In vivo studies on the pathogenic effects of canine and feline Helicobacter species

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IT DOESN'T MATTER WHO YOU ARE, IT'S WHAT YOU DO THAT TAKES YOU FAR

(MADONNA)
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>AB/PAS</td>
<td>Alcian blue/ periodic acid schiff</td>
</tr>
<tr>
<td>AGS</td>
<td>Gastric adenocarcinoma cell line</td>
</tr>
<tr>
<td>Alp</td>
<td>Adherence-associated lipoprotein</td>
</tr>
<tr>
<td>AP</td>
<td>Activator protein</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>Bab</td>
<td>Blood group antigen-binding adhesin</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cag PAI</td>
<td>cag pathogenicity island</td>
</tr>
<tr>
<td>Cag</td>
<td>Cytotoxin-associated protein</td>
</tr>
<tr>
<td>CC</td>
<td>Chief cell</td>
</tr>
<tr>
<td>CCUG</td>
<td>Culture Collection of the University of Göteborg</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony-forming units</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
</tr>
<tr>
<td>Fas-L</td>
<td>Fas-ligand</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>H.</td>
<td>Helicobacter</td>
</tr>
<tr>
<td>HP-NAP</td>
<td>H. pylori neutrophil-activating protein</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INS-GAS</td>
<td>Insulin-gastrin</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolt</td>
</tr>
<tr>
<td>Le</td>
<td>Lewis antigen</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LR</td>
<td>Limiting ridge</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa-associated lymphoid tissue</td>
</tr>
</tbody>
</table>
mg  Milligram
min  Minute
ml  Milliliter
mM  Millimolar
ND  Not determined
NF-κB  Nuclear factor-κB
nm  Nanometer
NSAID  Non-steroidal anti-inflammatory drug
OD  Optical density
Oip  Outer inflammatory protein
OMP  Outer membrane protein
OR  Odds ratio
p.i.  Post-infection
PAMP  Pathogen-associated molecular pattern
PAS  Periodic acid schiff
PC  Parietal cell
PCR  Polymerase chain reaction
PMN  Polymorphonuclear cell
PRR  Pattern-recognition receptor
RNA  Ribonucleic acid
ROS  Reactive oxygen species
Sab  Sialic acid binding adhesin
SPF  Specific pathogen free
TEM  Transmission electron microscopy
Th  T-helper
TLR  Toll-like receptor
TNF  Tumor necrosis factor
TRAIL  Tumor necrosis factor-related apoptosis inducing ligand
U  Units
Vac  Vacuolating cytotoxin
In 2005, the Nobel Price for Medicine was awarded to two Australian scientists, Barry Marshall and Robin Warren, for their discovery of the human gastric pathogen *Helicobacter pylori* and its association with gastroduodenal disease. The finding of this entirely new bacterial genus inspired many scientists around the world and its impact could be felt in every country. The isolation and study of this organism triggered a true revolution in the treatment of human gastritis and peptic ulceration and made this bacterium one of the most investigated bacterial species ever. This is reflected in the large number of *H. pylori* manuscripts that are published each year. Nevertheless, in spite of this intense investigation, a lot of controversy about the pathogenesis of *H. pylori* still exists. After Marshall’s discovery, interest in other spiral bacteria colonising the stomach of multiple animal species but also humans rose. To date, very few data are available on the actual clinical significance of so-called gastric non-*H. pylori* *Helicobacter* species and the mechanisms by which these bacteria elicit lesions in the host.
The *Helicobacter* genus consists of *Helicobacter* species found in the stomach (gastric helicobacters) and *Helicobacter* species isolated from the liver and intestinal tract (enterohepatic helicobacters) of man and animals. To date, 18 enterohepatic and 12 gastric *Helicobacter* species have been described (Van den Bulck, 2005). So far, three gastric *Helicobacter* species have not yet been cultured *in vitro* and are therefore designated “*Candidatus*” followed by a descriptive epithet (Murray and Stackebrandt, 1995). In this introduction, only the culturable gastric helicobacters will be discussed (Table 1).

1 Gastric *Helicobacter* species

1.1 Humans

Warren and Marshall (1983) described curved bacteria in stomach biopsies of human patients suffering from dyspepsia. These biopsies were culture positive for an S-shaped, Gram-negative, microaerophilic bacterium with 5 to 7 polar sheathed flagella. Originally, the organism was thought to be a member of the *Campylobacter* genus and was therefore named *Campylobacter (C.) pyloridis*, later corrected to *C. pylori*. It became clear that, although *C. pylori* resembles *Campylobacter* in many aspects, it differs in important features such as flagella, fatty acid content and 16S rRNA sequence. Therefore it was renamed *Helicobacter pylori*, the first member of the *Helicobacter* genus (Goodwin et al., 1989).

In addition to *H. pylori*, another *Helicobacter*-like organism was found in the stomach of patients suffering from dyspepsia (McNulty et al., 1989). This organism, tightly coiled with 10 to 20 bipolar sheathed flagella, was clearly distinguishable from *H. pylori* and showed more morphological resemblance with the gastric spiral bacteria found in animals. The bacterium was first referred to as “*Gastrospirillum hominis*”. However, following 16S rRNA sequencing, it was designated to the *Helicobacter* genus and the name “*H. heilmannii*” was proposed (Solnick et al., 1993). *In vitro* cultivation of “*H. heilmannii*” is virtually impossible with current methods, so its proper species designation remains controversial (Solnick, 2003).

It is now clear that “*H. heilmannii*” does not represent a single species. It rather reflects a species complex comprised of “*Candidatus H. suis*” (De Groote et al., 1999), found in the stomach of pigs and referred to as “*H. heilmannii*” type 1, and the spiral organisms found in the gastric mucosa of cats and dogs that are referred to as “*H. heilmannii*” type 2 i.e. *H. felis* (Lee et al., 1988), *H. bizzozeronii* (Hänninen et al., 1996), *H. salomonis* (Jalava et al., 1997)
(see below) and the recently described “Candidatus H. heilmannii” (O’Rourke et al., 2004b) and “H. cynogastricus” (Van den Bulck et al., In Press).

1.2 Animals

In 1986, Fox et al. isolated Campylobacter-like organisms from gastric lesions in a ferret and from two healthy ferrets. When it was later confirmed that these organisms belonged to the Helicobacter genus, the name C. mustelae was changed into H. mustelae (Goodwin et al., 1989). Instead of being spiral in shape, H. mustelae is a small rod that carries multiple sheathed flagella located at both poles as well as laterally (O’Rourke et al., 1992). Similar to H. pylori, H. mustelae has a very specific host range and is a natural colonizer of ferrets.

H. felis was first cultured by Lee et al. (1988) from the gastric mucosa of a cat. H. felis has since also been cultured from the stomach of dogs (Paster et al., 1991). This organism is tightly coiled and possesses 14 to 20 bipolar sheathed flagella. The presence of periplasmic fibrils that encase the bacterium was thought to be a unique feature of this gastric Helicobacter species until the isolation of “H. cynogastricus” from the canine mucosa. The latter bacterium indeed also possesses a periplasmic fibril (Van den Bulck et al., In Press). The fibrils of H. felis may disappear on subculture (Eaton et al., 1996a).

A spiral organism was isolated from the canine mucosa by Hänninen et al. (1996) that was morphologically different from H. felis. This organism lacked periplasmic fibrils around its thin body and had 10 to 20 bipolar flagella. DNA-DNA hybridisation studies showed clearly that it involved a new Helicobacter species, which was named H. bizzozeronii after Guilio Bizzozero, a pioneer in gastrointestinal microbiology. Not much later another Helicobacter species was isolated from dogs. This bacterium was named H. salomonis, in honor of the late Hugo Salomon (Jalava et al., 1997). It has 10 to 23 bipolar sheathed flagella and no periplasmic fibrils.

H. acinonychis (formerly called H. acinonyx) was cultured in the early nineties from a colony of cheetahs suffering from gastritis (Eaton et al., 1991b; Eaton et al., 1993). H. acinonychis has also been detected in two Sumatran tigers presenting gastritis (Schröder et al., 1998). In a recent study by Terio et al. (2005), the prevalence of different Helicobacter species was compared in cheetahs with and without gastritis. Not one single cheetah was infected with H. acinonychis which prompted the authors to assume that H. acinonychis was not representative of the Helicobacter species infecting the cheetah population as a whole.
Table 1. Characteristics of culturable gastric *Helicobacter* species

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Urease production</th>
<th>Catalase production</th>
<th>Alkaline phosphatase hydrolysis</th>
<th>Nitrate reduction</th>
<th>Indoxyl acetate hydrolysis</th>
<th>γ-glutamyl transferase production</th>
<th>Susceptible to Nalidixic acid</th>
<th>Cephalothin</th>
<th>Periplasmic fibrils</th>
<th>N° of flagella</th>
<th>Distribution of flagella</th>
<th>Growth with 1% glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pylori</em> (^{(1)})</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>-</td>
<td>4-8</td>
<td></td>
<td>Bipolar</td>
<td>-</td>
</tr>
<tr>
<td><em>H. mustelae</em> (^{(2)})</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>S</td>
<td>R</td>
<td>-</td>
<td>4-8</td>
<td></td>
<td>Peritrichous</td>
<td>-</td>
</tr>
<tr>
<td><em>H. felis</em> (^{(3)})</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>-</td>
<td>14-20</td>
<td>Bipolar</td>
<td>-</td>
</tr>
<tr>
<td><em>H. bizzozeronii</em> (^{(4)})</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>-</td>
<td>10-20</td>
<td>Bipolar</td>
<td>-</td>
</tr>
<tr>
<td><em>H. salomonis</em> (^{(5)})</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>R</td>
<td>S</td>
<td>-</td>
<td>10-23</td>
<td>Bipolar, ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>H. acynonychis</em> (^{(6)})</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>-</td>
<td>2-5</td>
<td>Bipolar</td>
<td>-</td>
</tr>
<tr>
<td>“<em>H. suncus</em>” (^{(7)})</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>6-12</td>
<td>Bipolar</td>
<td>-</td>
</tr>
<tr>
<td>“<em>H. cynogastricus</em>” (^{(8)})</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td></td>
<td>Bipolar</td>
<td>-</td>
</tr>
</tbody>
</table>

* these names have not yet been validated


+, positive reaction; -, negative reaction; ND = not determined; R = resistant, I = intermediately susceptible, S = susceptible
Cultures from the stomach of house musk shrews with chronic gastritis revealed a new *Helicobacter* species that was designated “*H. suncus*” (Goto et al., 1998). Further research is needed to elucidate the pathogenic role of this bacterium.

In 2002, a novel *Helicobacter* species was cultured from the stomach and feces of different dolphin species and a beluga whale. The name “*H. cetorum*” was proposed. Some animals had gastric ulcers but to date no conclusive evidence is available to suggest that “*H. cetorum*” is the causative organism of gastric ulcer disease in cetaceans (Harper et al., 2002).

## 2 *Helicobacter* related gastric disease

### 2.1 Histology and physiology of the stomach

The stomach is an expanded section of the digestive tube between the esophagus and small intestine. Its characteristic shape is shown in Fig. 1 along with terms used to describe the major histological regions of the stomach. The right side of the stomach is called the greater curvature and that on the left the lesser curvature.

![Fig. 1 Major histological regions of the human (left) and mouse (right) stomach.](image)

The mouse stomach (and that of the gerbil) is divided into a thin-walled forestomach and a thick-walled glandular stomach. This forestomach, which is not present in the human stomach, is covered with a stratified squamous epithelium and separated from the glandular stomach by the limiting ridge.
The stomach wall is made of a number of layers (Fig. 2). From inside to outside, the first main layer is the mucosa. This consists of an epithelium, the lamina propria underneath, and a thin smooth muscle layer called the muscularis mucosae. The second layer is the submucosa, consisting of dense irregular connective tissue. The following smooth muscle layer consists of a circular and longitudinal part and is covered by a serous membrane, the serosa.

Surface epithelium contains mucus-producing cells. They protect the mucosa from the acidic contents of the stomach. Mucins are oligomeric glycoproteins and MUC5AC and MUC6 are the major mucins in the stomach (Ho et al., 1995). On the mucosal surface funnel-shaped depressions (gastric pits) are found. Almost the entire mucosa is occupied by simple, tubular gastric glands which open into the bottom of the gastric pits (Fig. 3).

In contrast to the surface epithelium, the gastric glands have a specialized cellular composition and function in the different regions of the stomach. Cardiac glands are heavily branched tubular glands, which contain mainly mucus-producing cells. In the fundus, glands are composed of mucous, endocrine, parietal and chief cells (Fig. 3). Parietal (oxyntic) cells constitute the most conspicuous cell type because of their large size and acidophilic cytoplasm. A large number of mitochondria are present in these cells as well as intracellular canaliculi (Fig. 3). The latter are important structures in the mechanism of HCl production as parietal cells secrete the hydrochloric acid of the gastric juice. The acid is not present in the cell cytoplasm, but it is present in the canaliculi. Chief (zymogenic) cells are mainly located in the distal portion of the fundic glands. They produce pepsinogen, a precursor of the proteolytic enzyme pepsin, which is largely responsible for the stomach’s ability to initiate digestion of proteins. The glands of the antrum are more coiled than fundic glands, and they
may be branched. Endocrine cells, in particular gastrin-producing cells (G-cells), are more frequent in the antrum than in the fundus. D cells (producing somatostatin) and a few parietal cells may be present in the antrum but chief cells are usually absent.

The hormone gastrin causes an increase in the secretion of HCl and pepsinogen. It also causes increased motility in the stomach. Gastrin is released in the stomach in response to distention of the antrum, and in response to digestive products. It is inhibited by a pH less than 4, as well as the hormone somatostatin. The main functions of somatostatin are to inhibit HCl secretion and gastrin release.

![Fig. 3 Schematic drawing of the fundic gastric gland showing the different cell-types present. The insert shows in particular the ultrastructure of the parietal cell (adapted from Ross et al., 2002 and Stevens and Lowe, 2005).](image)
2.2 *Helicobacter* related gastric disease in humans

By 1984, it had become clear that there was a strong association between *H. pylori* and gastroduodenal disease. It would take several years, however, before there was sufficient evidence so that an etiologic role could be firmly established (Blaser, 1990).

*H. pylori* is ubiquitous and colonizes the stomach in about 50% of all humans (more frequently in developing countries as opposed to developed countries). In most individuals, *H. pylori* infection is asymptomatic. About 25% to 30% of infected individuals, however, will one day experience disease. The clinical outcome of *H. pylori* infection is diverse (Fig. 4) and dependent on the *H. pylori* strain (*i.e.* virulence factors), host susceptibility, environmental factors and their interactions.

![Diagram](image)

Fig. 4 *H. pylori* and its relationship with ulcers and gastric cancer. The green square represents the total population of uninfected persons in developed countries. Within is a circle representing the 30% who are infected with *H. pylori* (HP+). One third of the persons in the infected group develop a duodenal ulcer. Nearly all persons with a duodenal ulcer are infected. A gastric ulcer is usually caused by *H. pylori*, but about 30% of gastric ulcers occur in persons without *H. pylori* and can be related to aspirin and other non-steroidal anti-inflammatory drugs. Most gastric adenocarcinomas and lymphomas occur in persons with current or past infection with *H. pylori* (Adapted from Marshall, 2002).

The clinical symptoms of *H. pylori* infection are difficult to define. Any stomach symptom could possibly be caused by *H. pylori*. Dyspepsia is a constant gastric discomfort and/or pain that can be due to indigestion and heartburn. Heartburn can be related to *H. pylori* when some *H. pylori* patients produce excessive acid. Nausea associated with vomiting can be symptoms
of *H. pylori* infection. Blood present in the vomitus can be caused by a bleeding ulcer in the stomach. When *H. pylori* and gastritis cause delay in gastric emptying, patients may feel bloated (Sanders and Peura, 2002).

The majority of Western people with *H. pylori* gastritis display inflammation in the antrum, only few infected individuals have gastritis in the fundus (Dixon, 2001). Patients with antral-predominant gastritis are predisposed to duodenal ulcers, whereas patients with fundus-predominant gastritis and multifocal atrophy are more likely to develop gastric ulcers, gastric atrophy, intestinal metaplasia of the gastric mucosa, and ultimately gastric carcinoma.

The initial, acute phase of *H. pylori* infection is mostly subclinical. Infiltration of polymorphonuclear cells (PMNs) in the gastric epithelium and lamina propria edema can be found together with transient hypochlorhydria (Sobala *et al.*, 1991). Nevertheless, the acute phase of the infection is short and there is a gradual transition to chronic inflammatory infiltrates if the organism is not cleared by the host’s immune response. Acute gastritis is caused by every *H. pylori* strain but the clinical outcome is considered to vary with the *H. pylori* strain involved. Active chronic gastritis in *H. pylori* infection is primarily characterized by the presence of large numbers of lymphocytes and plasma cells in the mucosa and PMNs infiltrating the foveolar epithelium. In a later stage, formation of lymphoid follicles is observed and becomes a consistent feature in chronic *H. pylori* gastritis (Dixon, 2001). These lymphoid follicles constitute a mucosa-associated lymphoid tissue (MALT) and as such they provide the background tissue in which MALT-lymphoma arises.

*H. pylori* infection is also associated with atrophy and intestinal metaplasia of the gastric mucosa (Steadman *et al.*, 1988, Craanen *et al.*, 1992; Eidt and Stolte, 1994; Kuipers *et al.*, 1995; Dixon *et al.*, 1996). Atrophy can be defined as loss of glandular tissue that leads to thinning of the mucosa or can also be thought of as “a loss of functional cells”. Prolonged inflammatory processes in *H. pylori* infection may lead to glandular destruction and loss of parietal cells, the latter being replaced by mucous cells (mucous metaplasia). Atrophy is closely related to intestinal metaplasia (Guarner *et al.*, 2001). Metaplasia is defined as a potentially reversible change in which a fully differentiated cell type is replaced by another differentiated cell type (Dixon, 2001). When this other cell type resembles an intestinal phenotype, it is called intestinal metaplasia. Intestinal metaplasia as well as atrophy are regarded to predispose to malignancy (Sakaki *et al.*, 1995; Asaka *et al.*, 1997).

*H. pylori* infection is implicated in the development of two different gastric cancers: gastric adenocarcinoma (Peek and Crabtree, 2006) and gastric MALT lymphoma (Wotherspoon *et al.*, 1991; Parsonnet *et al.*, 1994, Stolte *et al.*, 2002; Farinha and Gascoyne,
The association with the gastric MALT lymphoma is strong and causal. Treatment of this cancer with *H. pylori* eradication leads to complete remission rates of between 35% and 100% (Wotherspoon *et al.*, 1993; Bayerdörffer *et al.*, 1995; Stolte *et al.*, 2002).

In 2002, gastric adenocarcinoma was the second leading cause of cancer-related death in the world (Peek and Blaser, 2002). Thankfully, stomach cancer incidence and mortality are declining throughout Europe and the rest of the world (Boyle and Ferlay, 2005). Two histologically distinct variants of gastric adenocarcinoma have been described. Diffuse-type gastric cancer consists of individual infiltrating neoplastic cells that do not form glandular structures, while intestinal-type adenocarcinoma consists of gland-like tubular structures with well-differentiated columnar epithelium. The latter type progresses through a series of histological steps initiated by the transition from normal mucosa to chronic superficial gastritis, which then leads to atrophic gastritis and intestinal metaplasia, and finally to dysplasia and adenocarcinoma (Fig. 5) (Correa, 1992; Sipponen and Marshall, 2000).

![Diagram](Image)

Fig. 5 General hypothesis of progression to intestinal-type adenocarcinoma (Correa, 1992).

*H. pylori* is responsible for the majority of duodenal and gastric ulcers. Gastric ulcers occur mostly at the transition zone between fundus and antrum (Stadelmann *et al.*, 1971), the area where the bacteria find their pH-optimum for growth, adhesion and subsequent inflammation. The presence of atrophy and intestinal metaplasia distal from this transition zone promotes ulcerogenesis. Individuals with enhanced acid secretion and *H. pylori* infection are predisposed for the development of duodenal ulcers (Harris *et al.*, 1996a). The excess of acid passing through the duodenum causes gastric metaplasia of duodenal epithelium (Wyatt *et al.*, 1987) and provides sites for colonization by *H. pylori* passing through the duodenum, as the organisms do not attach to native duodenal epithelial cells. Having colonized the
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metaplastic areas, *H. pylori* induces an active chronic inflammatory response. This leads to a weakening of mucosal defense against acid attack and erosion and ulceration may occur (Wyatt et al., 1990; Walker and Dixon, 1996). It has now been proven that by eradicating *H. pylori* it is possible to cure duodenal and gastric ulcer disease (Arkkila et al., 2005).

The prevalence of “*H. heilmannii*” among patients presenting dyspepsia is less than 5% (Hilzenrat et al., 1995; Mention et al., 1999; Ierardi et al., 2001; Boyanova et al., 2003). In contrast to *H. pylori* infection, the rare colonization of the gastric mucosa with “*H. heilmannii*”, mostly in the antrum, induces only a mild gastritis (Stolte et al., 1997). Nevertheless, gastritis is always present and characterized by the formation of lymphoid aggregates and chronic mucosal inflammation with patchy activity, whereby lymphocytes tend to diffuse into the foveolar lumen (Ierardi et al., 2001).

It has been described that “*H. heilmannii*”, like *H. pylori*, is associated with peptic ulcer disease (Yamamoto et al., 1999; Cales et al., 2000; Yoshimura et al., 2002; Sykora et al., 2003) and gastric cancer (Morgner et al., 1995). “*H. heilmannii*” has been implicated in low-grade MALT lymphoma in humans (Regimbeau et al., 1998). The incidence of MALT lymphoma associated with “*H. heilmannii*” infections is much higher than that with *H. pylori* (Morgner et al., 2000). This same group described the complete remission of the MALT lymphoma after eradication of “*H. heilmannii*” and these results were confirmed in a recently described case report by Thomas-Marques et al. (2005).

