2R in the second step. Reaction mixtures, temperature cycles and detection of PCR products have been described previously (Fox et al., 1998; Abu Al-Soud et al., 2003). Amplified products were analyzed by DGGE as described elsewhere (Abu Al-Soud et al., 2003). Helicobacter species-specific PCR was performed using primers for *H. ganmani, Helicobacter rodentium* and *Helicobacter bilis*. Detection of *H. ganmani* DNA was done using a semi-nested PCR targeting the 16-23S internal spacer region as described elsewhere (Tolia et al., 2004). The first-step PCR product of the Helicobacter genus-specific PCR assay was used as a template in PCR mixtures containing *H. bilis*- or *H. rodentium*-specific primers (Table 1). The specificity of the latter three assays was evaluated using DNA from *H. ganmani* (CCUG 43526 and 43527), *H. bilis* (CCUG 41387) and *H. rodentium* (MIT 95-2178).

**16S rDNA sequence analysis**

Sequencing of PCR-products (16S1F-GC, 16S2R) excised from DGGE gels was performed as described elsewhere (Abu Al-Soud et al., 2003). The primers 27F and 1494R were used to amplify the full length 16S rDNA from bacterial isolates, using PCR conditions described elsewhere (Robertson et al., 2001). Full length 16S rDNA was purified from agarose gels using Ultra-free-DA centrifuge tubes (Millipore, Bedford, MA, USA). Sequencing of both DNA strands was performed using ten sequencing primers (27F through 1494R, Table 1), the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit v3.0, and an ABI 310 DNA Sequencer (Applied Biosystems). Sequences were corrected and assembled using BioEdit software (mbio.ncsu.edu/BioEdit/bioedit.html). The closest known relative of partial and complete 16S rDNA sequences was determined using BLASTN 2.2.1 [ncbi.nlm.nih.gov/blast] (Altschul et al., 1997).

**Protein extraction and SDS-PAGE**

A whole cell extract of *H. ganmani* ES-4 (CCUG 47872), a mouse caecal isolate from the current study, was prepared by intense vortexing, freezing and thawing of harvested cells. Cell debris was removed by centrifugation and supernatant kept for immunoblot analysis. In addition, acid glycine extractions of cell surface proteins of the *H. ganmani* strain, *H. hepaticus* (CCUG 33637) and of *H. bilis* (CCUG 41387) was done as described elsewhere (Kornilov’ska et al., 2002). SDS-PAGE was performed under reducing conditions in a Criterion cell™ electrophoresis equipment (Bio-Rad, Hercules, CA, USA). Extracted proteins were separated in an 8-16% gradient gel with a 5% stacking gel (Criterion pre-cast gels, Bio-Rad). Proteins and *M. sternuroides* standards ranging from 14.4 to 97 kDa (Amersham Biosciences) were diluted in sample
buffer (0.5 M Tris-HCl, pH 6.8, 0.5% bromophenol blue, 8% glycerol, 4% SDS, 4% 2-mercaptoethanol), heated at 95°C for 3 min and separated for 20 min at 50 V and at 200 V until the blue line reached the end of the gel.

**Immunoblot assay**

Separated *Helicobacter* proteins were eletrophoret-
ically transferred to PVDF membranes (Micron Separation, Westborough, MA, USA) in a semidyed electroblotter (Ancos, Vig, Denmark) for 1.5 h at a constant current of 1.0 mA/cm\(^2\) (Utt et al., 2002). Membranes were saturated for 2x15 min in blocking buffers, rinsed and probed with mice sera diluted 1/50 under gentle agitation for 16 h at 8°C. After repeated washings, strips were probed with horse-radish peroxidase-labelled goat anti-mouse antibodies (DakoCytomation, Roskilde, Denmark) diluted 1/600 and incubated for 2 h at 8°C. Bound antibodies were detected by reaction in a 50 mM sodium acetate buffer containing 0.04% 3-amino-9-ethylcarbazole (Sigma-Aldrich, St. Louis, MO, USA) and 0.015% H\(_2\)O\(_2\) (Utt et al., 2002).