Van den Bulck et al. (2005) reported the prevalence of porcine, canine and feline helicobacters in human gastric “*H. heilmannii*” diagnosed biopsies. “*Candidatus H. suis*, *H. felis*, *H. bizzozeronii* and *H. salomonis* were present in 39.6%, 14.6 %, 4% and 21% of these samples, respectively. Not much is known regarding the pathology associated with these infections in humans, because only recently, tools have been developed to distinguish the different species involved (Baele et al., 2004; O’Rourke et al., 2004b). Only one report describes the presence of *H. felis* in a patient presented with dyspepsia (Germani et al., 1997). Another one reports the association of *H. felis*-like bacteria with human gastritis and ulceration (Lavelle et al., 1994). The only human “*H. heilmannii*” isolate cultured to date (Andersen et al., 1996) was later identified as *H. bizzozeronii* (Jalava et al., 2001). This bacterium was found in a patient with continuing dyspepsia.
2.3 *Helicobacter* related gastric disease in pet carnivores

*H. felis* has been found to colonize the stomach of cats and dogs. *H. salomonis* and *H. bizzozeronii* are found mainly in the dog and the latter is the predominant species in the canine stomach. These bacteria can be found throughout the entire stomach. In dogs, however, the antrum is the least colonized (Lecoindre *et al.*, 2000; Hwang *et al.*, 2002; Diker *et al.*, 2002). Mixed infections in dogs and cats are frequently found (Van den Bulck *et al.*, 2005). The clinical significance of these bacteria has been difficult to determine. Forty to 100% of the cats and dogs are infected with gastric spiral bacteria but this does not necessarily implicate disease. *Helicobacter* species have been associated with gastrointestinal disease in cats and dogs, although conclusive evidence is still lacking, as many animals carrying these bacteria do not present clinical symptoms (Happonen *et al.*, 1998).

In dogs, Peyrol *et al.* (1998) found no pathogenic significance for *H. bizzozeronii*. This was in contrast to *H. felis* that was seen adhering to chief and mucous cells and even occurred intracellularly, which could explain the observed cellular necrosis. Dogs naturally infected with *H. felis* had large numbers of lymphoid nodules throughout all regions of the gastric mucosa. In the subglandular region of all portions of the gastric mucosa, mild, diffuse lymphocytic infiltrates with small numbers of plasma cells and eosinophils were seen. Clinical signs such as loss of appetite and vomiting were noticed in these dogs (Haziroglu *et al.*, 1995). These results however contrast with an experiment conducted by Simpson *et al.* (1999a), who found no significant difference in clinical symptoms, inflammation and gastric function between control and experimentally *H. felis* infected dogs. The discrepancy between both studies could be due to host differences *i.e.* dog breed and age or bacterial strain differences. Occasionally, *H. felis* was observed within the canaliculi of gastric parietal cells (Lee *et al.*, 1992). The carnivorous pet helicobacters have a special affinity for parietal cells (Hermanns *et al.*, 1995; Happonen *et al.*, 1996). No such tropism is observed in human *H. pylori* infection, as the latter bacteria are rarely seen in association with parietal cells (Thomsen *et al.*, 1990; Taniguchi *et al.*, 1995).

In cats, *H. felis* infection is associated with predominantly antral lymphoid follicular hyperplasia and mild gastritis. Similar but less extensive lesions can be observed in uninfected cats (Simpson *et al.*, 2000; Scanziani *et al.*, 2001). Natural infection with *H. pylori* has been described in cats (Handt *et al.*, 1994). These *H. pylori*-infected cats showed lympho-plasmacytic infiltrates with small numbers of PMNs and formation of lymphoid follicles.
3 Pathogenesis of *Helicobacter* infections

The gastric colonization, inflammation and pathology observed in natural and experimental hosts after infection with *Helicobacter* species is the result of a complex interplay between the pathogen and the host immune response, with the gastric epithelial cell getting caught in the crossfire. Most of the research concerning *Helicobacter* virulence factors and the evoked host response is done with *H. pylori*, only little is known about the specific direct and indirect pathogenic effects of other gastric non-*H. pylori* helicobacters such as *H. felis* and *H. bizzozeronii*.

3.1 *Helicobacter* colonization

The successful life-lasting colonization of the stomach by *Helicobacter* species is achieved through a combination of bacterial factors namely urease to buffer the stomach acid, their helicoidal shape and highly motile flagella to cross the mucus layer and eventually, in *H. pylori* and *H. mustelae* infection, adhesion to the gastric epithelium. Moreover, the bacteria must overcome the host immune response.

3.1.1 Urease

Helicobacters can efficiently colonize the acidic environment of the stomach, which makes this bacterial genus unique. This colonization requires the presence of urease, an enzyme that hydrolyzes urea to ammonia and carbon dioxide. The produced ammonia neutralizes the hydrochloric acid of the stomach creating a neutral microenvironment surrounding the bacterium (Marshall *et al.*, 1990; Clyne *et al.*, 1995). Urease is mainly localised in the cytoplasm but becomes associated with the surface of the viable bacteria after autolysis of surrounding helicobacters (Phadnis *et al.*, 1996; Krishnamurthy *et al.*, 1998, Marcus and Scott, 2001). This external urease does not contribute to acid resistance, which is solely the function of the cytoplasmic urease (Scott *et al.*, 1998). *H. pylori* urease consists of two different subunits, UreA and UreB, which are encoded by two genes *ureA* and *ureB* (Hu and Mobley, 1990). These genes belong to a cluster of seven genes (*ureABIEFGH*) encoding for the whole active enzyme (Cussac *et al.*, 1992). Only recently it was shown that an *H. pylori* strain, unable to produce functional urease (the urease genes and the urease A and B subunits,
however, were present), was able to colonize and damage the gastric mucosa of Mongolian
gerbils (Mine et al., 2005). This seems to contrast with previous results from several authors
stating that urease is essential for colonization (Eaton et al., 1991a; Eaton and Krakowka,
1994; Karita et al., 1995; Iwao et al., 1999; Yoshiyama and Nakazawa, 2000). The
discrepancy between these results remains to be elucidated. Nevertheless, the role of urease is
not limited to colonization, the enzyme also plays a role in Helicobacter induced
inflammation (see below).

*H. mustelae* produces urease that is similar to the urease enzyme found in other
*Helicobacter* species (Dunn et al., 1991; Turbett et al., 1992; Bury-Moné et al., 2001). The
construction of a urease-negative mutant of this bacterium by Andrutis et al. (1995)
confirmed what had also been described in *H. pylori* (see above), namely that an urease-
negative mutant was unable to colonize the host’s stomach.

*H. felis* urease is composed of two subunits UreA and UreB, that are homologous but not
identical to those of *H. pylori* (Ferrero and Labigne, 1993; Gootz et al., 1994). It is unknown
if *H. felis* urease is required for colonization. Also *H. bizzozeroni* and *H. salomonis* are urease
positive. The complete urease gene cluster of *H. bizzozeronii* has been sequenced. This urease
is highly homologous with that of *H. felis* and *H. pylori* (Zhu et al., 2002).

3.1.2 Motility

Motility is essential for stomach colonization by helicobacters. *H. pylori* possesses a
unipolar bundle of two to six sheathed flagella (Geis et al., 1989). The flagella consist of a
body, hook and flagellar filament. The latter is covered by a sheath. This filament works as a
propeller, is about 3 μm long and has a club-like thickening at the distal end (Geis et al.,
1993). It is composed of two flagellin subunits namely the predominant FlaA and the minor
FlaB. Both subunits are necessary for full motility *in vitro* (Josenhans et al., 1995) and *in vivo*
(Eaton et al., 1996b). The sheath is suspected to play a role in acid protection, masking of
antigens and maybe adhesion (Jones et al., 1997a). The basal body of the flagella is embedded
in the bacterial cell wall and contains the proteins required for rotation and chemotaxis. The
hook links the body and the filament. Genes coding for the subunits FlaA and FlaB have been
sequenced and cloned by Leying et al. (1992) and Suerbaum et al. (1993), respectively. Other
genes involved in the biogenesis of the flagella, the assembly of the flagellar motor and the chemotaxis system have been characterized (Jenks et al., 1997; Kim et al., 1999; Foynes et al., 1999; O’Toole et al., 2000). The question whether flagella themselves or motility is needed by these pathogens was answered in a study by Ottemann and Lowenthal (2002). *H. pylori* mutants that had structurally wild-type but paralyzed flagella were constructed. It was shown that motility is important for the initial colonization of the stomach and also to attain full infection levels.

The degree of motility of different *H. pylori* strains correlates with the success of infection (Eaton et al., 1992; Eaton et al., 1996b). The use of mutants lacking specific flagellar proteins confirmed the importance of flagella and the resulting motility for *H. pylori* pathogenicity (Haas et al., 1993; Clyne et al., 2000). It has been postulated that *H. pylori* can actively swim towards the mucus layer. Various compounds are considered chemotactic for *H. pylori*, such as mucin (Turner et al., 1997) and urea (Nakamura et al., 1998). Intracellular urea hydrolyzed by cytoplasmic urease may supply the proton motive force required to drive the bacterial flagellar motor (Nakamura et al., 1998; Yoshiyama and Nakazawa, 2000).

*H. mustelae* flagella are also composed of the two flagellin subunits, FlaA and FlaB. Studies using mutants of the *flaA* and/or *flaB* gene showed that both subunits are necessary to obtain full motility and that they have a great influence on the density of colonization (Josenhans et al., 1995; Andrutis et al., 1997). In a study using subunit mutants, it was demonstrated that flagella do not play a direct role in promoting adherence of *H. mustelae* to gastric epithelial cells (Clyne et al., 2000).

In 1999, *H. felis* *flaA* and *flaB* genes were cloned and mutants were constructed (Josenhans et al., 1999). The authors described that the mutants were poorly motile *in vitro* and that the *flaA* mutant lost its ability to colonize the mouse stomach. Therefore, any impairment in motility would have consequences for gastric colonization by *H. felis*. To date, no information on *H. bizzozeronii* flagellin is available.
3.1.3 Adherence

The ability to attach to epithelial cells is an important feature for many bacterial pathogens colonizing mucosal surfaces. This is especially true for the gastric mucosa, which is subjected to a permanent stream of fluid by peristalsis, gastric emptying, continuous shedding and regeneration of the mucus layer. Adhesion is believed to be a critical initial step in the pathogenesis of many diseases.

Over 80% of the \textit{H. pylori} bacteria however are thought to remain in the mucus layer, with only a smaller number adhering to epithelial cells (Hessey \textit{et al.}, 1990). Also \textit{H. felis} is mainly found in the mucus layer and adherence to epithelial cells is only a rare phenomenon (Schreiber \textit{et al.}, 1999). \textit{H. mustelae} adheres firmly to the gastric epithelium, only few bacteria are seen lying in the mucus. \textit{H. pylori} and \textit{H. mustelae} adhesion to gastric epithelial cells results in pedestal formation (O’Rourke \textit{et al.}, 1992). This pedestal formation is due to rearrangement of the cell’s cytoskeleton (Segal \textit{et al.}, 1996).

\textit{H. pylori} colonizes the mucus layer by adherence to different mucins (Tzouvelekis \textit{et al.}, 1991; Hirmo \textit{et al.}, 1999). Lindén \textit{et al.} (2002) showed binding of \textit{H. pylori} to MUC5AC and that binding was affected by both host and microbe factors. MUC5AC glycoprotein has been designated as the primary receptor for \textit{H. pylori} in the human stomach (Van de Bovenkamp \textit{et al.}, 2003). \textit{H. pylori} also binds to the trefoil protein TFF1. This protein is present in mucus-secreting epithelial surface cells of the stomach and colocalizes with MUC5AC (Clyne \textit{et al.}, 2004).

There is little consensus on which \textit{H. pylori} adhesins are most important \textit{in vivo}. This controversy arises from the presence of \textit{H. pylori} strain differences and variable expression of \textit{H. pylori} adhesins and host cell receptors and of course the presence of numerous adhesins such as adherence-associated lipoprotein A and B (AlpA and AlpB; Odenbreit \textit{et al.}, 1999), blood group antigen-binding adhesin A (BabA; see below), sialic acid binding adhesin (SabA; Mahdavi \textit{et al.}, 2002), HopZ (Peck \textit{et al.}, 1999), heat shock proteins (Hsp; Huesca \textit{et al.}, 1998), lipopolysaccharide (LPS, see below) and many more. The best-characterized \textit{H. pylori} adhesin to date is BabA.

Blood group antigen-binding adhesin A (BabA) is an adhesin of \textit{H. pylori}, interacting with the blood-group antigen Le\textsuperscript{b} on gastric epithelial cells (Borén \textit{et al.}, 1993; Ilver \textit{et al.}, 1998). BabA has been shown to play an important role in facilitating bacterial colonization (Rad \textit{et al.}, 2002). Two corresponding genes encoding BabA have been cloned: \textit{babA1} and \textit{babA2}. Only the \textit{babA2} gene is functionally active. The expression of BabA correlates with the
presence of CagA (see below) (Ilver et al., 1998; Hennig et al., 2004). Moreover, babA2 is shown to be significantly associated with other H. pylori virulence genes such as oipA and certain alleles of vacA (see below). When coexpressed in the same H. pylori strain, these genes work synergistically in worsening inflammation (Zambon et al., 2003). A strong association between the babA2 gene and the presence of duodenal ulcers, gastric cancer and severity of gastric inflammation has been found (Gerhard et al., 1999; Prinz et al., 2001). Heterogeneity among H. pylori strains in expression of the BabA protein may be a factor that contributes to different clinical outcomes among H. pylori-infected humans (Hennig et al., 2004).

The question of whether H. pylori flagella per se are involved solely in motility or also in adherence of the organisms to the gastric mucosa remains to be further investigated. No difference was found in adherence to primary gastric epithelial cells when comparing H. pylori flaA and flaB isogenic mutants with the wild type strain (Clyne et al., 2000). Nevertheless, deficiency of certain H. pylori genes that play a role in the regulation of flagellar biosynthesis, such as fltQ and flbA, can affect the adhesion abilities of the bacterium. Foynes et al. (1999) found that isogenic fltQ mutants of H. pylori showed a reduced level of adherence to the human gastric adenocarcinoma cell line AGS. These studies showed that flagella do not play a direct role in promoting adherence of H. pylori to gastric epithelial cells, but that genes involved in the regulation of H. pylori flagellar biosynthesis may also regulate the production of an adhesin.

Laminin and collagen type IV have been postulated to be targets for H. pylori adherence (Trust et al., 1991). The initial recognition and binding of laminin by H. pylori may occur through LPS (Valkonen et al., 1994). Several studies support the role of LPS as an adherence factor for H. pylori (Edwards et al., 2000). However, a recent study revealed that LPS mediated binding only plays a minor role in adherence (Mahdavi et al., 2003).

Akanuma et al. (2002) reported that an H. pylori mutant strain lacking oipA (see below) could not colonize Mongolian gerbils and suggested that OipA played an important role in colonization of the gastric mucosa of gerbils. This is in contrast to humans where OipA negative H. pylori strains could be demonstrated in gastric biopsies (Yamaoka et al., 2000).

The alpA and alpB genes are not present in H. felis and H. mustelae (Odenbreit et al., 1999) and other putative adhesins of H. felis have not (yet) been described.
3.1.4 Evasion of the immune response

*Helicobacter* species are known to persist throughout the life of the host in the face of a significant inflammatory response. Hence, the bacteria have found a niche in which they can thrive despite obvious host responses.

The immune system is divided in two types of response. Innate immunity is comprised of hereditary components, which provide an immediate "first-line" of defense to pathogens. In contrast, adaptive (acquired) immunity is mediated by B and T lymphocytes, designed to develop a specific immunity to particular pathogens. This response takes days to develop, and so is not effective at preventing an initial invasion, but it will normally prevent any subsequent infection, and also aids in clearing up of longer-lasting infections.

*Evasion of the innate immune response*

After evasion of the defense mechanisms of the gastrointestinal tract (saliva, gastric mucus and gastric acid), the bacteria must escape phagocytosis by phagocytic cells such as macrophages and neutrophils. A strong infiltration of PMNs is associated with human *H. pylori* infection, nevertheless bacteria can resist PMNs phagocytosis in a manner that for some authors is *cag* pathogenicity island (*cag* PAI) dependent (see below) (Ramarao *et al.*, 2000a; Ramarao and Meyer, 2001) but for others, is considered to be *cag* PAI independent (Odenbreit *et al.*, 2001; Zheng and Jones, 2003). Moreover, *H. pylori* bacteria have been shown to survive in macrophage phagosomes (Odenbreit *et al.*, 2001), and this is dependent on their catalase activity (Basu *et al.*, 2004). Catalase is expressed at high levels (Hazell *et al.*, 1991) and is involved in the bacterial resistance against reactive oxygen species (ROS) released by PMNs (Ramarao *et al.*, 2000b). The latter enzyme is also present in *H. felis* (Lee *et al.*, 1988), *H. bizzozeronii* (Hänninen *et al.*, 1996) and *H. salomonis* (Jalava *et al.*, 1997). In addition there are at least two other less known enzymes involved in resistance against oxidative stress *i.e.* alkylhydroperoxide reductase (Lundström and Bölin, 2000) and thiol peroxidase (scavengase) (Wan *et al.*, 1997). The presence of alkylhydroperoxide reductase was confirmed in *H. felis*, *H. salomonis* and *H. acinonychis*. In *H. mustelae* it seems absent, although the corresponding gene (*AhpC*) is found (Lundström *et al.*, 2001).

Discrimination of potential pathogens from self-antigens is done with the use of a restricted number of receptors that recognize conserved motifs (termed pathogen-associated
molecular patterns or PAMPs) on pathogens that are not found in higher eukaryotes. These receptors are called pattern-recognition receptors (PRRs) and are present on antigen-presenting cells and a variety of other cells including gastric epithelial cells. Toll-like receptors (TLRs), which lead to activation of pro-inflammatory pathways, oxidative burst of neutrophils/macrophages and the release of reactive nitrogen intermediates, are an important family of these PRRs. Thirteen TLRs have been discovered from mammalian genomes (ten of which persist in humans) (Roach et al., 2005). The ligands for most of the TLRs have been identified. TLR2 (in association with TLR1 and TLR6) recognizes bacterial lipoprotein and peptidoglycans from Gram-positive bacteria (Takeuchi et al., 1999). TLR3 is considered to recognize double-stranded RNA (Alexopoulou et al., 2001). LPS from Gram-negative bacteria signals through TLR4 (Qureshi et al., 1999). TLR5 is known to recognize bacterial flagellin (Hayashi et al., 2001) and TLR9 plays an essential role in the recognition of bacterial and viral DNA (Hemmi et al., 2000; Krug et al., 2004). Studies on innate immune responses to H. pylori in epithelial cells have mainly focused on TLR4, TLR2 and TLR5 while TLR3, TLR7, TLR8 and TLR9 are principally involved in defense against viruses (Schnare et al., 2006).

H. pylori and H. felis LPS induce only a very weak cytokine response mediated by TLR4. TLR2 was proven to be the dominant innate immune receptor for recognition of Helicobacter species. As TLR2 is not expressed on the gastric epithelium, the bacteria can escape detection and elimination by the immune system as long as no other TLR2-expressing cells (such as PMNs) have infiltrated the stomach mucosa (Mandell et al., 2004). Recent reports conflict regarding the ability of TLR5 to recognize H. pylori flagellin. Some authors describe that TLR5 expressing epithelial cell lines do detect H. pylori flagellin (Smith et al., 2003; Torok et al., 2005). Conversely, evasion of TLR5 by H. pylori and H. felis flagellin was shown by Andersen-Nissen et al. (2005) and other authors (Lee et al., 2003; Gewirtz et al., 2004). The latter results suggest that TLR5 evasion may contribute to long-term persistence in individual hosts.

Evasion of the adaptive immune response

Humoral immunity is the aspect of immunity that is mediated by secreted antibodies, produced in the cells of the B lymphocyte lineage. Secreted antibodies bind to antigens on the surfaces of invading microbes, which flags them for destruction. Infection with H. pylori leads to robust production of IgG and IgA antibodies, both in the serum and within the gastric
mucosa (Wyatt and Rathbone, 1988; Blanchard et al., 1999b; Akhiani, 2005). However, it is still controversial whether local specific antibodies play a role in *H. pylori* resistance. Using a B-cell-deficient mouse model, Akhiani et al. (2004) demonstrated that *H. pylori* infection was completely cleared from mutant mice within the context of severe gastric inflammation. Conversely, wild-type mice remained colonized, but developed only mild gastritis. Thus, this study indicated that antibodies are not only dispensable for protection, but they promote bacterial colonization and impair gastric inflammation. Other previously reported studies, which used mice lacking factors of the T-helper (Th) 2 response (see below), came to the same conclusions (Blanchard et al., 1999a; Garhart et al., 2003).