Histopathology
Tissue samples, fixed in 4% buffered formaldehyde, were embedded in paraffin, cut in 4 µm thick sections and stained with haematoxylin and eosin. Stained sections were analyzed for histological lesions. The severity of the inflammatory infiltration was graded from none to severe inflammation using a similar system to the one developed for grading \textit{H. pylori} induced gastritis in guinea pigs (Sturegård et al., 1998).

Transmission electron microscopy
\textit{H. ganmani} ES-4 (CCUG 47872) cells were suspended and washed in PBS (pH 7.2) three times and centrifuged to a pellet which was pre-fixed in 0.1 M Sörensen phosphate buffer (pH 7.2) with 1.5% paraformaldehyde and 1.5% glutaraldehyde and post-fixed in 0.1 M Sörensen phosphate buffer (pH 7.2) with 1.0% osmium tetroxide. The sample was dehydrated with non-tox, embedded in agar 100 (Link, Stockholm, Sweden) and cut in 50-70 nm sections with a Reichert Supernova (Leica, Wien, Austria). Sections were contrasted with 4% uranyl acetate and 0.5% lead citrate and viewed in a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands).

Results
Faecal samples from the first group of IL-10-/- mice (n=6) were subjected to microbial culture under anaerobic and microaerobic conditions due to diarrhoeal illness in some animals. Growth consistent with HLO’s was detected under anaerobic, but not microaerobic conditions, in two of six faecal samples (from animals with diarrhoea). After sacrifice, samples from the liver, proximal and distal small bowel, caecum, proximal and distal colon were collected for culture. Ten of 36 cultured tissue samples, from four of six animals, demonstrated growth of HLO’s after seven to nine days of incubation. All isolates grew as discrete colonies with a tendency of swarming and were urease negative. Staining showed thin, Gram negative, slightly curved rods. Culture results of the first six animals analysed are outlined in Table 2. Transmission electron microscopy demonstrated spiral shaped organ-

Table 2. Culture, immunoblot and histology results from the six IL-10-/- animals in the first group.
All mice were positive for \textit{H. ganmani} by PCR.

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Culture of \textit{H. ganmani}</th>
<th>Immunoblot</th>
<th>Inflammation$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver small bowel caecum colon faeces</td>
<td>sero-conversion</td>
<td>liver small bowel caecum colon</td>
</tr>
<tr>
<td>1$^b$</td>
<td>- - + + +</td>
<td>positive</td>
<td>++ - +++ +</td>
</tr>
<tr>
<td>2$^b$</td>
<td>+ - + + +</td>
<td>positive</td>
<td>++ - +++ +</td>
</tr>
<tr>
<td>3</td>
<td>- - + - -</td>
<td>intermediate</td>
<td>+ - +++ +</td>
</tr>
<tr>
<td>4</td>
<td>- - - - -</td>
<td>negative</td>
<td>+ - + -</td>
</tr>
<tr>
<td>5</td>
<td>- - - - -</td>
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<td>++ - + +</td>
</tr>
<tr>
<td>6</td>
<td>+ + + + -</td>
<td>positive</td>
<td>++ - ++ +</td>
</tr>
</tbody>
</table>

$^*$Inflammation estimated to be none (-), mild (+), moderate (++) or severe (+++).

$^b$Mouse 1 and 2 had rectal prolapse.
isms with a morphology consistent with some Helicobacter. (Figure 1). Six isolates, probably representing one strain, were deposited at the Culture Collection, University of Gothenburg (CCUG 47870-47875).

All six faecal samples and 32 of 36 tissue samples investigated demonstrated amplification of PCR-products consistent with Helicobacter spp. The small bowel and liver from one mouse and the small bowel from two other mice were PCR-negative. DGGE analysis of all positive samples demonstrated a mobility pattern corresponding to H. ganmani, H. bilis and H. rodentium (Figure 2), which migrate similarly under the DGGE-conditions used. Sequencing of PCR products (~430 bp) amplified from mouse tissue samples as well as the isolated HLO’s using primers 16S1F and 16S2R, demonstrated complete homology of both strands in all samples (GenBank accession no. AY561830), and showed complete homology to H. ganmani sequences in GenBank. The full-length 16S rRNA-gene sequences from three isolates (CCUG 47871 to 47873, www.ccug.se), showed complete homology to H. ganmani 16S rDNA using the BLASTn GenBank search. No cross reactivity was found between the three species-specific PCR assays when they were applied to H. ganmani, H. rodentium and H. bilis. All Helicobacter genus PCR-positive samples were also positive using primers for H. ganmani, whereas they were PCR-negative for H. rodentium and H. bilis.