Most data indicate a primary role for T cells and more specific Th cells in *Helicobacter* infection (Blanchard et al., 1999a; Sutton et al., 2000; Gottwein et al., 2001). Th lymphocytes can be divided into two functional subsets, type 1 (Th1) and type 2 (Th2) T-helper cells, which are defined by distinct patterns of cytokine secretion. Th1 cells secrete cytokines including interleukin (IL)-2, tumor necrosis factor α (TNF-α) and gamma interferon (IFN-γ), and promote cell-mediated immune responses, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10 and induce B-cell activation and differentiation. Though the acquired immune response to *Helicobacter* infections includes both Th1 and Th2 cells, the local Th cell response in *H. pylori* and *H. felis* infection is generally of the Th1 type since the levels of IFN-γ, but not IL-4 and IL-5, have been shown to be increased in *Helicobacter*-induced gastritis (Mohammadi et al., 1996a; Mohammadi et al., 1997; Bamford et al., 1998; Sommer et al., 1998; Smythies et al., 2000; Eaton et al., 2001). The regulatory roles of Th1 and Th2 cells in immune protection against *Helicobacter* infections are incompletely understood. Both the Th1 response (Akhiani et al., 2002; Sawai et al., 1999) and the Th2 response (Mohammadi et al., 1997) have been linked to protection against these infections. The presence of Th1 cells in the gastric mucosa implies that cell-mediated immune responses are induced during infection with helicobacters. Given that the *Helicobacter* infection is an extracellular infection and that the biological niche for the organism is restricted to the lumen, a cell-mediated immune response is unlikely to be protective, a notion that is supported by the fact that infection with helicobacters persists for a lifetime.
**Molecular mimicry**

Molecular mimicry is another possible mechanism by which *H. pylori* could evade the immune response. Microbes can contain chemical structures that mimic normal host “self” proteins. This event is termed molecular mimicry. *H. pylori* can mimic Lewis (Le) antigens in its LPS O-polysaccharide chain (Monteiro *et al.*, 1998). LPS is a molecule composed of three moieties namely lipid A, the core oligosaccharide and the O-polysaccharide chain. LPS is present in the cell wall of Gram-negative bacteria. In most of these bacteria, LPS is a strong stimulator of the host immune response. In *H. pylori*, however, this is not the case and only low immunogenic activity can be assigned to *H. pylori* LPS (Muotiala *et al.*, 1992; Moran, 1995). The Lewis blood group antigen system is expressed on red blood cells but also on the gastric epithelial cells and is composed of type 1 antigens, Lewis a (Le\(^a\)) and Lewis b (Le\(^b\)), and type 2 antigens, Lewis x (Le\(^x\)) and Lewis y (Le\(^y\)). In Western populations most *H. pylori* strains express the type 2 antigens, whereas a small proportion express the type 1 antigens and simultaneous expression of both type 1 and type 2 Lewis blood groups is possible (Monteiro *et al.*, 1998). Nevertheless, not all *H. pylori* strains express Lewis antigen mimicry (Kocharova *et al.*, 2000). The exact role of Le antigens during *H. pylori* infections is unclear and controversial. One of the possible roles for LPS is that *H. pylori* evades the host immune response by molecular mimicry of its Le antigens with similar blood group antigens in the gastric mucosa. In subsequent studies, however, this hypothesis has been confirmed (Wirth *et al.*, 1997) as well as rejected (Taylor *et al.*, 1998; Heneghan *et al.*, 2000).

### 3.2 Helicobacter induced gastric pathology

Since the primary consequence of *H. pylori* infection is gastritis, many investigators have focused on the mechanisms involved in the gastric inflammatory response. Both bacterial factors and the host response are responsible for the induced inflammation seen in *Helicobacter* infection (Fig. 6).

#### 3.2.1 Influx of inflammatory cells

*Helicobacter* gastritis is characterised by the infiltration of neutrophils and mononuclear inflammatory cells (Warren and Marshall, 1983; Bayerdörffer *et al.*, 1992). The degree of
mucosal damage is correlated with neutrophil infiltration (Fiocca et al., 1994; Davies et al., 1994). These cells produce ROS and these are toxic to host tissues. Some *H. pylori* proteins affect the physiological state of the stomach e.g. urease, an enzyme that has not only a role in establishing colonization but may also help to recruit neutrophils and monocytes in the inflamed mucosa and may activate the production of proinflammatory cytokines (Harris et al., 1996b). The two best-described pro-inflammatory proteins in *H. pylori* pathology are *H. pylori* neutrophil-activating protein (HP-NAP) and the outer inflammatory protein (OipA).

![Molecular pathways linking *H. pylori* and inflammation/carcinogenesis](Asaka et al., 2001)

In 1993, an *H. pylori* factor was identified that was capable of promoting neutrophil adhesion to endothelial cells, and of inducing neutrophils to produce ROS *in vivo* and *in vitro* (Yoshida et al., 1993). This factor was termed *H. pylori* neutrophil-activating protein (HP-NAP) (Evans et al., 1995). The napA gene encoding for HP-NAP, has been found in all *H. pylori* isolates examined so far (Evans et al., 1995; Allen, 2001). Protein expression however is variable. This might be the reason for differences in the ability of individual strains of *H. pylori* to induce inflammation. A recent publication by Brisslert et al. (2005) suggested that HP-NAP is transported across the endothelial monolayer and has direct effects on the circulating neutrophils, inducing transendothelial migration of these cells. The ability of HP-NAP to rapidly induce ROS is an important factor in damage to host tissue.
However, this does not exclude that additional chemotactic factors could act in synergy with HP-NAP in mediating neutrophil chemotaxis \textit{in vivo}. The neutrophil chemoattractant interleukin-8 (IL-8) for example, that can be produced from gastric epithelial cells in response to \textit{H. pylori} infection, is also likely to contribute to neutrophil recruitment (Crabtree \textit{et al.}, 1994). \textit{H. pylori} IL-8 induction is related to the presence of certain genes of the \textit{cag} PAI both \textit{in vivo} and \textit{in vitro} (Orsini \textit{et al.}, 2000). \textit{CagA} (see below) is not one of these genes and deletion of \textit{cagA} does not affect the ability of \textit{H. pylori} to induce IL-8 secretion (Crabtree \textit{et al.}, 1995; Sharma \textit{et al.}, 1995). Nevertheless, some \textit{cag} PAI-negative strains have been shown to be able to induce IL-8 secretion suggesting the presence of factors other than \textit{cag} PAI involved in IL-8 induction (Nilsson \textit{et al.}, 2003). Yamaoka \textit{et al.} (2000) described the presence of an outer membrane protein that had the ability to induce IL-8 from gastric epithelial cells. This protein was designated outer inflammatory protein (OipA). \textit{H. pylori} contains either a non-functional or functional \textit{oipA} gene. In a following publication by Yamaoka \textit{et al.} (2002), the authors related \textit{H. pylori} density, gastric inflammation, and \textit{in vivo} gastric mucosal IL-8 production to the presence of the \textit{oipA} gene. Moreover, Zambon \textit{et al.} (2002) associated the \textit{oipA} gene with the development of peptic ulcer, gastric adenocarcinoma and intestinal metaplasia. Other studies, however, have challenged the role of OipA in IL-8 induction (Akanuma \textit{et al.}, 2002; Odenbreit \textit{et al.}, 2002). The adhesion factor BabA has also been shown to play a role in inducing IL-8 secretion and subsequently enhanced mucosal inflammation in humans (Rad \textit{et al.}, 2002).

Next to the specific bacterial factors, activation of epithelial TLR (see above) induces the expression of selectins, chemokines and chemokine receptors that regulate cell migration to the sites of inflammation (Iwasaki and Medzhitov, 2004). In general, the cascade of events occurring following ligation of the different TLRs involves the activation of a common set of adapter proteins and protein kinases, the best characterized of which lead to the activation of nuclear factor-κB (NF-κB) (Martin and Wesche, 2002). The transcription factor NF-κB participates in the expression of a wide variety of genes that are involved in the regulation of immune and inflammatory responses, apoptosis, proliferation and tumorigenesis. The induction of NF-κB, however, and subsequent increase in IL-8 production can be caused by numerous pathways other than TLR stimulation such as inflammatory cytokines, antigen specific T-and B-cell receptor complexes and cell stress (Hayden and Ghosh, 2004).
The type of Th response plays an important role in Helicobacter-induced inflammation (Akhiani et al., 2002; McCracken et al., 2005). The local Th cell response in H. pylori and H. felis infection is generally held to be of the Th1 type (see above). Naive T-cells are driven to differentiate into Th1 cells by several factors such as TNF-α and IL-12. The latter cytokine is produced by mononuclear cells in response to H. pylori (Guiney et al., 2003). Th1 cells are generally considered to be “proinflammatory” in the context of the digestive tract as the production of IFN-γ contributes to chronic inflammation (Sawai et al., 1999; Eaton et al., 2001). Moreover, gastric H. pylori-specific T-cell clones isolated from peptic ulcer patients are more often of the Th1 type than clones isolated from subjects with chronic gastritis only (D’Elios et al., 1997). Using the mouse model, it has been shown that the magnitude of the Th1 response correlates with the severity of gastric inflammation in mice (Mohammadi et al., 1996a; Sawai et al., 1999; Eaton et al., 2001). Conversely, IL-10 produced by Th2 cells is known to inhibit synthesis of Th1 cytokines (such as IFN-γ) (Fiorentino et al., 1989). Therefore, mice deficient in IL-10 production exhibited more severe gastritis than wild-type mice (Berg et al., 1998; Eaton et al., 2001). Thus in Helicobacter infections, a Th1 response correlates with severe gastric pathology, whereas a Th2 response seems crucial for the control of these harmful Th1 responses (Mohammadi et al. 1997).

3.2.2 Cytotoxic effects of Helicobacter infections

In the process of phagocytosis and activation of oxidative burst responses, ROS are released into the tissue by neutrophils. ROS can induce DNA damage and can lead to accumulation of genetic mutations, contributing to the pathogenesis of gastric cancer (Grisham et al., 2000). Previous studies have shown that H. pylori stimulates neutrophils to oxidative burst response (Mooney et al., 1991; Nielsen and Andersen et al., 1992; Unemo et al., 2005). This mechanism is a crucial factor in host defense (see above), but is also an important factor contributing to tissue damage in Helicobacter infections. H. felis is shown to be a very strong stimulator of human neutrophil chemotaxis but, in contrast to H. pylori, H. felis does not stimulate these cells to oxidative burst response (Hansen et al., 2001).

H. pylori is known to have a direct cytopathic effect on gastric epithelial cells. The most important and most widely investigated cytotoxic virulence factor of H. pylori is the cag pathogenicity island (cag PAI). Its presence is associated with an increased risk of peptic
ulcer and gastric cancer. This 40-kb gene cluster contains approximately 30 genes including the genes that encode a type IV secretion system (Censini et al., 1996). It has been described that *H. acinonychis* and *H. felis* lack the cag PAI gene (Xiang et al., 1995; Mohammadi et al., 1996b; Dailidiene et al., 2004). The cag PAI is found in most *H. pylori* strains, and although the risk of disease is increased with its presence, it is not essential for either ulcer formation or gastric cancer. Both pathologies have also been described in patients infected with cag PAI-negative *H. pylori* strains (Nilsson et al., 2003). Cytotoxin-associated protein A (CagA) was the first protein of the cag PAI that was identified and this protein is a major virulence factor (Blaser et al., 1995; Parsonnet et al. 1997). CagA is translocated into host cells and this process depends on a functional type IV secretion system (Backert et al., 2000; Odenbreit et al., 2000). This “molecular syringe” injects bacterial products into host cells. Inside the host cell, CagA becomes phosphorylated (Stein et al., 2000). After phosphorylation, the protein is believed to become part of a membrane-associated complex inside the host cell (Higashi et al., 2002). It is suggested that CagA induces rearrangements of the host cells cytoskeleton and may be involved in dysregulation of host cell functions, thereby contributing to pathogenesis (Selbach et al., 2003; Rieder et al., 2005).

Two cag-pathways that lead to IL-8 secretion are described, namely one that depends on nuclear NF-κB activation (Glocker et al., 1998) and another one depending on activator protein 1 (AP-1) (Naumann et al., 1999). To date, activation of NF-κB by *H. felis* has not been described. The presence of an intact cag PAI is not an absolute requirement for NF-κB activation and IL-8 secretion; other factors are also involved (see above).

*H. pylori* urease might cause damage to the host cells through the production of ammonia, an agent known to be toxic by itself on cultured cells and stomach tissue (Smoot et al., 1990; Mégraud et al., 1992; Tsujii et al., 1992; Sommi et al., 1996; Figura, 1997; Igarashi et al., 2001). Other known *H. pylori* enzymes involved in damaging host epithelium are phospholipase A2 (Langton and Cesareo, 1992) and alcohol dehydrogenase (Salmela et al., 1993).

**Apoptosis and proliferation**

The *H. pylori* colonized stomach contains more apoptotic cells than normal (Moss et al., 1996; Jones et al., 1997b). Apoptosis is a physiological suicide mechanism that preserves gastric mucosal homeostasis. A long-term increase in apoptotic rate could be the stimulus for lasting increase in cell proliferation; this hyperproliferation could promote the development of
neoplasia. Apoptosis is triggered by *H. pylori* as well as by various inflammatory mediators, such as TNF and IFN-γ. Also autoimmunity has been suggested as the cause of increased apoptosis (see below). Several studies have described the *in vitro* apoptosis-inducing capacity of *H. pylori* in which adhesion and *cag* PAI genes seem to play a crucial role (Wagner et al., 1997; Chen et al., 1997, Neu et al., 2002).

Several bacterial factors have been associated with the induction of apoptosis in host cells. These include *Helicobacter* urease, as well as the ammonia produced by this enzyme (Figura, 1997; Fan et al., 2000; Igarashi et al., 2001), LPS (Piotrowski et al., 1996; Durkin et al., 2006), γ-glutamyl transpeptidase (Shibayama et al., 2003) and vacuolating cytotoxin (VacA).

In epithelial cell lines, VacA has been shown to be associated with vacuolar degeneration and has also been shown to induce epithelial cell apoptosis *in vitro* (Leunk et al., 1988; Cover and Blaser, 1992; Kuck et al., 2001; Cover et al., 2003; Boquet et al., 2003) but its effect on host cells *in vivo* remains unclear. The *vacA* gene is present in most *H. pylori* strains, but the corresponding protein VacA may not be expressed in all cases. This may be partly due to sequence variation among *vacA* alleles (Forsyth et al., 1998). Xiang et al. (1995) did not detect the *vacA* gene and protein in *H. felis*. Although located on different loci of the chromosome, *cagA* is strongly linked with VacA cytotoxic activity. *H. pylori* strains from patients with overt disease have been shown to produce both CagA and VacA, whereas strains from asymptomatic carriers do not produce either VacA or CagA (Covacci et al., 1997). Nevertheless, this is not an absolute association as *cagA* deletion does not alter VacA expression or activity (Tummuru et al., 1994; Akopyants et al., 1998). Different studies have pointed out an association between peptic ulcer disease and vacuolating activity of *H. pylori* (Figura et al., 1989; Goossens et al., 1992; Weel et al., 1996) but also this association is not absolute (Graham and Yamaoka, 2000). De Bernard et al. (2004) described effects of VacA on host cells, such as a pro-inflammatory action by inducing degranulation of mast cells. This supports earlier findings by Telford et al. (1994), who inoculated mice with purified VacA and noted ulceration and gastritis.

Fas is a cell surface receptor and Fas-Ligand (Fas-L) is a cell surface protein. Target-cell killing by cytotoxic T-lymphocytes can be mediated through the interaction of Fas-L on the activated T-cell with Fas on the target cell, in this case the gastric epithelial cell (Takayama et al., 1995). *H. pylori* has been shown to stimulate the expression of both the receptor and the ligand and therefore contributes to the Fas/Fas-L system induced epithelial cell apoptosis.
(Jones et al., 1999; Wang et al., 2000a; Ishihara et al., 2001). However, *H. pylori* can augment apoptosis through IFN-γ production (Fan et al., 1998) and TNF-related apoptosis inducing ligand (TRAIL) (Wu et al., 2004) without the intervention of the Fas/Fas-L system.

*H. pylori* has been shown to have a direct inhibitory effect on the proliferation rate of different gastric epithelial cell lines (Knipp et al., 1996; Smoot et al., 1999). Ammonia produced by the bacteria was demonstrated to inhibit the growth of gastric epithelial cells (Matsui et al., 1995). Increased cell proliferation could be regarded as a protective reaction of the epithelium toward the cytopathic effects induced by *H. pylori* infection. This restoration process may be retarded in the presence of *H. pylori*. Conversely, Fan et al. (1996) reported enhanced proliferation of AGS cells after *H. pylori* infection.

**Autoimmune gastritis**

Loss of mucous- and acid-secreting cells (referred to as atrophy) in the stomach is a common phenomenon in *Helicobacter* infections. This cell loss can be due to the direct effect of the bacteria and/or the effects of the immune system (see above). Serum autoantibodies reacting with gastric mucosal antigens are frequently (60%) found in *Helicobacter*-infected patients (Faller and Kirchner, 2000; Negrini et al., 1991), this suggests that gastric fundus atrophy can be related to an autoimmune process driven by *Helicobacter* bacteria. Destruction of the glandular epithelium can be mediated through autoantibodies or by autoimmune T-cell attack (Bergman et al., 2001). Most of these autoantibodies are directed against the parietal cell’s gastric proton pump (D’Elios et al., 2004) and have been thought to be due to molecular mimicry (Negrini et al., 1996). It has been suggested that *H. pylori* induces a gastric autoimmune response by inducing anti-Le antibodies that bind to its LPS and also to gastric epithelium. These autoimmune reactions to antigens in the gastric mucosa may play a role in the pathogenesis of gastritis and gastric atrophy (Negrini et al., 1991; Negrini et al., 1996; Faller et al., 1996). A study in mice showed that immunisation of the animals with *H. pylori* leads to formation of anti-Le\textsubscript{xy} antibodies, which react with the gastric H\textsuperscript{+},K\textsuperscript{+}-ATPase present in the parietal cells’ canaliculi (Appelmelk et al., 1996). In humans however, autoantibodies against H\textsuperscript{+},K\textsuperscript{+}-ATPase are formed but the reactivity is not directed against Lewis epitopes (Faller et al., 1996; Amano et al., 1997; Claeys et al., 1998). Therefore human anti-H\textsuperscript{+},K\textsuperscript{+}-ATPase autoantibodies associated with *H. pylori* infection do not involve Lewis mimicry. In fact, they do not involve molecular mimicry at all (Claeys et al., 1998; Faller et al., 1998).
A similar mechanism has been described in *H. mustelae*. The latter bacterium expresses another blood group antigen A, which can be found both on the bacterium and on the ferret’s gastric epithelium (Monteiro et al., 1997; Croinin et al., 1998; Hynes et al., 2004). Croinin et al. (2001) reported that ferrets naturally infected with *H. mustelae* generate antibodies that react with parietal cells, but these autoantibodies are due to another process, such as gastric inflammation, rather than molecular mimicry. These findings correspond to what is found in humans infected with *H. pylori* (see above).

Sakagami et al. (1996) did not find increased levels of anti-H⁺,K⁺-ATPase autoantibodies in *H. felis* infected mice. Research on *H. felis* and *H. bizzozeronii* LPS revealed the absence of Lewis antigen molecular mimicry. The chemical composition, and hence structure, of *H. felis* LPS differs significantly from that of *H. pylori*, despite some structural similarities in the core region (Hynes et al., 2004). This interesting feature warrants further research. To date no LPS analysis has been performed on *H. salomonis*.

*Alterations in acid and gastric hormone levels*

The acute phase of *Helicobacter* infection leads to transient hypochlorhydria secondary to parietal cell dysfunction that could last for a few days to a few months (Sobala et al., 1991). *H. pylori* LPS *in vivo* and another unknown protein *in vitro* were shown to have an inhibitory effect on the parietal cell function (Cave and Vargas, 1989; Ootsubo et al., 1997). Also *H. felis* has been shown to inhibit acid secretion from rabbit parietal cells *in vitro* (Vargas et al., 1991) but this was, similar as for *H. pylori*, independent of its LPS (Padol et al., 2001).

Chronic *H. pylori* infection, however, can lead to enhanced or decreased acid production. The mechanism involved is unknown. Increased gastrin production is one of the possible explanations. Gastrin secretion is stimulated by *H. pylori* induced proinflammatory cytokines on G-cells (Lehmann et al., 1996). Also by inhibiting somatostatin-producing D-cells, hypergastrinemia is established (Moss et al., 1992). The effect of gastrin on acid homeostasis depends on the location of the *H. pylori*-induced inflammation. In antral-predominant gastritis (most frequently seen), high gastrin levels lead to increased acid output (El-Omar et al., 1995). This hyperchlorhydria is the main cause of duodenal ulcer formation. In fundus-predominant gastritis, *H. pylori* and the present inflammation (Th1) inhibit parietal cells (Beales and Calam, 1998), consequently followed by increased gastrin levels. This continuous gastrin stimulus is unable to raise the acid production and leads to an ungoing proliferative
stimulus of the parietal cells (El-Omar et al., 1997). This eventually results in atrophy of the glands and increases the risk of ulceration and adenocarcinoma (Peek and Blaser, 2002).

Dogs naturally infected with *H. felis* and *H. bizzozeronii*-like organisms did not show marked perturbations in the gastric-secretory axis. These animals had normal gastrin-stimulated acid output without the hypergastrinemia encountered in humans. Notwithstanding the presence of multiple bacteria, no significant inflammation was seen in these dogs and the presence of a direct effect of the bacteria on acid secretion was questioned (Simpson et al., 1999b).

4 **In vivo models of Helicobacter gastritis**

Studies with bacteria, tissue cultures, and computer simulation can provide us with useful information on certain human and animal diseases, but the complexity of living organisms requires research and testing on animals that are analogous to humans to attain reliable and effective results. An animal model may be considered homologous if the symptoms and the course of the condition shown by the animal are identical to those found in humans. Few experimental disease models completely mimic the etiology, course, and pathology of the human target disease but may be useful to study certain aspects or treatments of it. Models never provide final answers, but only offer approximation (Wright, 1997).

Numerous articles and reviews concerning *Helicobacter* animal experimentation have been published (Lee, 1999; O’Rourke and Lee, 2003; Pritchard and Przemeck, 2004; Whary and Fox, 2004). The use of animal models in *Helicobacter* research allows us to investigate more in detail the mechanisms by which these bacteria cause gastric disease and provides us with an *in vivo* equivalent of *in vitro* obtained results. Natural as well as experimental animal models of *Helicobacter* infection are available depending on the bacterial host specificity. This overview focuses mainly on *in vivo* models of non-*H. pylori* *Helicobacter* associated inflammation but some findings on *in vivo* modelling of *H. pylori* infection are discussed as well.
4.1 Mouse models

Mice are by far the most commonly used laboratory animals in research in general and in *Helicobacter* research in particular. Thanks to genetic engineering many different mouse strains and mutants are available, each with the purpose to provide better insight in a particular characteristic of *Helicobacter* pathogenesis.

4.1.1 Modelling the infection

Cantorna and Balish (1990) were the first to investigate colonization of germ-free mice with *H. pylori* but did not succeed in recovering any bacteria. By using immunocompromised mice colonization was obtained (Karita et al., 1991). Few years later, Marchetti et al. (1995) succeeded in establishing an *H. pylori* infection in specific-pathogen-free (SPF) CD1 mice and wild type CD1 and BALB/c mice. In the mean time, another bacterium closely related to *H. pylori*, namely *H. felis*, was found to easily colonise the mouse’s stomach (Dick et al., 1989; Lee et al., 1990), thus providing a suitable animal model for further *Helicobacter* research. In 1997, an *H. pylori* strain was described with high colonizing ability in C57BL/6 mice (Lee et al., 1997). This *H. pylori* strain (designated the Sydney strain, SS1) became a standard strain for use in animal experimentation. Nevertheless, *H. felis* rodent models are still widely used to study *H. pylori* infection (Fox et al., 1996; Mohammadi et al., 1996b; Sakagami et al., 1996; Berg et al., 1998; Ferrero et al., 2000) in spite of its major limitation namely that it most likely does not express the virulence determinants that are important in *H. pylori* infection (Xiang et al., 1995).

4.1.2 Modelling infection-associated inflammation

*H. felis* was found to colonize the stomach of Swiss Webster outbred mice in large numbers (Lee et al., 1990). The bacteria were mainly located in the mucus layer and deep in the gastric pits. At 2 weeks post-infection (p.i.), an acute inflammatory response was found with primarily eosinophils and neutrophils. Three weeks p.i., the PMNs response was more pronounced with formation of microabscesses and increased number of lymphocytes. Eight weeks p.i., large lymphoid nodules were present in the submucosa and multiple small microabscesses were still present in the pyloric mucosa (Lee et al., 1990). In contrast to the
previous results, Enno and co-workers (1995) did not find much inflammation in *H. felis* infected BALB/c mice sacrificed up to 19 months p.i., but found large lymphoid aggregates in the mucosa and submucosa after 22 months of infection. These aggregates were reminiscent of MALT lymphoma found in human *H. pylori* infection. Sakagami *et al.* (1996) compared different mouse strains in their response to *H. felis* infection and found BALB/c mice to present only very mild antral gastritis with heavy bacterial colonization (these mice are called nonresponders). SJL and C57BL/6 mice, however, had the most severe gastritis, mainly in the fundus, and this was accompanied with atrophic changes at 6 months p.i. (these mice are called responders). The authors also described an inverse relationship between the severity of gastritis and the degree of *H. felis* colonization. In the same year, another study on the short-term infection (11 weeks) of different mouse strains inoculated with *H. felis* was reported by Mohammadi *et al.* (1996b). The differences in pathology between mouse strains were comparable with the results of Sakagami *et al.* (1996) although no atrophy had yet developed. In 2000, a report was published concerning long-term *H. felis* infection in Swiss outbred mice. The animals developed both glandular and lymphoid tissue lesions due to chronic *H. felis* infection at 13 months p.i.. It was suggested that the T-helper phenotype of the host influences glandular lesion formation or lymphoma formation in this model of infection (Roth *et al.*, 1999; Ferrero *et al.*, 2000). All these studies emphasize the importance of the host in disease outcome following gastric *Helicobacter* infection. The *Helicobacter* species and *H. pylori* strain that is used was also shown to influence the colonization and inflammation level in a particular mouse strain (Van Doorn *et al.*, 1999; Mähler *et al.*, 2002). To date, no comparative pathology study between different *H. felis* strains has been described.

Glandular lesions seen in *Helicobacter* infected C57BL/6 mice comprise apoptosis and random loss of parietal and chief cells. *H. felis* bacteria were found inside parietal cell canaliculi although there was no evidence of cellular damage (Wang *et al.*, 1998).

Dial *et al.* (2000) described the gastrin and somatostatin homeostasis in *H. felis* infected mice. In contrast to human *H. pylori* infections (see above), experimental infection of C57BL/6 mice lead to hypogastrinemia apparently due to a direct effect of *H. felis* on the G-cells or due to the *Helicobacter* induced inflammatory cytokines. Hypochlorhydria was not only the result of decreased gastrin output but also of the atrophic loss of parietal cells. Apparently, the animal host used in *Helicobacter* infection studies plays a crucial role in gastrin regulation. Human, rat, gerbil and ferret react with hypergastrinemia to *Helicobacter* infection, while C57BL/6 mice react with hypogastrinemia (Dial *et al.*, 2000).
There are also gender differences in the gastric inflammatory and epithelial response to *H. felis* in the murine model. This was investigated by Court *et al.* (2003), who found that in female mice compared to male mice, infection with *H. felis* resulted in an earlier onset of chronic gastric inflammation, epithelial hyperplasia and oxyntic cell loss.

“*H. heilmannii*” infection in mice has also been investigated through inoculation of the animals with gastric scrapings or homogenised biopsy material (Eaton *et al.*, 1995; Peterson *et al.*, 2001). O’Rourke *et al.* (2004a) described the infection of BALB/c mice with ten different “*H. heilmannii*”-like isolates obtained from humans and animals. “*H. heilmannii*” could be readily seen in large numbers in fundus and antrum. The mice developed gastric MALT lymphoma after 6 months of infection. The prevalence of pathological changes was notably higher than that seen in the *H. felis*-BALB/c infection model. As in the latter model, no active chronic gastritis was observed.

Early attempts to establish *H. acinonychis* infection in BALB/c mice failed (Eaton *et al.*, 1993). By using IL-12β-deficient C67BL/6 mice that are known to be more permissive to *H. pylori* infection (Hoffman *et al.*, 2003), colonization with *H. acinonychis* was obtained (Dailidiene *et al.*, 2004). The bacteria were recovered from the mice and used to inoculate wild-type C67BL/6 and BALB/c mice. *H. acinonychis* was found to be capable of persistent mixed infection with *H. pylori* and genetic recombination between both species was described.

After isolation of “*H. suncus*” from the stomach of the house musk shrew, experimental challenge of “*H. suncus*” free animals was done (Goto *et al.*, 2000). The stomach was examined histologically to compare “*H. suncus*” free musk shrews with experimentally infected and spontaneously infected shrews. Gastritis was observed in fundic and pyloric glands of the stomach from spontaneously and experimentally infected animals. The same gastric lesions seen in the stomach of spontaneously infected animals were observed microscopically in the stomach of experimentally infected animals, but no lymphoid follicle formation was present in the latter.

Up to now, no mouse or other animal model had been described to investigate *H. bizzozeronii* and *H. salomonis* pathogenicity.
4.1.3 Modelling infection-associated cancer

*H. felis* infection has been used in mice to model gastric cancer (Table 2). The accelerated development of gastric carcinoma in insulin-gastrin (INS-GAS) transgenic FVB mice infected with *H. felis* was reported to be associated with hypergastrinemia (Wang et al., 2000b). These transgenic mice express human gastrin in the pancreas. Hypergastrinemia initially is associated with increase in the number of parietal cells, but long-term stimulation leads to atrophy and progression to gastric cancer. *H. felis* synergized with hypergastrinemia in these mice and accelerated formation of lesions. The same was also found for *H. pylori* infection (Fox et al., 2003). Fox et al. (2002) were the first to describe the occurrence of *Helicobacter*-associated gastric adenocarcinomas in wild-type C57BL/6 mice after 15 months of *H. felis* infection. Infection for over 15 months induced complete absence of parietal and chief cells in the latter mouse model and 100% of the mice displayed adenocarcinoma in the fundus. Eradication of *H. felis* in C57BL/6 mice restored the normal mucosal architecture of the stomach and inhibited gastric cancer progression (Cai et al., 2005).

4.2 Gerbil models

In 1996, Hirayama et al. established an *H. pylori* Mongolian gerbil model. Further research on this infection model reported chronic active gastritis, intestinal metaplasia and formation of gastric ulcers (Matsumoto et al., 1997; Takahashi et al., 1998; Ikeno et al., 1999). Long-term infection (> 62 weeks) of Mongolian gerbils, provided the first experimental evidence that *H. pylori* infection alone can result in the development of gastric carcinoma (Watanabe et al., 1998; Honda et al., 1998; Zheng et al., 2004). Since this discovery, the use of gerbils in *Helicobacter* research has increased considerably, especially as this model is proven to respond more rapidly and more aggressively to *H. pylori* infection than mice (Matsumoto et al., 1997; Court et al., 2002). The gerbil model has also been used to study gastrin and acid output after *H. pylori* infection. In the gerbil model hypergastrinemia is observed together with hypochlorhydria (Takashima et al., 2001).

Court et al. (2002) were the first to inoculate gerbils with *H. felis* and to date no other *H. felis* studies in gerbils have been conducted. Court et al. (2002) compared the effects of *H. pylori* and *H. felis* on gastric epithelial cell proliferation in mice and gerbils. At 4 weeks, gerbils infected with *H. pylori* or *H. felis* developed antral gastritis and both *H. pylori* and *H.
*felis* infected animals showed significantly increased antral epithelial cell proliferation. Epithelial cell proliferation induced by *H. felis* in gerbils was twice that when stimulated by *H. pylori*. In the mouse model, minimal inflammatory changes and no epithelial cell proliferation were observed for both bacterial species 4 weeks p.i.. At 8 weeks of infection, mice infected with *H. felis* had higher levels of inflammation than *H. pylori* infected mice and *H. felis*, but not *H. pylori*, induced significantly increased epithelial cell proliferation in the cardia and fundus. This study mainly focused on the effect of *H. felis* on gastric epithelial cell proliferation; no detailed description of the induced inflammation was given.

**Table 2.** Summary of key rodent models of *Helicobacter* associated gastric cancer (adapted from Rogers and Fox, 2004)

<table>
<thead>
<tr>
<th>Rodent</th>
<th>Infective agent</th>
<th>Tumor</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice</td>
<td><em>H. felis</em></td>
<td>Gastric adenocarcinoma</td>
<td>Bacteria lack vacA and PAI</td>
</tr>
<tr>
<td>INS-GAS FVB mice</td>
<td><em>H. felis</em> and <em>H. pylori</em></td>
<td>Gastric adenocarcinoma</td>
<td>Hypergastrinemia promotes tumorigenesis</td>
</tr>
<tr>
<td>Mongolian gerbil</td>
<td><em>H. pylori</em></td>
<td>Gastric adenocarcinoma</td>
<td>Closely mimics gastric disease</td>
</tr>
<tr>
<td>BALB/c mice</td>
<td>Several <em>Helicobacter</em> species</td>
<td>Gastric MALT lymphoma</td>
<td>Usually requires 18-24 months</td>
</tr>
</tbody>
</table>

### 4.3 Other animal models

In comparison with mouse models, rats have been used for the study of *Helicobacter* pathogenesis only to a limited extent. Fox *et al.* (1991a) were the first to describe *H. felis* colonization of gnotobiotic rats causing small foci of lymphocytes and eosinophils throughout the subglandular region of the antrum by two weeks p.i.. At 8 weeks p.i., there were increased numbers of lymphocytes and eosinophils in the subglandular areas and some focal aggregates of lymphocytes extended from the submucosa to the luminal surface. Unlike the fundic and antral gastritis seen in *H. felis*-infected germfree mice, the gastritis in rats was primarily confined to the antrum.

In 1998, for the first time a rat model of chronic *H. pylori* colonization was described (Li *et al.*, 1998). Earlier reports with *H. pylori* in rats only observed the effect of the bacteria shortly after inoculation and no colonization was obtained (Ross *et al.*, 1992; Li *et al.*, 1997). In the *H. pylori* rat model, a moderate chronic antral gastritis and predominant antral colonization was found after 2 months and 1 year of infection (Li *et al.*, 1999).
As this animal model has been used extensively for gastric acid secretion studies (Ryberg et al., 1990), it allowed more in depth research on the effect of *Helicobacter* bacteria on gastric acid secretion. Danon et al. (1998) used *H. felis* and “*H. heilmannii*” inoculated rats to study gastrin release and gastric acid secretion. The bacteria colonized the gastric antrum of rats in large numbers and induced mild gastritis but colonization by these bacteria did not alter gastrin and acid secretion. Gastric motility abnormalities are believed to be related to *Helicobacter* infection (Minocha et al., 1994). This was investigated by Duval-Araujo et al. (2000) by using a “*H. heilmannii*” rat model. They found alterations in gastric motility (increased gastric emptying), gastrin and somatostatin levels compared to non-infected rats.

*H. mustelae* shares many biochemical, molecular and phenotypic characteristics with *H. pylori*. Moreover, the gastric inflammatory response seen in naturally and experimentally infected ferrets mimics the sequelae of human *H. pylori* infection (Fox et al., 1990; Fox et al., 1991b; Fox et al., 1991c; Fox et al., 1997; Erdman et al., 1997). Therefore, experimental and natural infections of ferrets with *H. mustelae* have been used to study potential virulence factors that influence colonization of the stomach and the development of gastritis, ulcers, and gastric cancer. The bacteria are associated with hypergastrinemia in ferrets, which may be related to the pathogenesis of peptic ulcer disease (Perkins et al., 1996). The presence of urease activity, intact flagellin proteins and surface rings in this bacterium were all proven to be important for colonization of the ferret stomach (Andrutis et al., 1995; Andrutis et al., 1997; Patterson et al., 2003).

To date, guinea pigs have only been used in experimental *H. pylori* infections (Shomer et al., 1998; Rijpkema et al., 2001; Sjunnesson et al., 2003; de Jonge et al., 2004; Lueth et al., 2005). After 4 weeks of infection, gastritis consisted of a mixed population of lymphocytes, eosinophils and other PMNs mainly in the antrum. Fifteen weeks p.i., MALT developed. The presence of prominent eosinophils infiltration is due to the presence of a well-characterized IL-8 homologue in guinea pigs (Collins et al., 1993). This is an important advantage over rat and mouse models in *Helicobacter* research, as in the latter models an exact murine homologue for human IL-8 has yet to be found (Call et al., 2001). Other advantages of the guinea pig model are the animal’s dietary requirement for vitamin C and the anatomic features of its stomach. As in humans, vitamin C is present in high concentrations in the gastric juice of guinea pigs. Because vitamin C has been speculated to play a role in human *H. pylori* related gastric carcinogenesis (Correa et al., 1995), this animal model could prove
useful in the study of *H. pylori*-vitamin C interaction. Moreover, the guinea pig stomach lacks a non-glandular region and is thus anatomically more similar to the human stomach than is the stomach of other rodents (Shomer *et al.*, 1998).

To investigate the pathogenic significance of helicobacters in pet carnivores, experimental infections have been conducted in cats and dogs. Gnotobiotic dogs have been experimentally infected with *H. pylori* (Radin *et al.*, 1990) and *H. felis* (Lee *et al.*, 1992), SPF dogs with *H. felis* (Simpson *et al.*, 1999a), and SPF cats with *H. felis* (Simpson *et al.*, 2000) and *H. pylori* (Fox *et al.*, 1995; Perkins *et al.*, 1998). A conventional *H. pylori* dog model was established by Rossi *et al.* (1999).

Lee *et al.* (1992) inoculated gnotobiotic Beagle dogs with *H. felis* that were euthanized one month post-infection. They found that *H. felis* colonized all areas of the stomach and was associated with the presence of lymphofollicular gastritis mainly in the fundus. A similar study was done by Simpson *et al.* (1999a) but instead of gnotobiotic dogs, SPF beagles were used. In contrast to the study by Lee *et al.* (1992), they did not find a significant difference in severity of mucosal infiltration in infected and uninfected dogs and most of the inflammatory infiltrate was found in the antrum. Moreover, similar to what was found in dogs with a natural *H. felis* infection, the gastric secretory axis was unchanged in both experimentally infected and control dogs. *H. pylori* experimental infection in conventional dogs was associated with clinical signs such as vomiting and diarrhea. Histologically, PMNs infiltration was found initially but was rapidly replaced by chronic follicular gastritis and lymphoid follicles after a few weeks of infection (Rossi *et al.*, 1999).

In SPF cats infected with *H. felis*, lymphoid follicular hyperplasia, atrophy, and fibrosis were observed mainly in the antrum. Mild mononuclear inflammation was detected in both infected and uninfected cats, but was more extensive in infected cats, with pangastric inflammation, eosinophilic infiltrates, and cardia gastritis observed only in infected animals (Simpson *et al.*, 2000). Neutrophils were not found in the latter study. Moreover, similar to what was found in experimentally and naturally *H. felis* infected dogs, the gastric secretory axis was unchanged in both infected and control cats. In contrast to the previous study, *H. pylori* infected SPF cats had multifocal antral gastritis consisting of lymphoid aggregates and moderate diffuse infiltration of polymorphonuclear leukocytes in the subglandular region of the antrum (Fox *et al.*, 1995; Perkins *et al.*, 1998).
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Today, *H. pylori* is a well-recognized pathogen that chronically infects up to 50% of the world’s human population and is proven to be associated with gastritis, peptic ulcer disease and gastric cancer. Other bacteria morphologically distinct from *H. pylori* are occasionally found colonizing the human stomach. These spiral-shaped bacteria are collectively designated “*H. heilmannii*”. Gastric inflammation in patients infected with “*H. heilmannii*” is less severe than with *H. pylori*. Nevertheless, these bacteria have been associated with gastrointestinal symptoms and gastric cancer. Reports have been published demonstrating that the microorganisms histologically identified as “*H. heilmannii*” often turn out to be *Helicobacter* species found in cats and dogs such as *H. felis*, *H. bizzozeronii* and *H. salomonis*. The pathogenic significance of these species in humans, cats and dogs remains unclear and is still a matter of controversy and debate. The latter to a great extent is rooted in the fact that the various research groups use different host species, strains within host species, bacterial species and strains within bacterial species, generating data which are often very difficult to compare.

The general aim of the present thesis hence was to investigate the differential pathological effects of pet carnivore *Helicobacter* species and strains on the gastric mucosa of experimental hosts under standardized conditions.

The specific aims of this thesis were:

- to highlight the importance of research on pet-borne *H. felis* infections associated with human gastric ulcer disease.
- to compare virulence differences between pet carnivore *Helicobacter* species and strains by involving different mouse strains.
- to establish a Mongolian gerbil model for infection with *H. bizzozeronii* and to compare pathogenic effects of *H. bizzozeronii* to those of *H. felis* in a short term infection model
- to study the evolution of the pathogenic effect of *H. felis* and *H. bizzozeronii* in the gerbil model and investigate bacterium-epithelial cell interactions.
CHAPTER 1

Peptic ulcer disease associated with *Helicobacter felis* in a dog owner


*Adapted from European Journal of Gastroenterology & Hepatology, In Press*
Summary

The aim of this study was to investigate the identity of the “H. heilmannii”-like bacteria found in the stomach of a human patient suffering from stomach ulcers and her asymptomatic pet dog. An elderly woman was referred for gastroscopy because of right hypochondrial pain, nausea, anorexia and vomiting. Gastric ulcers were observed and histology revealed the presence of multiple “H. heilmannii”-like bacteria. Multiplex PCR identified the bacteria as *H. felis*. Her pet dog was also examined gastroscopically. Only mild gastric lesions were found in the dog but PCR showed the presence of *H. felis* as well as *H. bizzozeronii* and “*Candidatus* H. heilmannii”. This report associates *H. felis* infection in humans with severe gastric ulceration. Moreover, the suggestion can be made that the patient contracted *H. felis* from her dog.
Introduction

The Gram-negative bacterium *H. pylori* is present in the stomach of approximately 50% of the world’s population and is one of the most common causes of chronic gastritis. In some gastric biopsies, *Helicobacter* bacteria morphologically different from *H. pylori* are observed (Solnick *et al*., 1993). These bacteria have been referred to as “*H. heilmannii*”. “*H. heilmannii*” infections have been associated with gastritis (Debongnie *et al*., 1998), gastric lymphoma (Morgner *et al*., 2000), ulcers (Cales *et al*., 2000) and some unusual histopathological features (Ierardi *et al*., 2000). Its prevalence is rare with a reported range of between 0.25% and 1.7%. “*H. heilmannii*” infections have been postulated to be of zoonotic origin with various animal species serving as reservoirs (Meining *et al*., 1998; Thomson *et al*., 1994; van Loon *et al*., 2003; Lavelle *et al*., 1994; Dieterich *et al*., 1998; Trebesius *et al*., 2001; Yoshimura *et al*., 2002). It is now clear that what was originally named “*H. heilmannii*” does not represent a single species, but a species complex comprised of “*Candidatus H. suis*” (De Groote *et al*., 1999), found in the stomach of pigs and referred to as “*H. heilmannii*” type 1, and the spiral organisms found in the stomach of cats and dogs that are assigned “*H. heilmannii*” type 2 i.e. *H. felis*, *H. bizzozeronii*, *H. salomonis* (Jalava *et al*., 1997) and the recently described “*Candidatus H. heilmannii*” (O’Rourke *et al*., 2004) and *H. cynogastricus* (Van den Bulck *et al*., In Press). The prevalence of helicobacters in the stomach of dogs is high (Van den Bulck *et al*., 2005). Nevertheless, the clinical significance of these infections in these animals is still a matter of debate.

Although there is epidemiological evidence that direct contact with animals is a risk factor for humans to contract these infections (Meining *et al*., 1998) and several case reports have documented this (Thomson *et al*., 1994; van Loon *et al*., 2003; Lavelle *et al*., 1994; Dieterich *et al*., 1998), the evidence has remained circumstantial, mainly because the methods to identify these gastric non-*H. pylori* helicobacters to the species level were lacking. We recently developed a multiplex PCR that allows to simultaneously identify several of these gastric non-*H. pylori* helicobacters up to the species level (Baele *et al*., 2004).

Here we report a case of a histologically diagnosed “*H. heilmannii*” infected patient presenting with stomach ulcers and her histologically diagnosed “*H. heilmannii*” infected dog which in both cases, after multiplex PCR, proved to harbour the same *Helicobacter* species, namely *H. felis*. 
Case report

A 76-year-old woman was admitted to the hospital St-Pierre, Ottignies (Belgium) presenting right upper quadrant pain, nausea, anorexia and vomiting. She was treated with an amoxicillin and clavulanate combination (500 mg) and butylscopolamine (10 mg) twice a day for one week, but symptoms persisted. She exhibited 5 kg weight loss in one month. There was no history of non-steroidal anti-inflammatory drugs (NSAIDs) ingestion.