Histological analysis of culture positive mice in the
first group displayed inflammation in caeca, colon and liver of varying severity, most pronounced in the caecal area. In three of the six animals, all culture-positive for *H. ganmani* from caecum biopsies, we found severe typhlitis with cystic dilatation of glandular structures, irregular crypt architecture and a massive infiltration of lymphocytes and granulocytes in the caecal mucosa (Figure 3A).

**Figure 3.** Pictures A-C showing histology changes for IL-10 ^{-/-} ^ mice colonised with *H. ganmani*. 
A. Caecal mucosa of a mouse with intense inflammatory reaction in lamina propria, development of reaction centres, reactive changes within the colonic epithelium with destruction of glandular structures and thickening of the mucosa (magnification x40, bar 200 µm). Insert demonstrates lymphocytic infiltrate (magnification x400, bar 20 µm).

Inflammation in the colon was generally milder than in the caecum but three animals showed a more pronounced colitis with mucosal and submucosal inflammatory infiltrates consisting of both lymphocytes and granulocytes (Figure 3B). Liver specimens displayed diffuse inflammation with small aggregates of inflammatory cells and four of the six animals had larger lymphoid infiltrates in the liver consisting mainly of lymphocytes with an appearance, suggesting lymphoma, similar to what was reported previously by Wang *et al* (2000) for *H. pylori* (Table 2, Figure 3C).

C57BL/6 mice infected with *H. ganmani* proteins with *M*~s~ between 14 and 120 kDa (Figure 4A). These three sera were from *H. ganmani* culture-positive animals, of which all demonstrated moderate to severe typhlitis and two suffered from rectal prolapse and diarrhoea.
Intermediate antibody reactivity was found with two sera (Figure 4A, strips 3 and 5) and one serum showed only reactivity to one band and was interpreted as negative. This sero-negative mouse was also culture-negative. One of the animals with an intermediate immune reaction displayed typhlitis and the other two a mild inflammation of the caecum and colon (Table 2). Immunoblot using each of the two protein extraction methods gave similar results (data not shown). In addition, an immunoblot with *H. ganmani* antigens was performed with sera from wild-type mice known to be PCR-positive for *H. bilis* and *H. hepaticus*, respectively, demonstrating some weak bands, *i.e.* no cross reactivity against proteins of *H. ganmani*. Picture B show immunoblots with sera from the *H. ganmani* culture positive mice (first study) probed on strips with antigens of *H. hepaticus* and *H. bilis* showing bands mainly in the 45-65 kDa region, corresponding in size to some heat shock proteins, often cross-reactive between the various *Helicobacter* spp. Strips marked with PC represent positive control sera from mice colonized with *H. hepaticus* and *H. bilis* respectively. Arrows indicate proteins of high specificity for these two species.

(4B). Weak antibody reactivity to bands in the 45-65 kDa region was observed, most probably corresponding to some minor cross reactivity between some heat shock proteins.

Additionally 18 mice of the breeding colony
An extended investigation of 18 additional animals from the same breeding colony was further analysed for *H. ganmani* status using *Helicobacter* genus-specific PCR-DGGE, *H. ganmani*-, *H. rodentium*-, and *H. bilis*-specific PCR assays as well as immunoblot. Tissue specimens were also analysed for histopathological changes. All animals were positive with *Helicobacter* species-specific as well as *H. ganmani*-specific PCR in the caecum, and proximal and distal colon. Tissue specimens of the small bowel from ten animals were PCR-posi-
tive with *Helicobacter* genus-, as well as *H. ganmani*-specific PCR. None of the tissue specimens became PCR-positive either for *H. rodentium* or *H. bilis*. The additional breeding colony animals demonstrated a similar histological pattern as found in the first group (Table 3).