Physical abdominal examination revealed right upper quadrant and paraumbilical pain, positive Murphy’s sign and normal peristalsis. Blood cell counts as well as biochemical blood analysis were normal. Endoscopic appearance of the oesophagus, the gastric corpus mucosa, and the duodenum was normal. However, ulcers were present in the antrum (Fig. 1). Biopsy specimens were taken from the fundus, antrum and ulcer area. Rapid urease testing was positive for fundic biopsies (HUT-Test®, Astra-Zeneca, Wedel, Germany). A cytological smear was prepared. The specimens were fixed in 10% formalin, embedded in paraffin, and cut into sections that were stained with Giemsa, haematoxylin and eosin (H&E) and periodic acid schiff (PAS) staining. A few paraffin sections were taken for DNA extraction (DNeasy Tissue Kit, Qiagen, Hilden, Germany) and subsequent PCR. Light microscopic examination of H&E stained sections showed chronic antral gastritis with infiltration of neutrophils in the area where the ulcers were present. Giemsa staining of the paraffin sections and the cytological smear revealed the presence of spiral-shaped microorganisms in fundus and antrum, consistent with the characteristic features of “H. heilmannii” (Andersen et al., 1999) (Fig. 2, Fig. 3). “H. heilmannii” is easy to distinguish from H. pylori by microscopy because of the long corkscrew shape of “H. heilmannii” in contrast to the S-shape of H. pylori. Since contact with animals is a known risk factor for contracting “H. heilmannii” infections (Meining et al., 1998), the clinician questioned the patient about possible contacts with animals. It appeared that the patient lived in close contact with her dog. To identify the spiral-shaped bacteria to species level, a multiplex PCR (Baele et al., 2004) was used that allows the discrimination between H. felis, H. bizzozeronii, H. salomonis and “Candidatus H. suis”, based on the transfer RNA intergenic spacers of Helicobacter species and the urease gene of H. felis. Another urease-based species-specific PCR was used to identify “Candidatus H. heilmannii” (O’Rourke et al., 2004). PCR of the biopsy DNA extract showed the presence of H. felis. The patient was successfully treated with the proton pump inhibitor pantoprazole 40 mg and the antibiotics amoxicillin 1g and clarithromycin 500 mg twice a day for 7 days reducing the dose of pantoprazole to 40 mg once a day for the next 28 days. Re-evaluation by
endoscopy and biopsy one month after the first endoscopy revealed scar tissue on the site of previously localised antral ulcers and the absence of spiral bacteria.

Her dog was a 7-year-old spayed female wire-hair Dachshund that was clinically healthy. This dog was presented to the Faculty of Veterinary Medicine, Ghent University (Belgium) for gastroscopy. No macroscopic abnormalities were found in the oesophagus, antrum and duodenum. Endoscopic examination of the fundus revealed a small erosion near the lesser curvature. The greater curvature showed mucosal oedema with mild signs of gastritis. Biopsies were taken from the fundus, antrum and duodenum and fixed in 10% buffered formalin for histology. Sections were cut and stained with H&E, Giemsa and PAS. Additional gastric tissue samples were obtained for both PCR and rapid urease test (CUTest®, Temmler Pharma, Marburg, Germany). The fundic and antral urease tests were positive within less than an hour. Antral, fundic and duodenal histological examination revealed a normal morphology. In the mucous and gastric pits of the fundus, Giemsa staining showed the presence of long, tightly coiled spiral microorganisms, consistent with the features of “H. heilmannii” (Fig. 4). DNA from the canine gastric biopsies was isolated using the DNeasy Tissue Kit (Qiagen), according to manufacturer’s instructions. PCR identified the bacteria in the biopsies as belonging to three different species namely H. felis, H. bizzozeronii and “Candidatus H. heilmannii”. The dog was treated with a 10-day triple therapy consisting of amoxicillin 20mg/kg and metronidazole 10 mg/kg twice a day, and omeprazole 0.7 mg/kg once a day. Endoscopic re-evaluation six months after the completion of the treatment still revealed the presence of mild gastritis. The rapid urease test was positive within the hour and histology showed normal gastric mucosa with the presence of “H. heilmannii”-like bacteria. PCR detected only the presence of “Candidatus H. heilmannii”. Two-week triple-therapy was instituted but amoxicillin was replaced by azithromycin 11 mg/kg once a day. Two weeks after cessation of the second treatment, repeated endoscopy and histology still showed some very mild gastritis in the fundus but without the presence of Helicobacter bacteria. Rapid urease tests and PCR on biopsy samples yielded negative results.

Discussion

To our knowledge this is the first documented report in which a patient presenting stomach ulcers was found H. felis positive. Germani et al. (1997) described the presence of H. felis in an individual presenting dyspepsia but no mention was made of the presence of gastric ulcers.
“H. heilmannii”-like bacteria have been observed in gastric tissue samples of humans suffering from gastric erosions but these bacteria were not identified to the species level.

Recently, a multiplex PCR was developed to discriminate between the closely related species \textit{H. felis}, \textit{H. bizzozeronii}, \textit{H. salomonis} and “\textit{Candidatus Helicobacter suis}” in one reaction mixture (Baele \textit{et al.}, 2004), in contrast to previously described PCRs, in which only one species or an assembly of species could be identified (Germani \textit{et al.}, 1997). Moreover, a PCR to identify the recently described “\textit{Candidatus H. heilmannii}” was developed (O’Rourke \textit{et al.}, 2004). These tests allowed us to identify morphologically “\textit{H. heilmannii}”-like bacteria to the species level.

\textit{H. felis} was demonstrated in gastric biopsy specimens from a patient exhibiting gastric ulcers as well as in gastric biopsies specimens of her pet dog. Since there was intense contact between the owner and her pet, transmission of \textit{H. felis} might have occurred between the dog and the patient. This in accordance with several other published reports discussing the possible \textit{Helicobacter} transmission between house-hold dogs and their owners (Meining \textit{et al.}, 1998; Thomson \textit{et al.}, 1994). Nevertheless, definite proof of animal to man transmission is still lacking. Van den Bulck \textit{et al.} (2005) reported the prevalence of pet carnivore helicobacters in human gastric “\textit{H. heilmannii}” diagnosed biopsies. \textit{H. felis} was present in 14.6 % of these samples. From these, 8.9 % were single infections and 5.7 % were mixed infections with other \textit{Helicobacter} species.

In pet carnivores the prevalence of \textit{H. felis} is high. Nevertheless the pathogenic importance of this bacterium in cats and dogs is still unclear (Happonen \textit{et al.}, 1998). However, in experimental rodent models \textit{H. felis} can induce strong inflammatory responses comparable with \textit{H. pylori} infection in humans (Lee \textit{et al.}, 1990). In contrast to \textit{H. pylori}, not much is known about possible virulence factors of \textit{H. felis} and the mechanism by which this bacterium causes gastric inflammation and cellular damage remains unclear.

Only \textit{H. felis} was found in the patient although her pet dog was also infected with \textit{H. bizzozeronii} and “\textit{Candidatus H. heilmannii}”. However, \textit{H. bizzozeronii} (Van den Bulck \textit{et al.}, 2005) and “\textit{Candidatus H. heilmannii}” have been proven to be able to colonize the human stomach. Variation between individuals in susceptibility to the different \textit{Helicobacter} species may be a plausible explanation but this remains to be elucidated.

Treatment of the patient and her pet with proton pump inhibitors and antimicrobials cleared the \textit{H. felis} infection in both of them. It also cleared the \textit{H. bizzozeronii} infection in the dog. The effect of triple therapy on eradication of canine gastric helicobacters has been previously described and proved to be effective although reinfection is likely to occur
Happonen et al., 2000). “Candidatus H. heilmannii” DNA however was detected in the dogs stomach six months after completion of the first treatment with amoxicillin. Only after a second treatment with azithromycin the infection was eradicated. It is not clear if these findings are due to decreased susceptibility of the “Candidatus H. heilmannii” strain to amoxicillin or that the bacterium was indeed cleared but that, after the first treatment, reinfection occurred.
References


Figures

**Fig. 1** Gastroscopic image from the human patient showing one of the gastric ulcers found.

**Fig. 2** Giemsa staining of the human patient’s stomach biopsy demonstrating spiral organisms on the surface of the gastric epithelium. Bar = 10 μm.

**Fig. 3** “*H. heilmannii*”-like bacteria present in the cytological smear of the human patient’s stomach biopsy. Bar = 10 μm.
Fig. 4 Giemsa staining of the canine stomach biopsy showing multiple spiral organisms in the gastric foveolae. Bar = 10 μm.
CHAPTER 2

The inflammatory response in the mouse stomach to Helicobacter bizzozeronii, Helicobacter salomonis and two Helicobacter felis strains


Adapted from Journal of Comparative Pathology (2005), 133, 83-91
Abstract

The inflammatory response in the mouse stomach was evaluated as a means of distinguishing different non-\textit{H. pylori} \textit{Helicobacter} strains in terms of virulence. Mice of four strains (BALB/c, SJL, C57BL/6 and CFW) were infected intragastrically with four bacterial strains (\textit{H. felis} ATCC 49179 and CCUG 37471, \textit{H. bizzozeronii} and \textit{H. salomonis}). The animals were killed for gastric examination at 3, 9 or 16 weeks p.i. \textit{H. salomonis} could not be detected by the polymerase chain reaction (PCR), but the other three organisms were detected in all stomach samples at all timepoints. SJL mice consistently showed particularly severe gastric inflammation regardless of bacterial strain. Lymphocytes and occasionally neutrophils were seen in submucosa and lamina propria mucosae. BALB/c mice showed the least severe inflammatory changes. \textit{H. bizzozeronii} differed from the two \textit{H. felis} strains in producing less striking pathological changes in mice. Of the two \textit{H. felis} strains, ATCC 49179 produced the more severe inflammatory changes in SJL mice.
Introduction

Approximately two decades ago, *H. pylori* was conclusively identified as the leading cause of acute gastritis and peptic ulceration in man (Marshall et al., 1985a,b). Over the years, many reports have been published concerning the association of *H. pylori* not only with gastritis, but also with gastric adenocarcinoma and lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) (Group, 1993; Parsonnet et al., 1994; Veldhuyzen van Zanten and Sherman, 1994; Marshall, 1995). Soon after the identification of *H. pylori*, other bacteria, morphologically distinct from *H. pylori*, were described in the stomach of human patients with gastritis (Heilmann and Borchard, 1991). These bacteria were later identified as belonging to the *Helicobacter* genus, but because they all had a similar morphology and could not be cultivated they were collectively and provisionally named “*H. heilmannii*” (Solnick et al., 1993). “*H. heilmannii*” is found in human beings (0.2 – 0.6%) and also in a wide range of animal hosts such as cats, dogs, pigs and cattle (Lee et al., 1988; Eaton et al., 1996; De Groote, 2000). Through 16S rDNA sequence analysis, two different types of “*H. heilmannii*” were identified. Type 1 was phylogenetically closely related to, if not identical with “*Candidatus* H. suis”, a bacterium found in pig stomachs (De Groote et al., 1999). Type 2 was closely related to helicobacters encountered in the stomach of cats and dogs, namely *H. felis* (Lee et al., 1988; Paster et al., 1991), *H. bizzozeronii* and *H. salomonis* (Hänninen et al., 1996; Jalava et al., 1997). The prevalence of these bacteria in the stomach of dogs varied from 61% to 100% (Eaton et al., 1996). Andersen et al. (1996) were the first to culture a “*H. heilmannii*”-like strain from the human stomach; this organism however was later identified as *H. bizzozeronii* (Jalava et al., 2001). These observations have led to speculation that animals may serve as a source of infection for human beings.

*Helicobacter* species and strains may differ in virulence, as has been noted in *H. pylori*, in which such differences have been attributed to the presence or absence of virulence genes (Doig et al., 1999). The virulence genes of the non-*H. pylori* gastric helicobacters, however, are largely unknown. The purpose of the present study was to use the degree of colonization and inflammation in the stomach of mice as the basis of a method for distinguishing various species and strains of canine and feline gastric helicobacters.
Materials and Methods

Animals

Specific Pathogen-Free (SPF) male mice, aged 6 weeks, of four strains (BALB/c, C57BL/6, SJL and CFW) were obtained from Harlan NL, Horst, the Netherlands. They were barrier-maintained in a room with a controlled environment and housed in groups of six in filter-top cages on autoclaved (121°C, 15 min) wood shavings. The animals were fed an autoclaved diet containing 18% protein (Teklad Global Rodent diet; Harlan NL) and received autoclaved water ad libitum. The experimental protocol was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

Bacterial Strains and Inocula

The strains used were \textit{H. bizzozeronii} CCUG 35545, \textit{H. salomonis} CCUG 37845 and \textit{H. felis} CCUG 37471, all isolated from canine gastric mucosa and kindly provided by P. Vandamme; and \textit{H. felis} ATCC 49179, isolated from feline gastric mucosa and kindly provided by R. Ferrero. In our laboratory, the strains were subcultured two times. They were grown on brain-heart infusion (BHI) agar to which Skirrow supplement (Oxoid Ltd, Basingstoke, UK), vitamins (Vitox®; Oxoid), amphotericin B (Fungizone®; Bristol-Myers Squibb, New York, USA) and horse blood 10% were added. The plates were incubated (37°C, 48 h) in jars under microaerophilic conditions, created by evacuating 80% of the normal atmosphere and introducing a gas mixture of 84% N$_2$, 8% H$_2$ and 8% CO$_2$. The bacteria were harvested from the plates and suspended in BHI broth with Skirrow supplement, vitamins (Vitox®) and horse serum 10%. The final concentration was adjusted to an optical density of 1.0 at 660 nm corresponding to approximately $10^8$ colony-forming units (cfu)/ml (Corthésy-Theulaz \textit{et al.}, 1995).

Experimental procedure

Each of the four mouse strains was treated in the following manner. Four groups of 18 animals were each inoculated with one of the four bacterial strains, and each group was accompanied by nine uninoculated controls (Lee \textit{et al.}, 1990; Sakagami \textit{et al.}, 1996). The inoculations were made intragastrically with a ball-tipped gavage needle after a 12h fasting period. Each mouse received 0.4 ml of bacterial suspension on three occasions at 48-h intervals. At 3, 9 and 16 weeks p.i., six infected and three control animals were killed by
cervical dislocation after isoflurane anaesthesia. The stomach of each mouse was resected and samples were taken for PCR analysis and histopathological examination (see below).

**PCR analysis**

Firstly, all four bacterial strains were examined for the presence of the *CagA* and *VacA* genes. The detection of these virulence factors was conducted as described by Xiang *et al.* (1995), *H. pylori* strain SS1 being used as positive control (Lee *et al.*, 1997).

From each mouse, samples of the gastric fundus and antrum were taken for DNA extraction and multiplex PCR analysis, as described elsewhere (Baele *et al.*, 2004). This PCR test discriminates between *H. felis*, *H. bizzozeronii* and *H. salomonis*, on the basis of the transfer RNA intergenic spacers of *Helicobacter* species and the urease gene of *H. felis*. Briefly, PCR reactions were performed in a volume of 10 µl containing a final primer concentration of 0.1 µM for each of the fluorescently (TET™, NED™, HEX™; Applied Biosystems, Foster City, USA) labelled oligonucleotides and 0.1 µM for each of the unlabelled primers, a concentration of 40 µM of each deoxynucleoside triphosphate (Amersham Pharmacia Biotech, Little Chalfont, England), 3 mM MgCl₂, Polymerase Taq platinum (Invitrogen Life Technologies, Carlsbad, USA) 0.03 U/µl, and 1x PCR buffer (Invitrogen Life Technologies). Two microlitres of template DNA were added to the vials. An initial denaturation of 5 min at 95°C was followed by three cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, and subsequently 35 cycles with the following conditions: 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, with a final extension at 72°C for 7 min.

The PCR products were analysed by capillary electrophoresis on 1 µl of PCR product. Samples were identified as *H. felis* when a TET-labelled fragment of 137 bp and a NED-labelled fragment of 434 bp were obtained. Samples were identified as *H. bizzozeronii* when a TET-labelled amplicon of 136 bp and a HEX-labelled fragment of 373 bp were obtained, whereas the presence of only a TET-labelled amplicon of 134 bp resulted in *H. salomonis* identification.

**Histological Examination**

A longitudinal strip of tissue from the oesophagus to the duodenum was fixed in 10% phosphate-buffered formalin, processed by standard methods and embedded in paraffin wax. Sections (5 µm) were cut and stained with haematoxylin and eosin (H&E) and Giemsa stain.
H&E-stained sections were examined “blindly” by two investigators, to grade the intensity of gastric inflammation, by the scoring system of Sakagami et al. (1996), with minor modifications (Table 1).

<table>
<thead>
<tr>
<th>Grade</th>
<th>Inflammatory changes</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>No increase in inflammatory cells</td>
</tr>
<tr>
<td>1</td>
<td>Mild infiltration of lymphocytes and/or neutrophils in the lamina propria</td>
</tr>
<tr>
<td>2</td>
<td>Uniform infiltration of the lamina propria by lymphocytes, plasma cells, eosinophils and scattered neutrophils, with no leucopedesis in the regions of the gastric pits</td>
</tr>
<tr>
<td>3</td>
<td>Moderately dense infiltration of the lamina propria by lymphocytes and plasma cells and/or moderate numbers of neutrophils in the lamina propria, with microabscesses in the region of gastric pits</td>
</tr>
<tr>
<td>4</td>
<td>Dense lymphocyte and plasma cell infiltrates and/or extensive neutrophil infiltrates in the lamina propria, with obvious cryptitis</td>
</tr>
</tbody>
</table>

Giemsa-stained sections were likewise examined “blindly” by two investigators to determine the distribution and degree of gastric colonization by the different *Helicobacter* strains. Cardia, fundus and antrum were each graded for colonization (on a 0 to 4 scale) by the grading system of Weltzin et al. (1997). The bacteria per longitudinal section were counted and scored as 0 (no bacteria), 1 (1-20 bacteria), 2 (21-50 bacteria), 3 (51-100 bacteria) or 4 (> 100 bacteria).

Data Analysis

Interobserver agreement was analysed by kappa statistics. The theoretical kappa and weighted kappa values are from -1 (no agreement at all) to +1 (complete agreement). Disagreements between the observers have different weights whether they differ by one grade or more. Values below 0.5 represent poor, those between 0.5 and 0.75 good and values over 0.75 excellent interobserver agreement. Only values greater than 0.5 were considered to indicate acceptable reliability (Tepeš et al., 1999). The *Helicobacter* strains and also the mouse strains were compared in terms of colonization and inflammation scores by a proportional odds model with cumulative logits (OR). Time was also added to the model as a fixed effect. Due to the fact that time had a significant effect and that there was a significant interaction between *Helicobacter* strain and mouse strain, separate analyses were done for each mouse strain at each of the three timepoints.
Results

PCR Analysis and Degree of Colonization

Analysis for the presence of CagA and VacA virulence factors in the four Helicobacter strains yielded negative results.

None of the control mice were Helicobacter-positive. All mice from all four mouse strains inoculated with H. felis or H. bizzozeronii were positive histologically (H&E and Giemsa stains) and by the PCR at all timepoints, but the results for H. salomonis were completely negative. The bacteria, if present, were primarily located in the gastric pits (Fig. 1) and scattered throughout the mucous layer covering the surface epithelium. They were also observed in close association with the parietal cells (PCs), especially in the transition zone between cardia and fundus (Fig. 2).

Results of the colonization scores are shown in Fig. 3. The antrum was consistently more heavily colonized than the fundus or cardia, regardless of mouse strain or bacterial species (data not shown). At 16 weeks only a few bacteria were seen in SJL mice. CFW mice showed the highest degree of colonization, followed by BALB/c, C57BL/6 and SJL mice ($P<0.0001$). Throughout the post-infection period, the colonization scores of H. bizzozeronii in all stomach regions of all mouse strains were higher than those of H. felis ($P<0.001$). There was no significant difference in bacterial colonization between the two H. felis strains.

The kappa values for Helicobacter colonization indicated good interobserver agreement (0.65; 95% CI 0.58 - 0.72).

Histological Analysis

The control animals had a well developed gastric mucosa with normal foveolate surface. No abnormal inflammatory cell reaction was noticed.

In all inoculated mice, the degree of inflammation was moderate to severe in the fundus. Little inflammation was seen in the antrum. Infiltration of inflammatory cells occurred in the submucosa and lamina propria. There were no signs of mucosal atrophy and infiltration of neutrophils was negligible although eosinophil infiltration was sometimes encountered in CFW mice at 16 weeks p.i.. In some cases, cellular debris was present in the crypts (microabscesses), mainly in the fundus. The scores representing the severity of inflammation are depicted in Fig. 4.

Throughout the post-inoculation period, the gastritis scores of BALB/c mice were significantly lower than those of C57BL/6 (OR: 0.238, $P=0.0002$) or SJL (OR: 0.098,
mice; no significant difference was found between BALB/c and CFW mice ($P=0.34$). In BALB/c mice, at 3 and 9 weeks, scattered lymphocytes were found along the limiting ridge (i.e., the transition zone between forestomach and stomach), regardless of bacterial strain (Fig. 5). At 16 weeks more pronounced lymphoid infiltrates were seen, especially in BALB/c mice infected with *H. felis* strain ATCC 49179, although this difference was not statistically significant.

In SJL mice, gastritis was particularly severe (SJL-C57BL/6 OR: 2.417, $P=0.019$; SJL-CFW OR: 7.097, $P<0.0001$; SJL-BALB/c OR: 10.14, $P<0.0001$). At 3 weeks p.i., a uniform infiltration of the lamina propria by lymphocytes was noted, mainly around the limiting ridge but to a lesser extent in the fundus. Occasionally (3/18 mice), microabscesses were found. The gastritis score differed significantly between the two *H. felis* strains at 3 weeks (F-CS OR: 0.04, $P=0.04$). The monomorphonuclear cell infiltration in the fundus was much more pronounced with *H. felis* ATCC 49179. The inflammation caused by *H. bizzozeronii* was significantly milder than that caused by *H. felis* ATCC 49179 (B-CS OR: 0.0094, $P=0.01$) but not significantly different from that caused by *H. felis* strain CCUG 37341 ($P=0.32$). At 9 weeks, SJL mice again differed from the other mouse strains in showing more severe inflammation, but there was no significant difference between the three bacterial strains in terms of inflammation produced. At 9 weeks, microabscesses had developed, mainly in the crypt lumina of the fundus, in half of the SJL mice (one of which died from natural causes), regardless of bacterial strain (Fig. 6). By 16 weeks, the number of SJL mice showing abscesses had declined, at which timepoint they were present only in mice infected with *H. felis* CCUG 37471. At 16 weeks, inflammation in SJL mice infected with *H. bizzozeronii* was more severe than in *H. felis*-infected mice, albeit not significantly. In *H. felis*-infected SJL mice at 16 weeks, infiltration by mononuclear cells was slightly greater than at 9 weeks, and equal to that seen at 16 weeks in C57BL/6 mice.

In C57BL/6 mice at 3 weeks, the degree of inflammation in mice infected with *H. felis* CCUG 37471 was greater than in ATCC 49179-infected animals. However, this difference, which was not significant ($P<0.1329$), was no longer apparent at 9 and 16 weeks. At the latter timepoint, two of 18 C57BL/6 mice infected with *H. felis* ATCC 49179 showed microabscesses.

At 3 and 16 weeks, CFW mice had slightly higher inflammation scores ($P<0.0291$ and $P<0.0001$, respectively) than BALB/c mice, while at 9 weeks lower inflammation scores were given ($P<0.0006$). In CFW mice, no significant differences between bacterial strains at any timepoint were noticed.
When taking all mouse strains and all timepoints into account, the degree of inflammation produced by *H. bizzozeronii* was significantly lower than that produced by *H. felis* CCUG 37471 (OR: 0.320; 95% CI: 0.1640 – 0.622; *P*<0.0008) or *H. felis* ATCC 49179 (OR: 0.420; 95% CI: 0.220 – 0.810; *P*<0.0009). No statistical difference was seen between the two *H. felis* strains, except at 3 weeks in SJL mice. With two exceptions (infections in mice of strain CFW; and *H. bizzozeronii* infection in BALB/c mice), inflammation scores in this study increased progressively with time (Fig. 7).

The kappa values for chronic (0.76; 95% CI 0.71 - 0.81) and active (0.75; 95% CI 0.63 - 0.87) inflammation showed excellent interobserver agreement.

**Discussion**

Jalava *et al.* (1999) assessed 24 different *H. felis* strains, including the two used in the present study, to assess their ability to colonize BALB/c and C57BL/6 mice. The present study confirmed the colonization of BALB/c and C57BL/6 mice by *H. felis* strains CCUG 37471 and ATCC 49179, and extended these findings by including two additional mouse strains (SJL and CFW) and *H. bizzozeronii*.

The study showed a clear difference between *H. bizzozeronii*-induced inflammation and the inflammation produced by *H. felis* in the same mouse strain: overall, inflammation resulting from *H. bizzozeronii* infection was less than that produced by the *H. felis* strains. This difference was especially striking in SJL mice 3 weeks after infection between *H. bizzozeronii* and *H. felis* ATTC 49179. Moreover, 3 weeks p.i. *H. felis* ATCC 49179 produced more inflammation than the other *H. felis* strain used, with a significant difference in SJL mice. The study demonstrated that the gastritis produced in the four mouse strains by *H. felis* (strain CCUG 37471 or ATCC 49179) or *H. bizzozeronii* consisted of a mild, chronic but active inflammation dominated by lymphocytes; the severity of inflammation depended on the mouse strain.

The degree of colonization was inversely proportional to the severity of the gastritis. This was clearly shown in SJL mice 16 weeks p.i.. This accords with, and extends the findings of Sakagami *et al.* (1996), who suggested that inflammation might increase the resistance of the stomach to *Helicobacter* infection.

The degree of inflammation is dependent on host immune response and bacterial virulence factors. Roth *et al.* (1999) demonstrated the importance of the host T-cell response initiated by *Helicobacter* infections in subsequent gastric pathology. The fact that BALB/c mice have
been described genetically as T-helper (Th) 2 responders and C57BL/6 as Th1-driven (Gorham et al., 1996; Hazlett et al., 2000) might explain the difference between these mouse strains as reflected by the degree of inflammation produced. T-helper cell responsiveness in SJL mice is age- and gender-related. In these mice, Th1 cells are induced by immunization of older (≥ 10 weeks) male SJL mice, whereas immunization of young male SJL mice (≤ 8 weeks) results in the preferential induction of Th2 cells (Cua and Stohlman, 1997). The SJL mice used in this study were 9 weeks old at the first histological evaluation; possibly, therefore, the Th1 response was partly responsible for the higher inflammation scores seen in SJL and C57BL/6 mice.

In addition to the determining role of the host immune response as stated above (Mohammadi et al., 1996), specific Helicobacter virulence factors may also be an important determinant of Helicobacter-associated infection. Several studies of H. pylori have associated the expression of CagA (usually coexpressed with VacA) with severe inflammation and the development of ulcers (Xiang et al., 1995; Van Doorn et al., 1999). H. felis ATCC 49179 does not possess the H. pylori cagA and vacA gene (Xiang et al., 1995). We adopted the same primersets as Xiang et al. (1995) for the testing of H. felis strain CCUG 37471, H. bizzozeronii and H. salomonis and also encountered negative results. Due to the high specificity of the H. pylori PCR assay used, however, we cannot exclude that cagA and vacA homologues are present in these Helicobacter species. Nevertheless, the fact that the inflammatory outcome depended to a considerable degree on the bacterial strain indicates that yet unknown genes may play a decisive role in the inflammatory process.

The clinical significance of these non-H. pylori helicobacters for dogs and cats is still unclear and a matter of debate. The intraspecies variation in pathogenetic potential might explain the observed differences in the severity of gastritis seen among animals. Using electron microscopy, Peyrol et al. (1998) described H. bizzozeronii and H. felis trapped in canine PC canaliculi throughout the fundic region. In the present study bacteria were also seen in close association with PCs but only at the cardia-fundus transition zone. Peyrol et al. (1998) found a clear difference in pathogenicity between H. bizzozeronii and H. felis in naturally infected beagle dogs, the former being non-pathogenic. In the present study, H. bizzozeronii produced relatively slight inflammation in the early stages of infection, progressing later to a severe influx of mononuclear cells.

Inoculation of four different mouse strains with H. salomonis did not result in gastric infection detectable by the PCR. This confirms and extends the observations of Stoffel et al. (2000), who failed to infect BALB/c mice with H. salomonis. In an as yet unpublished study,
we failed to infect Mongolian gerbils with *H. salomonis*. Inoculation with *H. bizzozeronii*, however, resulted in colonisation of the gerbil stomach. Moreover, in agreement with Court *et al.* (2002), we succeeded in infecting gerbils with *H. felis*. The species of the host probably plays an important role in the colonizing ability of these bacteria. *H. felis* and *H. bizzozeronii* infections have been detected in a wide range of hosts (natural or experimental), while *H. salomonis* infection has so far been encountered only in dogs (Jalava *et al.*, 1997) and by PCR in rabbits (Van den Bulck *et al.*, 2005a). In a recent study, a quarter of human patients diagnosed with a “*H. heilmannii*” infection were in fact *H. salomonis*-positive (Van den Bulck *et al.*, 2005b).

In conclusion, in the present study, the degree of inflammation produced was shown to depend on the host as well as on the strain of non-*H. pylori* *Helicobacter*. To our knowledge, the detection of differences in helicobacter virulence by means of a mouse inflammation model has not been described previously.
References


Figures

**Fig. 1** CFW mouse 9 weeks after infection with *H. bizzozeronii*, showing numerous bacteria in the antral pits (arrow). Giemsa stain. Bar = 50 μm.

**Fig. 2** CFW mouse 9 weeks after infection with *H. bizzozeronii*, showing close association with PCs (arrow). Giemsa stain. Bar = 10 μm.
Fig. 3 Mean (n=6) colonization scores (cardia, antrum, fundus) of the different bacterial strains (CS, *H. felis* ATCC 49179; F, *H. felis* CCUG 37471; B, *H. bizzozeronii* CCUG 35545) in the different mouse strains (BALB/C, CFW, C57BL/6, SJL) during the course of infection, i.e., at weeks 3, 9 and 16 p.i..
Fig. 4 Mean (n=6) inflammation scores (cardia, antrum, fundus) of the different bacterial strains (CS, *H. felis* ATCC 49179; F, *H. felis* CCUG 37471; B, *H. bizzozeronii* CCUG 35545) in the different mouse strains (BALB/C, CFW, C57BL/6, SJL) during the course of infection, i.e., at weeks 3, 9 and 16 p.i.
Fig. 5 Scattered lymphocytes (arrow) along the limiting ridge in a BALB/c mouse, 3 weeks after inoculation with *H. felis* ATCC 49179. H&E stain. Bar = 100 μm.

Fig. 6 Microabscess (arrow) in an SJL mouse, 9 weeks after inoculation with *H. felis* ATCC 49179. H&E stain. Bar = 100 μm.
Fig. 7 Mean inflammation scores of the different bacterial strains (CS, *H. felis* ATCC 49179; F, *H. felis* CCUG 37471; B, *H. bizzozeronii* CCUG 35545) in the four mouse strains (BALB/C, CFW, C57BL/6, SJL) during the course of infection.
CHAPTER 3
Chapter 3.1

Helicobacter felis and Helicobacter bizzozeronii induce gastric parietal cell loss in mongolian gerbils

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Abstract

Non-\textit{H. pylori} \textit{Helicobacter} infections are associated with gastritis, gastric ulcers and MALT lymphomas in man. Approximately 50\% of these are caused by helicobacters commonly found in dogs and cats, including \textit{H. felis}, \textit{H. bizzozeronii} and \textit{H. salomonis}. In contrast to \textit{H. pylori}, the virulence mechanisms of these species are unknown. In this study the virulence of \textit{H. felis}, \textit{H. bizzozeronii} and \textit{H. salomonis} was investigated in Mongolian gerbils. Female SPF gerbils were inoculated intragastrically with \textit{H. felis}, \textit{H. bizzozeronii} or \textit{H. salomonis} and sacrificed three weeks later. Fundus and antrum samples were taken for bacterial detection by PCR. A longitudinal strip covering all stomach regions was taken for histology. Gastric colonization, inflammation, apoptosis, loss of parietal cells (PCs) and cell proliferation were assessed. Controls and \textit{H. salomonis} inoculated gerbils were negative in PCR. \textit{H. felis} and \textit{H. bizzozeronii} inoculated animals were positive. \textit{H. felis} inoculated animals showed loss of PCs extending from the limiting ridge into the fundus. A high cell proliferation rate was noticed in the mucosal area devoid of PCs. A dense band of apoptotic cells and large numbers of \textit{Helicobacter} bacteria were seen at the transition zone between affected and normal PCs. In \textit{H. bizzozeronii} infected gerbils, this was less pronounced. Focal apoptotic loss of gastric epithelial cells was spatially associated with the presence of bacteria especially in \textit{H. felis} and to a lesser extent in \textit{H. bizzozeronii} infected gerbils. This loss of cells may lead to intestinal metaplasia.
Introduction

*Helicobacter* species are spiral, flagellated, Gram-negative bacteria with worldwide prevalence, that colonize the gastrointestinal tract of human beings and animals. *H. pylori* in humans is associated with gastritis, gastric ulcerations (Marshall *et al.*, 1985), gastric adenocarcinoma and lymphoma of mucosal-associated lymphoid tissue (MALT lymphoma) (Group, 1993). In a small percentage of cases (0.2 – 0.6%), other bacteria, morphologically distinct from *H. pylori*, are found in the stomach of people suffering from gastritis (Heilmann and Borchard, 1991). These bacteria also belong to the *Helicobacter* genus. Due to their similar morphology and unculturable status, they were collectively and provisionally named “*H. heilmannii*” (Solnick *et al.*, 1993). “*H. heilmannii*” is not only found in humans but also in a wide range of animal hosts such as cats (Lee *et al.*, 1988), dogs (Eaton *et al.*, 1996), pigs and cattle. Through 16S rDNA sequence analysis, two different types of “*H. heilmannii*” could be identified in human gastric biopsies. Type 1 is phylogenetically closely related if not identical to “*Candidatus* *H. suis*”, a bacterium found in the stomach of pigs (De Groote *et al.*, 1999). Type 2 closely affiliates to helicobacters encountered in the stomach of cats and dogs, namely *H. felis*, *H. bizzozeronii* and *H. salomonis* (Jalava *et al.*, 1997). Andersen and colleagues (1996) were the first to actually culture a “*H. heilmannii*”-like strain from the stomach of an infected person, which was later identified as *H. bizzozeronii* (Jalava *et al.*, 2001). Recent research concerning the molecular identification of non-*H. pylori* spiral organisms in human gastric samples showed “*Candidatus* *H. suis*” and *H. salomonis* as the most prevalent ones. *H. felis* and *H. bizzozeronii* were present, though less common in human gastric samples (Van den Bulck *et al.*, 2005). It has been postulated that “*H. heilmannii*” is indeed zoonotic in nature (Dieterich *et al.*, 1998), but solid proof of this hypothesis is still lacking.

*H. pylori* strains may differ in virulence. More virulent strains can cause damage to gastric epithelial cells which has been attributed to the presence or absence of certain virulence genes (Marshall *et al.*, 1998). The virulence mechanisms of non-*H. pylori* gastric helicobacters however are hitherto largely unknown nor is it clear whether the same differences in terms of virulence are present among these organisms. The Mongolian gerbil is used to study *H. pylori* induced stomach ulcers and gastric carcinogenesis (Hirayama *et al.*, 1996; Watanabe *et al.*, 1998). The gerbil model appears to be more susceptible to *H. pylori*-induced gastric carcinogenesis (Ikeno *et al.*, 1999) and inflammatory changes may progress more rapidly and aggressively than in other laboratory animal models or in man (Hirayama *et al.*, 1996;
Matsumoto et al., 1997). Court et al. (2002) were the first to describe the successful colonization of Mongolian gerbils with an \textit{H. felis} strain. \textit{H. bizzozeronii} and \textit{H. salomonis} however were not included. In the present study, we used a Mongolian gerbil model to examine and compare the virulence of \textit{H. felis}, \textit{H. bizzozeronii} and \textit{H. salomonis} and their effect on the oxyntic mucosa.

\textbf{Materials and methods}

\textit{Animals}

Twenty-three specific pathogen-free (SPF) female gerbils (Elevage Janvier, Le Genest St Isle, France) of six weeks old were barrier-maintained in a room with controlled environment and housed in pairs in filtertop cages on autoclaved (121 °C, 15 min) wood shavings. They were fed an autoclaved diet containing 18\% protein (Teklad Global Rodent Diet, Harlan NL, Horst, the Netherlands) and received autoclaved water ad libitum. The experimental protocol was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

\textit{Bacterial strains and inocula}

The bacterial strains used in this study were \textit{H. bizzozeronii} CCUG 35545, \textit{H. salomonis} CCUG 37845 (both kindly provided by P. Vandamme) and \textit{H. felis} ATCC 49179 (kindly provided by R. Ferrero). They were grown on brain-heart infusion (BHI; Oxoid ltd, Basingstoke, UK) agar to which Skirrow supplement (Oxoid Ltd), vitamins (Vitox®, Oxoid Ltd), amphotericin B (Fungizone®, Bristol-Myers Squibb, New York, USA) and 10\% horse blood were added. The plates were incubated (37 °C, 48 h) in flushing jars under microaerophilic conditions, created by evacuating 80\% of the normal atmosphere and introducing a gas mixture of 84\% N\textsubscript{2}, 8\% H\textsubscript{2} and 8\% CO\textsubscript{2}. The bacteria were harvested and the final concentration was adjusted to an optical density of 1.0 at 660 nm corresponding to approximately $10^8$ cfu/ml (Corthésy-Theulaz et al., 1995).

\textit{Experimental procedure}

Ten animals were inoculated three times (with 48 h time interval) with 0.4 ml of an \textit{H. felis} ATCC 49179 suspension. The same procedure was conducted with \textit{H. bizzozeronii} CCUG 35545 and \textit{H. salomonis} CCUG 37845, except that five animals were inoculated with each bacterial strain. Inoculation was performed intragastrically using a ball-tipped gavage needle.
Three gerbils were inoculated with sterile culture medium and served as uninfected controls. All the animals were killed 3 weeks after the first inoculation using isoflurane anaesthesia followed by cervical dislocation. The stomach was resected and samples were taken for polymerase chain reaction (PCR) analysis and histological examination as described below.

**PCR analysis**

Samples of fundus and antrum (ca. 4 mm²) from controls, *H. felis, H. bizzozeronii* and *H. salomonis* inoculated animals were taken for DNA extraction (DNeasy Tissue Kit, Qiagen, Hilden, Germany) and multiplex PCR analysis as described elsewhere (Baele et al., 2004). This PCR test allows the discrimination between *H. felis, H. bizzozeronii* and *H. salomonis*, based on the transfer RNA intergenic spacers of *Helicobacter* species and the urease gene of *H. felis* (Table 1). Briefly, PCR reactions were performed in a volume of 10 μl containing a final primer concentration of 0.1 μM of each of the fluorescently (TET™, NED™, HEX™; Applied Biosystems, Foster City, USA) labelled oligonucleotides, 0.1 μM of each of the unlabelled primers, 40 μM of each deoxynucleoside triphosphate (Amersham Pharmacia Biotech, Little Chalfont, England), 3 mM MgCl₂, Polymerase Taq platinum (Invitrogen Life Technologies, Carlsbad, USA) 0.03 U/μl, and 1x PCR buffer (Invitrogen Life Technologies). Two microlitres of template DNA were added to the vials. An initial denaturation of 5 min at 95 °C was followed by three cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C, and subsequently 35 cycles with the following conditions: 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C, with a final extension at 72 °C for 7 min.

The PCR products were analysed by capillary electrophoresis on 1 μl of PCR product. Samples were identified as *H. felis* when a TET-labelled fragment of 137 bp and a NED-labelled fragment of 434 bp were obtained. Samples were identified as *H. bizzozeronii* when a TET-labelled amplicon of 136 bp and a HEX-labelled fragment of 373 bp were obtained, whereas the presence of only a TET-labelled amplicon of 134 bp resulted in *H. salomonis* identification.

**Histological examination**

A longitudinal strip of tissue from the oesophagus to the duodenum was fixed in 10% phosphate buffered formalin, processed by standard methods, and embedded in paraffin. Seven consecutive 5 μm sections were cut and stained with the following techniques. One section was stained with haematoxylin and eosin (H&E), one with Giemsa and one with periodic acid schiff (PAS). Immunohistochemical staining of the *Helicobacter* bacteria was
Table 1. Oligonucleotide primers used in the multiplex PCR.

<table>
<thead>
<tr>
<th>Primer and label</th>
<th>Sequence</th>
<th>Source or reference (nt positions)</th>
<th>Target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3B (TET)</td>
<td>5′ AGG TCG CGG GTT CGA ATC C</td>
<td>Welsh and McClelland (1991)</td>
<td>tRNA genes</td>
</tr>
<tr>
<td>HT135R-tail</td>
<td>5′ tail- ACC AAC TGG GCT AAG CGA CC</td>
<td>De Groote et al. (2000)</td>
<td>tRNA genes</td>
</tr>
<tr>
<td>Bi1F (HEX)</td>
<td>5′ AAC CAA YAG CCC CAG CAG CC</td>
<td>H. felis urease, nt 936 – 955</td>
<td>Urease gene H. bizzozeronii</td>
</tr>
<tr>
<td>Bi2R</td>
<td>5′ TGG TTT TAA GGT TCC AGC GC</td>
<td>H. felis urease, nt 1309 – 1290</td>
<td>Urease gene H. bizzozeronii</td>
</tr>
<tr>
<td>Fe1F (NED)</td>
<td>5′ TTT GGT GCT CAC TAA CGC CCT C</td>
<td>H. felis urease, nt 966 – 987</td>
<td>Urease gene H. felis</td>
</tr>
<tr>
<td>Fe3R</td>
<td>5′ TTC AAT CTG ATC GCG TAA AG</td>
<td>H. felis urease, nt 1403 – 1382</td>
<td>Urease gene H. felis</td>
</tr>
</tbody>
</table>

*Nucleotide (nt) positions are based on H. felis urease X69080*
done on the next section using a polyclonal genus specific rabbit anti-\textit{H. pylori} antibody (1/320, overnight, 21 °C; Dakocytomation Denmark A/S, Glostrup, Denmark) followed by a biotinylated goat anti-rabbit IgG antibody (1/500, 30 min, 21 °C) and peroxidase streptavidin complex (Dakocytomation Denmark A/S) (De Groote \textit{et al.}, 2000). Apoptosis was visualised on the following section using immunohistochemical staining for activated caspase-3 (R&D Systems Europe Ltd, Oxon, UK) as described by Van Cruchten \textit{et al.} (2003). On the sixth section, replicating cells were shown by immunohistochemical staining for Ki67 using a mouse monoclonal anti-Ki67 antibody (1/50, overnight, 21 °C; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). PCs were identified on the last section by immunohistochemical staining using a mouse monoclonal antibody specific for the hydrogen potassium ATPase (1/200, 1 h, 21 °C; Abcam Ltd, Cambridge, UK) (Suzuki \textit{et al.}, 2005; Cai \textit{et al.}, 2005). Primary antibodies for Ki67 and for hydrogen potassium ATPase were followed by a biotinylated goat anti-mouse IgG antibody (1/200, 30 min, 21 °C; Dakocytomation Denmark A/S). Slides were developed with diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St-Louis, USA) and H$_2$O$_2$ and counterstained with haematoxylin.

\textbf{Results}

The control animals and the \textit{H. salomonis} infected gerbils were negative in PCR. No bacteria and no inflammatory changes of any kind were detected in the histological sections of these animals. The gastric samples of all the gerbils inoculated with \textit{H. felis} and \textit{H. bizzozeronii} yielded positive results in PCR.