Table 3. Immunoblot and histology results of the additional 18 IL-10-/- mice from the breeding colony

<table>
<thead>
<tr>
<th>Mouse no</th>
<th>Immunoblot</th>
<th>liver</th>
<th>Inflammation</th>
<th>caecum</th>
<th>colon</th>
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<tbody>
<tr>
<td>1*</td>
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<td>++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
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</table>

*Inflammation estimated to be none (-), mild (+), moderate (++) or severe (+++). Mice no 2, 5, 7, 9, 14 and 16 were negative by immunoblot and showed normal histology.

Sero-conversion against antigens of *H. ganmani* was demonstrated in 11 of 18 (61%) sera. Nine of 11 (82%) sero-converted animals demonstrated lesions of the caecum, colon or liver, whereas two apparently sero-positive mice had no detectable lesions. Serum of one animal with mild inflammation of the caecum was sero-negative for *H. ganmani* (Table 3).

**Discussion**

The breeding colony of mice investigated in the current study was tested for *Helicobacter* colonization one year earlier, using *Helicobacter* genus-specific PCR on faecal samples. No *Helicobacter* species were detected and the animals did not show any IBD-like symptoms. Following this screening, mice were transferred from the quarantine room into our animal house for continued breeding. Eight months later the animals began to present symptoms. The breeding colony was kept in a separate cage locker with an individual air supply. *Helicobacter* genus-PCR screening of water, clean bedding, chow, water bottles and cages did not demonstrate any possible source of *Helicobacter*.

The mouse colony was re-tested and *H. ganmani* was detected in all mice. This shows the difficulty to keep mice free of *Helicobacter* gut colonization and the importance of regular screening for *Helicobacter* spp. in breeding colonies as well as in individual experimental setups.

*H. hepaticus* causes hepatitis and liver tumours in immunocompetent mice, particularly male A/JCr mice (Whary et al., 1998), and *H. bilis* is associated with hepatitis in selected strains (Fox et al., 1995) but for *H. ganmani* few observations are available. Natural colonisation of *Helicobacter* species was investigated in IL-10-/- mice (Zhang et al., 2005). DNA of *H. ganmani* and *H. hepaticus* was detected and the severity of typhlocolitis depended on the colonizing *Helicobacter* species. In that study no culture or analyses of the immune responses were performed. Recently, Taylor and co-
workers reported that enterohepatic Helicobacter species commonly infect mouse research colonies in both commercial and academic animal houses; however, no H. ganmani was detected in any of the 79 animals examined (Taylor et al. 2007).

Enteric Helicobacter spp., in particular H. hepaticus and H. bilis, have been shown to cause typhlitis, colitis and hepatitis in immunodeficient mice (Shomer et al., 1997; Franklin et al., 1998; Kullberg et al., 1998). Helicobacter-induced lesions in these models exhibit severe typhlitis with less severe colitis and hepatitis. The histological lesions observed in these studies are similar to the lesions found in the present study with pronounced typhlitis and less severe colitis and hepatitis.

To investigate a possible causative link between H. ganmani and IBD in IL-10 -/- mice, an experimental infection model, using Helicobacter-free mice, is needed (Shen et al., 1997; Roberson et al., 2001). Only a few strains of H. ganmani are available today through public type culture collections but as the number of available isolates increases it will be possible to study whether different strains of H. ganmani vary in virulence. Recent findings of H. ganmani-like 16S rDNA sequences in liver samples from children with chronic liver disease, but not in controls (Tolia et al., 2004), indicates that further studies are needed to confirm a possible role of this microbe in various human and animal enterohepatic diseases (Nambiar et al., 2006). Thus, a zoonotic potential of this species is plausible.

No single infectious agent has been clearly associated to human IBD, i.e. ulcerative colitis and Crohn’s disease related to an exaggerated mucosal immune response directed towards the mucosal microflora (Bouma et al., 2003). Thus, a zoonotic potential of this species is plausible.

Conclusions
Our findings suggest that chronic infections with H. ganmani should be added to the list of Helicobacter spp. associated with chronic colitis and hepatitis in IL-10 -/- mice. It seems necessary to test for Helicobacter gut colonization before starting experimental infections in immunodeficient mouse strains.

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