In the H&E stained sections from \textit{H. felis} and \textit{H. bizzozeronii} infected gerbils, the inflammation mainly consisted of lymphocytic and neutrophilic infiltrates located in the mucosa/submucosa of the antrum and in the forestomach-stomach transition zone (i.e. limiting ridge). \textit{H. felis} consistently induced a more pronounced cellular reaction (sometimes present as lymphoid follicles) than \textit{H. bizzozeronii} both at the limiting ridge and in the antrum. Extensive loss of PCs was consistently seen on the H$^+$-K$^+$-ATPase stained sections in \textit{H. felis} infected gerbils. This was not noticed in the control, uninfected gerbils. The PC loss extended from the limiting ridge into the fundus. This loss of PCs was also present in three out of five \textit{H. bizzozeronii} inoculated gerbils but was less extensive (Fig. 1). In the area where gastric epithelial cells were lost, replacement by glands lined with mucous cells sometimes occurred (Fig. 2). In the serial sections, stained for apoptosis, a band of apoptotic cells was found at the transition between the zone of complete PC loss and the zone with normal PCs.
(Fig. 3A, Fig. 4A). This was the case in all *H. felis* infected gerbils and in the three *H. bizzozeronii* infected gerbils showing PC loss. Staining for *Helicobacter* bacteria showed bacteria mainly in the antrum. In the fundus, high numbers of bacteria were exclusively found in the transition zone of normal/affected PCs (Fig. 5) and therefore also in the region with the apoptotic cells. The bacteria were also seen in close proximity to PCs (Fig. 3B, Fig. 4B). Ki67 staining showed intense cell proliferation in the area devoid of gastric epithelial cells at all levels of the mucosa whereas cell proliferation in control animals was limited to a narrow proliferative zone at the neck of the glands (Fig. 6). There was also a high density of proliferating cells at all mucosal levels of the antrum.

**Discussion**

In this study, gastric colonization was for the first time successfully achieved with *H. bizzozeronii* in Mongolian gerbils. The attempt to colonize gerbils with *H. salomonis* was unsuccessful.

The inflammatory changes at three weeks after *H. felis* inoculation confirm the results of Court *et al.* (2002). In addition, we found loss of PCs and a striking spatial association of *Helicobacter* bacteria and apoptotic cells. *H. bizzozeronii* infection was also associated with gastric inflammation, apoptosis and a loss of PCs at three weeks p.i. but to a much lesser extent than that seen in *H. felis* infection. In the fundus, no bacteria were seen except at the transition zone between affected and normal PCs in both *H. felis* and *H. bizzozeronii*-infected gerbils. The spatial association of the bacteria with the apoptotic cells suggests that the bacteria directly or indirectly induce the loss of PCs. It is unclear whether the extensive PC loss is exclusively due to apoptosis. Probably secondary necrosis may also play a role. In a study conducted by Xia *et al.* (2000) the term “antralization” was proposed to describe the loss of PCs and the presence of antral-type mucosa in the fundus in humans infected with *H. pylori*. This “antralization” could also be noticed in the *H. felis* and *H. bizzozeronii* infected gerbils. In this respect *H. felis* infection in Mongolian gerbils may be a valuable model for the study of *H. pylori* induced “antralization” in the human stomach.

Several hypotheses may be proposed to explain this striking loss of PCs. The virulence factors associated with gastric epithelial cell damage have been studied thoroughly with *H. pylori*. The intact *cag* pathogenicity island (*cag* PAI) in *H. pylori* is associated with the development of gastric pathology (Nilsson *et al.*, 2003). The *cagA* gene is part of this *cag* PAI and plays a key role in disease pathogenesis together with *vacA*, the latter producing a
vacuolating cytotoxin. In a study conducted by Peek et al. (2000) however, it was shown that neither cagA nor vacA are required for induction of inflammation in the gerbil model. Another gerbil study examined the inflammatory virulence of cagE, also a member of the cag PAI, indicating a causative role of H. pylori CagE protein in the pathogenesis of inflamed gerbil gastric mucosa (Akanuma et al., 2002; Ogura et al., 2000). It has been clearly shown that H. pylori infection induces cell apoptosis (Wagner et al., 1997) and stimulates cell proliferation in the gastric epithelium (Fan et al., 1996). Most in vitro and in vivo studies however, have shown no association between CagA expression and apoptosis. The underlying mechanisms by which H. pylori alters cell turnover thus remain poorly understood (Xia and Talley, 2001).

Gene products of the cag PAI are also involved in activation of IL-8 gene expression in gastric epithelial cells. IL-8 attracts granulocytes and monocytes, leading to release of products that affect cell death. The virulence factors of non-H. pylori Helicobacter species leading to gastric epithelial damage are unknown. Using PCR primers developed for H. pylori, neither the cag PAI nor the vacA gene could be amplified from H. felis ATCC 49179 (Xia and Talley, 2001). Using the same primersets as Xiang et al. (1995), we did not obtain amplicons with H. bizzozeronii either (data not shown). Due to the high specificity of the H. pylori PCR assay used, however, we cannot exclude that cagA and vacA homologues are present in these Helicobacter species.

Another bacterial mechanism that could contribute to Helicobacter pathogenicity is molecular mimicry. Molecular mimicry signifies that if the colonizing strain expresses an epitope on its surface (e.g. Lewis x) that is shared by the host PCs, the host could be at an increased risk for developing crossreactive immunopathology and immune mediated PC loss (Falk et al., 2000). H. felis and H. bizzozeronii however do not exhibit molecular mimicry (Moran et al., 1999; Hynes et al., 2004).

There is a remarkable tendency in the literature to suggest that non-H. pylori helicobacters do not cause gastric PC damage, although H. felis and H. bizzozeronii have been observed in the PC canaliculi of cats and dogs (Peyrol et al., 1998). It has been suggested that H. felis can switch off acid secretion by producing an acid-inhibitory protein resulting in an increase in pH of the canaliculus without causing further damage to the PC (Vargas et al., 1991). Degenerative changes in canine PCs have, however, been reported, but it is uncertain as to whether Helicobacter species colonize degenerating PCs or induce their degeneration (Happonen et al., 1998). Furthermore it has been stated that H. felis almost never adheres to epithelial cells (Taylor et al., 1992).
The loss of PCs observed in the present study in association with both *H. felis* and *H. bizzozeronii* could be the result of cytokines released by activated inflammatory cells. Another possible explanation is that this could be due to an as yet unknown virulence factor of *H. felis* and *H. bizzozeronii*. The loss of gastric glands and the subsequent mucous metaplasia may lead to ‘functional atrophy’ followed by hypochlorhydria. The presence of bacteria in the transition zone comprising normal/affected PCs could indicate that this is the location corresponding to their most favourable pH.

Based on the observations seen at three weeks p.i., long term *H. felis* and *H. bizzozeronii* infection could lead to total functional depletion of the gastric glands, favouring the bacterial colonisation of the whole fundic area. The elevated gastric epithelial cell proliferation, as a reaction to this functional cell loss, could eventually result in the development of gastric cancer. Long-term infection studies with *H. felis* and *H. bizzozeronii* in Mongolian gerbils are in progress to test this hypothesis.
References


Fig. 1 H⁺-K⁺-ATPase staining of (A) a control gerbil, indicating differential staining between purple chief cells and brown stained PCs (Bar insert = 50 μm). (B) Gerbil infected with *H. felis* ATCC 49179 with severe PC loss and “antralisation” (arrow). (C) Section of an *H. bizzozeronii* CCUG 35545 infected gerbil with PC loss near the limiting ridge, though less severe than that seen in the *H. felis* ATCC 49179 infected gerbil. Bar = 100 μm.
**Fig. 2** PAS stained section of (A) a control, uninfected gerbil and (B) a serial section of the in fig. 1B shown *H. felis* ATCC 49179 infected gerbil showing “antralization”. Bar = 100 μm.
Fig. 3 Two serial longitudinal sections of an *H. felis* ATCC 49179 infected gerbil showing (A) apoptosis visualized by staining activated Caspase-3 and (B) *Helicobacter* bacteria stained immunohistochemically with anti-*H. pylori* antibody. The highest apoptotic cell density and bacteria were found in the transition zone between the area of PC loss (L) and normal (N) PCs (arrows). Bar = 100 μm. Notice the proximity of bacteria to PCs (insert). Bar = 10 μm.
Fig. 4 Two serial cross-sections of an \textit{H. felis} ATCC 49179 infected gerbil showing (A) apoptosis visualized by staining activated Caspase-3 and (B) \textit{Helicobacter} bacteria stained immunohistochemically with anti-\textit{H. pylori} antibody. The highest apoptotic cell density and bacteria were found in the transition zone between the area of PC loss (L) and normal (N) PCs (arrows). Bar = 100 \textmu m. Notice the proximity of bacteria to PCs (insert). Bar = 10 \textmu m.
Fig. 5 Serial sections of an *H. felis* ATCC 49179 infected gerbil showing (A) PCs loss with H⁺-K⁺-ATPase staining and (B) *Helicobacter* bacteria immunohistochemically stained with anti-*H. pylori* antibody. Notice the bacteria being present in the transition zone normal/affected PCs. Bar = 100 μm.
Fig. 6 Ki67 staining of (A) a control, uninfected gerbil and (B) an *H. felis* ATCC 49179 infected gerbil. There is an increased proliferation rate in the area of gastric epithelial cell loss. Bar = 100 μm.
Chapter 3.2

The pathogenic effect of *Helicobacter felis* and *Helicobacter bizzozeronii* on the gastric mucosa in mongolian gerbils: a sequential pathological study


*Adapted from Journal of Comparative Pathology, In Press*
Summary

In contrast to *H. pylori*, little is known about the pathogenic mechanisms of gastric non-*H. pylori* *Helicobacter* species. Nevertheless the *H. felis* animal model is often used to study various aspects of gastric *Helicobacter* infections. This study in Mongolian gerbils was performed to obtain a greater insight into bacteria-host interactions in *H. felis* and *H. bizzozeronii* infections. The animals were inoculated with *H. felis* or *H. bizzozeronii* and sacrificed at different timepoints p.i.. Stomach tissue was taken for light and transmission electron microscopy (TEM) and polymerase chain reaction (PCR). Parietal cells (PCs), apoptosis, cell proliferation and nuclear factor-κB (NF-κB) activation were visualised immunohistochemically. From day eleven p.i. onwards, *H. felis* inoculated animals showed inflammation consisted of neutrophilic granulocytes mainly in the antrum and lymphocytic infiltrates around the limiting ridge (LR) and throughout the stomach mucosa and submucosa. Moderate to severe loss of PCs extending from the limiting ridge into the fundus was seen. A front of apoptotic cells, spiral bacteria, cell proliferation, and NF-κB activation were seen at the transition zone between affected and normal PCs. TEM revealed the interaction of *H. felis* flagellae and fibrils with PCs and chief cells. Moreover, *H. felis* was seen in proximity and inside necrotic cells. At 10 weeks p.i., some *H. felis* infected gerbils had completely lost their fundic glands and showed mucous metaplasia of the epithelium. *H. bizzozeronii* made no flagellar contact with epithelial cells. It was associated with only mild PC loss. The mechanism by which *H. felis* induces PC necrosis and apoptosis remains unclear. The observed flagellar contact and NF-κB activation could play an important role in *H. felis*-associated inflammation.
CHAPTER 3.2

Introduction

*H. felis* and *H. bizzozeronii* are phylogenetically closely related to the human gastric pathogen *H. pylori*, yet have a number of characteristics that distinguish them from the latter. Their morphology is different from *H. pylori* but similar to that of “*H. heilmannii*”, which occasionally can be found in the human stomach and has been implicated as a cause of gastritis and mucosa-associated lymphoid tissue (MALT) lymphoma (Morgner *et al*., 2000). It is now clear that what was originally named “*H. heilmannii*” does not represent a single species. It rather reflects a species complex comprised of “*Candidatus H. suis*” (De Groote *et al*., 1999), found in the stomach of pigs and referred to as “*H. heilmannii*” type 1, and the spiral organisms found in the gastric mucosa of cats and dogs that are referred to as “*H. heilmannii*” type 2 i.e. *H. felis*, *H. bizzozeronii*, *H. salomonis* (Jalava *et al*., 1997) and the recently described “*Candidatus H. heilmannii*” (O’Rourke *et al*., 2004) and “*H. cynogastricus*” (Van den Bulck *et al*., In Press). “*H. heilmannii*” infections in humans have been postulated to be a zoonosis with various animal species serving as reservoirs (Yoshimura *et al*., 2002; Sykora *et al*., 2003; van Loon *et al*., 2003). Recently, human gastric biopsy samples were subjected to a multiplex polymerase chain reaction (PCR), allowing the identification of *H. felis*, *H. bizzozeronii*, *H. salomonis* and “*Candidatus H. suis*”. “*Candidatus H. suis*” (36.6%) and *H. salomonis* (21.2%) were found to be the most prevalent non-*H. pylori* Helicobacter species while *H. felis* and *H. bizzozeronii* were identified in 14.6% and 4% of the samples respectively (Van den Bulck *et al*., 2005).

In contrast to *H. pylori*, very little is known about the virulence and pathogenic mechanisms of these gastric so-called non-*H. pylori* Helicobacter species. In *H. felis*, only a few virulence genes have been characterized (Josenhans *et al*., 1999). Nevertheless, the murine *H. felis* model has been used widely to study various aspects of gastric Helicobacter infections such as pathogenesis, antibiotic treatment and vaccine development. One may question to what extent the mechanisms by which *H. pylori* causes gastric lesions are comparable to those of *H. felis*.

Approximately a decade ago, the Mongolian gerbil was introduced in *H. pylori* research (Hirayama *et al*., 1996). Severe pathologies are seen in the *H. pylori*-infected Mongolian gerbil with ulcers being formed in most animals (Matsumoto *et al*., 1997). Using this animal model, it was for the first time possible to provide evidence that *H. pylori* infection alone could induce tumour formation (Watanabe *et al*., 1998). Regarding *H. felis*, Court *et al.* (2002) were the first to report the successful infection of gerbils with *H. felis*. 

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We recently conducted a preliminary study on the gastric pathology induced by *H. felis* and *H. bizzozeronii* in the gerbil model (De Bock *et al.*, 2006). Our results showed loss of PCs near the cardia after 3 weeks of infection, especially in the *H. felis* infected gerbils. The aim of the present study was to investigate at different time points after experimental infection, the differential pathogenic effect of *H. felis* and *H. bizzozeronii* on the gastric mucosa of the Mongolian gerbil in order to gain more insight into the pathogenic mechanisms leading to inflammation.

**Material and Methods**

**Animals**

Sixty-five specific pathogen-free (SPF) female gerbils (Elevage Janvier, Le Genest St Isle, France), aged six weeks, were barrier-maintained in a room with controlled environment and housed in pairs in filter-top cages on autoclaved (121 °C, 15 min) wood shavings. They were fed an autoclaved diet containing 18% protein (Teklad Global Rodent Diet, Harlan NL, Horst, the Netherlands) and received autoclaved water ad libitum. The experimental protocol was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

**Bacterial Strains**

The bacterial strains used in this study were *H. bizzozeronii* CCUG 35545 (kindly provided by P. Vandamme) and *H. felis* ATCC 49179 (kindly provided by R. Ferrero). They were grown on brain-heart infusion (BHI; Oxoid ltd, Basingstoke, UK) agar to which Skirrow supplement (Oxoid Ltd), vitamins (Vitox®, Oxoid Ltd), amphotericin B (Fungizone®, Bristol-Myers Squibb, New York, USA) and 10% horse blood were added. The plates were incubated (37 °C, 48h) in flushing jars under microaerophilic conditions, created by evacuating 80% of the normal atmosphere and introducing a gas mixture of 84% N₂, 8% H₂ and 8% CO₂. The bacteria were harvested and suspended in BHI broth. The final concentration was adjusted to an optical density of 1.0 at 660 nm, corresponding to approximately $10^8$ colony forming units (cfu) /ml (Corthésy-Theulaz *et al.*, 1995).

**Experimental Protocol**

The animals were divided in two groups of 25 animals each for the inoculation with *H. felis* and *H. bizzozeronii* respectively, and one group of 15 animals that served as controls.
The animals were inoculated three times (at 48 hours intervals) with 0.4 ml bacterial suspension. The control group was inoculated with BHI broth. Inoculation was performed intragastrically, under isoflurane (Isoflo®, Abbot, Illinois) anaesthesia, using a ball-tipped gavage needle. At 7, 11, 21, 35 and 70 days after the first inoculation, five *H. felis* infected animals, five *H. bizzozeronii* infected animals and three control animals were killed by cervical dislocation following isoflurane anaesthesia. The stomach of each gerbil was resected and samples were taken for PCR analysis, histopathological and ultrastructural examination as described below.

**PCR Analysis**

From each gerbil, samples of the gastric fundus and antrum (ca. 4 mm²) were taken for DNA extraction (DNeasy Tissue Kit, Qiagen, Hilden, Germany) and multiplex PCR analysis as described elsewhere (Baele et al., 2004). This PCR test allows the discrimination between *H. felis* and *H. bizzozeronii* based on the transfer RNA intergenic spacers of *Helicobacter* species and the urease gene of *H. felis*.

**Histological and Ultrastructural Examination**

Two longitudinal strips of tissue from the oesophagus to the duodenum along the greater curve of the stomach were cut. The different stomach regions *i.e.* cardia, antrum and fundus were examined, with special interest in the regions where *Helicobacter* bacteria were seen.

One strip was examined macroscopically before being fixed in 10% phosphate buffered formalin, processed by standard methods, and embedded in paraffin wax for light microscopy. Eight consecutive 5 μm sections were cut. The first section was stained with haematoxylin and eosin (H&E), the second one with Giemsa stain and the third with periodic acid Schiff (PAS). H&E-stained sections were examined to score the intensity of gastric inflammation (infiltration of mononuclear cells and neutrophils) and mucous metaplasia according to the Updated Sydney System (Dixon et al., 1996). Immunohistochemical staining of *Helicobacter* bacteria was done on the fourth section using a polyclonal genus specific rabbit anti-*H. pylori* antibody (1/320, overnight, 21 °C; Dakocytomation Denmark A/S, Glostrup, Denmark) (De Groote et al., 2000). Apoptosis was visualised on the fifth section using immunohistochemical staining for activated caspase-3 (R&D Systems Europe Ltd, Oxon, UK) as described by Van Cruchten et al. (2003). On the sixth section, replicating cells were identified by immunohistochemical staining for Ki67 using a mouse monoclonal anti-Ki67 antibody (1/50, overnight, 21 °C; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK).
PCs were identified on the seventh section by immunohistochemical staining using a mouse monoclonal antibody directed against the beta-subunit of hydrogen potassium ATPase (1/200, 1h, 21°C; Abcam Ltd, Cambridge, UK) (Suzuki et al., 2005). On section number eight, nuclear factor-κB (NF-κB) activation was visualised using a rabbit polyclonal anti-NF-κB p65 antibody (1/500, overnight, 4 °C; Santa Cruz Biotechnology, Santa Cruz, USA) (Isomoto et al., 2000). Primary antibodies for Ki67 and for hydrogen potassium ATPase were followed by a biotinylated goat anti-mouse IgG antibody (1/200, 30 min, 21°C; Dakocyтомation Denmark A/S). The primary antibodies for H. pylori and NF-κB p65 were followed by a biotinylated goat anti-rabbit IgG antibody (1/500, 30 min, 21 °C; Dakocyтомation Denmark A/S). After rinsing, the sections were incubated with avidin-biotin-peroxidase complex and the colour was developed in diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St-Louis, USA) and H2O2. Nuclei were counterstained with haematoxylin.

The second longitudinal tissue strip was used for Transmission Electron Microscopy (TEM) to study the interaction of the bacteria with the gastric mucosa. After dividing the longitudinal strip into 2 mm² tissue pieces with a razor blade, the numbered samples were fixed in formaldehyde 4% containing 1% CaCl2 (w/v) in 0.121 M Na-cacodylate adjusted to pH 7. The samples were washed and postfixed in 1% OsO4. After osmication, the samples were dehydrated in graded ethanol solutions, infiltrated and embedded in LX112 resin (Ladd Research, Burlington, USA). Semithin sections (2 μm) were cut and stained with toluidine blue. Finally, selected regions were chosen for ultrathin sectioning (90 nm) using an ultratome (Ultracut E, Reichert-Jung). The sections were stained with uranyl acetate and lead citrate solutions before examining under a Jeol EX II transmission electron microscope at 80 kV.

Data analysis

The overall inflammation scores were compared between H. felis and H. bizzozeronii by a proportional odds model with cumulative logits incorporating time, bacterial species and their interaction as categorical fixed effects. All timepoints (11, 21, 35 and 70 days p.i.) were compared to 7 days p.i., adjusting the global significance level of 0.05 by Bonferroni’s multiple comparisons technique to the significance level of 0.0125.
Results

Macroscopic Appearance

The gastric mucosa of all non-infected gerbils and of those infected for 7 and 11 days showed no visible changes (Fig. 1A). At 21 days p.i., the reddish fundic mucosa of two *H. felis* infected gerbils was altered close to the forestomach, being replaced by a paler mucosa. At 35 days, this was visible in three out of five *H. felis* inoculated animals (Fig. 1C) but in none of the *H. bizzozeronii* inoculated gerbils (Fig. 1B). At 70 days p.i., three out of five *H. felis* inoculated animals showed this pale mucosa throughout the fundic region. The stomach tissue of *H. bizzozeronii* inoculated gerbils had still no visible changes at 70 days (Fig. 1D).

Bacterial colonization

Giemsa staining, immunohistochemical staining and PCR revealed no detectable *Helicobacter* organisms in control animals throughout the entire experiment. The colonization level of the infected animals is shown in table 1. Antral biopsies were more often PCR positive than fundus tissues. At 7 days p.i., PCR of the antrum revealed positive results for all inoculated animals although few animals were *Helicobacter* positive by Giemsa staining. From day 11 through day 35, all *H. felis* and *H. bizzozeronii* infected animals were positive in the fundus and/or antrum by PCR and by staining, or by PCR alone. At day 70, two *H. felis* inoculated gerbils gave negative results in PCR and in histology. In the other three animals, only very few bacteria could be seen histologically.
Table 1 Colonization of the gastric mucosa of control, *H. felis* and *H. bizzozeronii* infected Mongolian gerbils determined by Giemsa staining, anti-*H. pylori* antibody immunohistochemistry and multiplex PCR. [No of positive animals /total No of animals]

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Note. C = control animals, F = *H. felis* inoculated animals, B = *H. bizzozeronii* inoculated animals; Giemsa: detection of bacteria in Giemsa stained sections; anti-HP: detection of bacteria in anti-*H. pylori* antibody stained sections.

**Histology**

At all time points, the gastric mucosal specimens from control gerbils had normal histomorphology. Ki67 positive stained cells were limited to a narrow proliferative zone at the neck of the glands. PAS staining revealed normal distribution of mucus epithelial cells in the fundus and antrum. Some scattered caspase-3 positive cells were noticed in the fundus and antrum. No nuclear staining was seen corresponding with NF-κB activation.

The histological findings of gastric mucosal specimens in *H. felis* and *H. bizzozeronii*-infected gerbils are shown in Table 2. The overall inflammation scores varied significantly over time (*P*<0.0001) and also differed significantly between *H. felis* and *H. bizzozeronii* (odds ratio = 0.01, *P*<0.0001). The interaction between time and bacterial species was not significant (*P* = 0.53). All timepoints, except 11 days p.i., differed significantly from 7 days p.i.. The same significant results were obtained for the fundus and antrum inflammation scores.
At 7 days p.i., four out of five *H. felis* inoculated gerbils showed very mild lymphocytic infiltration around the limiting ridge (LR) i.e. the transition zone between the squamous epithelium of the forestomach and the glandular stomach (Fig. 2A). In that area, one of these four animals showed mild loss of PCs (Fig. 2B). Bacteria were present in the same region (Fig. 2C). Inflammation was not detected in *H. bizzozeronii* inoculated gerbils before day 21 p.i.

At day 11, PC loss and more pronounced infiltration of lymphoid cells in the cardiac region (Fig. 3) and neutrophilic infiltration in the antrum were noted in *H. felis* infected gerbils.

At twenty-one days p.i. (Fig. 4), the PC loss was seen in four out of five *H. felis* infected gerbils, with one animal showing PC loss that extended much further than seen at 11 days from the LR into the fundus. Also NF-κB activation, apoptosis, extensive cell proliferation (at all levels of the mucosa) and mucous metaplasia were noticed in the region of PC loss. At this time point, *H. bizzozeronii* infected animals showed mainly moderate inflammation in fundus and antrum. Only one of these gerbils had mild PC loss around the LR and NF-κB activation in the transition zone normal/damaged PCs.

At day 35 p.i., many polymorphonuclear cells were scattered throughout the mucosa and submucosa of fundus and antrum in all *H. felis* inoculated gerbils, occasionally forming mucosal microabscesses. Lymphoid follicles were especially conspicuous in the submucosa, but they were also found in the deep portion of the propria mucosae (Fig. 5). The majority of these animals showed more extensive PC loss than seen in other animals at previous timepoints and four had replacement of gastric epithelial cells by elongated mucin producing glands (mucous metaplasia). NF-κB activation was present in the area of PC loss. One animal had complete loss of PCs throughout the fundus. One *H. bizzozeronii* inoculated gerbil showed mild lymphocytic infiltrations at the LR and all five *H. bizzozeronii* inoculated gerbils had mild neutrophilic cell infiltration in the antral mucosa.

Seventy days p.i in *H. felis* inoculated gerbils, three out of five animals lost almost all PCs throughout the fundus and showed replacement by mucous glands (Fig. 6). Intense neutrophilic infiltration was present throughout the stomach mucosa. Two animals showed mainly submucosal infiltration of lymphocytes with mild or no PC loss. No NF-κB activation was found at this timepoint in any of the five animals examined. In the antrum of most *H. bizzozeronii* inoculated gerbils, mild to moderate infiltration of neutrophils and lymphocytes was seen. One animal had PC loss accompanied with apoptosis and increased cell proliferation.
Transmission Electron Microscopy

In the gastric mucosa of *H. felis* inoculated gerbils, the spiral shaped bacteria could be easily distinguished by the presence of periplasmic fibrils encasing the bacterium (Fig. 7D). To examine the bacteria-host interaction, stomach tissue samples taken at day 21 p.i. were used as in these samples the bacteria were clearly found in the transitional zone between normal and damaged PCs. In this zone, the bipolar flagellae and the periplasmic fibrils of the bacteria were seen to be closely positioned against parietal and chief cell microvilli (Fig. 7, Fig. 8). The ultrastructure of the flagella of *H. felis* (internal filament surrounded by a sheath) was clear on cross sections (Fig. 8 insert). *H. felis* was frequently observed in the proximity of PCs that showed damage to the plasma membrane or necrotic changes of the organelles, and in close association with cellular debris (Fig. 7D). Apoptotic PCs were rarely seen and almost no bacteria were found in their vicinity. Neutrophilic granulocytes and lymphocytes were abundant, and some neutrophilic granulocytes contained phagocytosed *Helicobacter* bacteria in their cytoplasm.

TEM of *H. bizzozeronii* inoculated gerbils at day 21 p.i. showed no presence of necrotic PCs nor bacterial flagellae adhering to the host (Fig 9).
Table 2. Histological findings in the gastric mucosa of control, *H. felis* and *H. bizzozeronii* infected Mongolian gerbils. [No of animals with histological changes/total No of animals]

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<th>Presence mucous metaplasia</th>
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Note. C = control animals, F = *H. felis* inoculated animals, B = *H. bizzozeronii* inoculated animals; n = number of animals per group; 0* = none, 1* = mild, 2* = moderate, 3* = severe
Discussion

In the present study, we confirmed the loss of PCs observed in our previous short-term experiment in which Mongolian gerbils were inoculated with *H. felis* and *H. bizzozeronii* and euthanised three weeks p.i. (De Bock et al., 2006). By euthanizing gerbils at different timepoints after inoculation, a greater insight was obtained in the present study regarding the kinetics of *H. felis* and *H. bizzozeronii* induced gastric mucosal damage. Moreover, TEM revealed details of the interactions of the bacteria with the host cells. At 7 days p.i., we observed the first mild signs of inflammation around the LR in *H. felis*-infected animals. There was also PC loss and presence of few bacteria. At the subsequent timepoints in *H. felis* inoculated gerbils, inflammation became more severe in the fundus and especially in the antrum. More PCs were lost and mucous metaplasia was noticed. At 70 days after *H. felis* inoculation, almost total loss of the functional fundic mucosa was observed in some animals but not in others, indicating individual host differences. One gerbil had normal gastric histomorphology and was *H. felis* negative. One animal showed almost no PC loss but was indeed *H. felis* positive in PCR. Conversely, another animal had severe gastritis and PC loss but was found to be *H. felis* negative in PCR. Loss of PCs may lead to hypochlorhydria. At this timepoint only few bacteria were observed in histological sections suggesting at least partial elimination of the bacteria. The same has been described for human *H. pylori* gastritis where the bacteria seem to disappear due to hypochlorhydria (Clyne et al., 1995). Mucous metaplasia in *H. felis* infected gerbils was already seen in two gerbils at 3 weeks p.i. This has also been described in *H. pylori* infected gerbils from week 4 p.i. onwards (Ikeno et al., 1999). Mucous metaplasia is generally regarded as a condition that predisposes to malignancy. *H. felis* infection of Mongolian gerbils for more than the 10 weeks of the present study merits further investigation. Also, the individual host differences observed after 70 days p.i. need further study in order to understand the type of host response leading to protective immunity versus the type of response leading to further damage. Although mild fundic and moderate antral inflammation, together with PC loss, were seen in *H. bizzozeronii* infected gerbils, this bacterium seems to be far less pathogenic than *H. felis* in the Mongolian gerbil model. This corresponds with previous findings in the mouse model by De Bock et al. (2005).

Apoptotic loss of gastric epithelial cells was seen immunohistochemically, but TEM revealed a more important role for primary necrosis in parietal and chief cell loss. We found a remarkable tendency of *H. felis* to interact with the gerbil chief cells and PCs through its flagellae and periplasmic fibrils. The interacting structures that were seen on TEM were
indeed flagella and periplasmic fibrils, as their ultrastructure corresponds with the ultrastructural description (Peyrol et al., 1998). To date, periplasmic fibrils have only been found on two gastric Helicobacter species, namely H. felis and the recently described H. cynogastricus (Van den Bulck et al., In Press). It has been suggested that these fibrils are contractile proteins involved in motility but their exact function remains to be elucidated (Josenhans et al., 1999). Flagella have been reported to play a role in adherence in Salmonella infections (Allen-Vercoe and Woodward, 1999) and in one study on Campylobacter pathogenesis (Yao et al., 1994). H. felis adherence to gastric epithelial cells is rarely observed (Taylor et al., 1992) and in the present study, only loose contact between H. felis flagellar structures and epithelial cells was found. Peyrol et al., (1998) however, did find adhesion and intracellular processing of H. felis in both canine mucous and chief cells and observed host cell necrosis, as also seen in the present study. The mechanism by which H. felis induces necrotic and/or apoptotic cell loss is still unclear. It could concern a direct effect of the bacteria on the host cells or it could be mediated by attraction of inflammatory cells into the mucosa.

At 21 and 35 days p.i., the highest number of NF-κB positive nuclei was found. The positive nuclei were only present in the transition zone between normal and damaged gastric mucosa. In that same area H. felis was present. The position and morphology of the immunopositive cells suggested that these were PCs (Koyama et al., 2000). NF-κB plays a pivotal role in regulating genes involved in inflammation, cell proliferation, and apoptosis (Barnes, 1997; Barket and Gilmore, 1999). NF-κB contributes to the activation of proinflammatory cytokines, which attract monocytes and macrophages towards the site of bacterial colonization. H. pylori can activate NF-κB in gastric epithelial cells (Keates et al., 1997; Münzenmaier et al., 1997); this activation is cag pathogenicity island (cag PAI) dependent (Glocker et al., 1998; Foryst-Ludwig and Naumann, 2000). Hitherto there is no conclusive evidence for the presence of a cag PAI equivalent in H. felis. Our study is the first to demonstrate local and time-dependent NF-κB activation in PCs after H. felis infection. Smith et al. (2003) and Torok et al. (2005) reported that H. pylori flagellin can activate NF-κB via Toll-like receptor (TLR) 5 in epithelial cell lines. Schmauß er et al. (2004) confirmed TLR5 expression in gastric epithelium in vivo and thereby implied that TLR5 is involved in the inflammatory response to H. pylori. Recently, however, other conflicting reports were published demonstrating evasion of TLR5 by H. pylori flagellin (Lee et al., 2003; Gewirtz et al., 2004) and H. felis flagellin (Andersen-Nissen et al., 2005). In this study, we demonstrated contact of H. felis flagellae and periplasmic fibrils with gerbil gastric epithelial cells in the
same region as immunohistochemical NF-κB positive nuclei, possibly indicating that *H. felis* flagellin and fibril interaction with unknown epithelial cell receptors could result in NF-κB activation in PCs. The latter hypothesis is endorsed by the observation that *H. bizzozeronii* was found not to adhere to gastric epithelial cells and no NF-κB activation expression was seen except for 1 animal at 21 days. NF-κB activation however can also be induced by other mechanisms than bacterial adherence. Bacterial lipopolysaccharide can stimulate epithelial cells in rapid production of proinflammatory cytokines that can activate NF-κB (Naumann, 2000). *In vitro* studies may help to further elucidate the direct interactions of *H. felis* and *H. bizzozeronii* with parietal and other cells of the gastric epithelium and thus provide a basis to explain the apparent difference in virulence between these two *Helicobacter* species.
References


Figures

Fig. 1 Longitudinal tissue strips taken along the greater curve of the gerbil stomach. (A) Control. (B) 35 Days after *H. bizzozeronii* inoculation a normal gastric mucosa is seen. (C) 35 Days after *H. felis* inoculation. Replacement of the reddish fundic mucosa by a paler mucosa extending from the limiting ridge into the fundus is noticed (double arrow). (D) At 70 days after inoculation of *H. bizzozeronii* (Bi) still no gross changes are seen, while at this timepoint in *H. felis* (Fe) inoculated gerbils the reddish fundic mucosa has completely disappeared and is replaced by a paler mucosa. FS = forestomach, LR = limiting ridge (arrowheads), F = fundus, An = antrum
Fig. 2 Three serial longitudinal sections of the stomach of a seven days *H. felis* ATCC 49179 infected gerbil showing (A) very mild infiltration of lymphocytes (H&E stain) and (B) mild loss of PCs near the limiting ridge (hydrogen potassium ATPase staining). (C) *Helicobacter* bacteria (arrows) stained immunohistochemically with anti-*H. pylori* antibody. Bacteria are seen in the same region as PC loss. Bar A,B,C = 100 μm, bar insert C = 10 μm.
Fig. 3 H&E stained section of the stomach of an eleven days *H. felis* ATCC 49179 infected gerbil showing more pronounced lymphocytic infiltration. Bar = 100 μm.
Fig. 4 Three serial longitudinal sections of the stomach of a 21 days *H. felis* ATCC 49179 infected gerbil showing (A) infiltration of neutrophils and lymphocytes (H&E stain), (B) *Helicobacter* bacteria (arrows) stained immunohistochemically with anti-*H. pylori* antibody and (C) NF-κB activation (arrows: nuclear localisation) in the same region namely the transition zone normal/affected fundic mucosa. Before activation, NF-κB resides in the cytoplasm and must translocate to the nucleus to function. Immunopositive cells are represented with morphological features of PCs. Bars A,B,C = 100 μm, bar insert B = 10 μm, bar insert C = 50 μm.
Fig. 5 H&E stained section from the stomach of a gerbil at 35 days p.i. with *H. felis* ATCC 49179 showing the presence of a lymphoid follicle near the limiting ridge and submucosal infiltration of lymphocytes accompanied by loss of normal epithelial architecture. Severe infiltration of neutrophils was also found in the antrum (not shown). Bar = 100 μm.
Fig. 6 At seventy days p.i. with *H. felis* ATCC 49179, almost all functional PCs are lost. (A) Some hydrogen potassium ATPase stained remnants are still present (arrows). (B) Replacement of gastric epithelium by mucous glands is noticed on the PAS stained section. Bar A, B = 100 μm.
Fig. 7 Electron micrographs. (A) Multiple *H. felis* bacteria are seen, some of them making contact (arrows) with the PCs with their polar flagellae and at the site of the periplasmic fibrils. (B,C) Cross-section of *H. felis* in a PC canaliculus, showing contact of the flagella (B) and periplasmic fibrils (C) with the PCs’ microvilli (arrows). (D) *H. felis* surrounded by necrotic PC debris and a rare apoptotic cell. Periplasmic fibrils are indicated with arrowheads. Bars: A,C,D = 1 μm, B = 0.5 μm. PC = parietal cell, HF = *H. felis*. 
**Fig. 8** Electron micrographs. (A) *H. felis* flagellae make contact with chief cell microvilli (arrows). The insert shows the ultrastructure of the flagella on cross section. (B) Close contact of *H. felis* with cell surface microvilli (arrows). Bars A,B = 1 μm, bar insert A = 0.5 μm.

**Fig. 9** Electron micrograph of *H. bizzozeronii* indicating bacteria lying free in the gastric gland lumen without epithelial contact. Bar = 1 μm.
GENERAL DISCUSSION
Not much is known about the role of non-*H. pylori* helicobacters in human gastric pathology and the mechanisms by which these bacteria cause gastric disease. It was not until recently that identification of gastric non-*H. pylori* *Helicobacter* organisms to the species level was possible by adopting a multiplex PCR test enabling the simultaneous identification of *H. felis*, *H. bizzozeronii*, *H. salomonis* and “*Candidatus* H. suis” in gastric tissue (Baele et al., 2004). This test has revealed that *H. felis* and *H. bizzozeronii* are responsible for 11.3% of the “*H. heilmannii*” positive human gastric biopsies (Van den Bulck et al., 2005b). This fully justifies further research on their putative pathological effects on the gastric mucosa.

At the onset of our research, there was only anecdotal evidence that *H. felis* actually is pathogenic in humans (Germani et al., 1997). In chapter one we describe the association of *H. felis* infection with the presence of multiple gastric ulcers in a human patient. The concurrent *H. felis* infection in the patient’s dog urged us to suggest a zoonotic link. Zoonotic potential has been attributed to several animal helicobacters (Meining et al., 1998; Svec et al., 2000). Definite proof, however, is still lacking. The fact that other *Helicobacter* species (“*Candidatus* H. heilmannii” and *H. bizzozeronii*) were found in the stomach of the dog but not in the patient may imply differences in host susceptibility to specific *Helicobacter* species. These differences in host susceptibility to *Helicobacter* infections were also shown in our (chapter 2) and other mouse studies, in which mice strains with different immunological background were used (see below). Another explanation could be that there are differences between *Helicobacter* species in terms of infectivity: “*Candidatus* H. heilmannii” and *H. bizzozeronii* perhaps being less transmissible. *H. bizzozeronii* and “*Candidatus* H. heilmannii” have, however, been proven to be able to colonize the human stomach. The data in our study may hence reflect differences in strains beyond the species level.

In view of the above, we recommend that clinicians treating patients, which are diagnosed histologically ‘*H. heilmannii*’ positive, should inquire about possible close contact of their patients with pet animals. The above-cited multiplex PCR now allows the identification of *Helicobacter* isolates to the species level. In case the patient and his/her pet are infected with the same gastric *Helicobacter* species, one may propose treatment of the animal as well as of the patient.

Humans seem to be highly susceptible to *H. salomonis* infection, since *H. salomonis* is highly prevalent in human gastric biopsies (Van den Bulck et al., 2005b). This is rather peculiar, as this bacterial species is only rarely found in cats and dogs (Van den Bulck et al., 2005b). It is possible that another animal species acts as a reservoir host for these bacteria or
perhaps that man constitutes the natural host. These questions remain to be elucidated. Notwithstanding the fact that *H. salomonis* is commonly found in human biopsies, extensive research on this organism is lacking, due to its fastidious growth and the fact that to date no suitable animal model has been established to determine this organism’s putative pathogenic role. In the gerbil and mouse model described in this thesis, *H. salomonis* inoculation did not lead to colonization of the stomach (De Bock *et al.*, 2005; De Bock *et al.*, 2006). Because natural infection with *H. salomonis* has been found in the rabbit stomach as well (Van den Bulck *et al.*, 2005a), we have tried to experimentally infect rabbits with *H. salomonis* by intragastric inoculation but without success (unpublished results). Other cultivation methods for *H. salomonis* and/or pre-treatment of the animals with gastric acid inhibitors could be useful in further efforts to establish an *H. salomonis* animal model. Stoffel *et al.* (2000) pretreated mice with omeprazole 30 minutes prior to *H. salomonis* inoculation. Nevertheless, they did not succeed in establishing an *H. salomonis* infection in these mice. This could be due to the fact that proton pump inhibitors (such as omeprazole) have a slow onset of action and require several doses to achieve a maximum effect (Hatlebakk and Berstad, 1996). H2 receptor antagonists (such as cimetidine and ranitidine) (Adachi *et al.*, 2005) or sodium alginate (Dettmar *et al.*, 2006) lower the stomach acidity within a few hours after administration. The use of these latter products or a longer pretreatment protocol with proton pump inhibitors could have better effects on the establishment of an *H. salomonis* infection in experimental animal models.

In contrast to *H. pylori*, only few genes of *H. felis* have been characterized (Josenhans *et al.*, 1999) and to date no bacterial virulence mechanism responsible for *H. felis* pathogenicity in animal models has been described. Many *H. pylori* virulence components, such as the *cag* PAI and *vacA* (Xiang *et al.*, 1995), apparently are not present within the genome of *H. felis*. Primers designed for detection of *H. pylori* virulence genes have been tested on diverse strains of *H. felis*, *H. bizzozeronii* and *H. salomonis*. No amplicons were obtained for *vacA*, *babA2*, *babB*, *sabA*, *oipA*, *napA*, *hopZ*, *iceA* nor for the genes of the *cag* PAI (unpublished results and personal communication B. Flahou, 2006). Due to the high specificity of the *H. pylori* PCR assay used, however, we cannot exclude that *cag* PAI and other *H. pylori* gene homologues are present in these *Helicobacter* species and remain undetected in the above PCR. In the supposition that these virulence genes actually are missing in *H. felis*, it is intriguing that a bacterium belonging to the same genus as *H. pylori* and responsible for, to a large extent, similar gastric lesions, seems to differ considerably from *H. pylori* with respect
to its virulence genes. Moreover, research on *H. felis* LPS revealed a LPS structure significantly different from that of *H. pylori*, an observation which may have important implications when comparing the inflammation in *H. felis* animal models with that in *H. pylori* infected humans, as LPS has a significant immunomodulating activity (Hynes *et al.*, 2004).

*H. felis* is often used in animal models to study the pathogenesis of *Helicobacter* infections in general. In the early nineties, *H. felis* was even used as an alternative to experimental *H. pylori* infection in the mouse as *H. pylori* did not seem to colonize mice. After the establishment of the *H. pylori* mouse infection model in 1995, some researchers continued to use the rodent *H. felis* model, because gastric damage is more severe in mice infected with *H. felis* compared to infection with *H. pylori*. Nevertheless, as mentioned above, many *H. pylori* virulence factors have not yet been found in the *H. felis* genome. This may limit the usefulness of this animal model to study interactions between clinically important *H. pylori* constituents and the induced host response. Therefore, we can question to what extent extrapolation of results obtained with *H. felis* in the mouse model to human *H. pylori* infection is justified.

Using a mouse model, we investigated differences in virulence between two *H. felis* strains and we determined the influence of the host on disease outcome. Moreover, we have established a mouse model to study *H. bizzozeronii* pathogenicity and we compared *H. bizzozeronii* induced inflammation to that induced by *H. felis* (De Bock *et al.*, 2005).

This study confirmed the importance of the host and the type of host response in the inflammatory response to an *H. felis* infection as described previously (Sakagami *et al.*, 1996; Mohammadi *et al.*, 1996; Ferrero *et al.*, 2000). In *Helicobacter* infections, a Th1 response correlates with severe gastric pathology, whereas a Th2 response seems crucial for the control of these harmful Th1 responses (Mohammadi *et al.*, 1997; Bergman *et al.*, 2001; Portal-Celhay and Perez-Perez, 2006). Indeed, in our study, mouse strains known to exhibit a dominant Th1 response (C57BL/6 and SJL) developed significantly more inflammation than the Th2 responder BALB/c and the balanced Th1/Th2 responder CFW. This was the case for both *H. felis* strains and the *H. bizzozeronii* strain.

We were the first to compare the degree of inflammation induced by two *H. felis* strains in a mouse model. *H. felis* ATCC 49179 was isolated in Australia from the stomach of an adult cat (ATCC database [www.atcc.org](http://www.atcc.org)) and *H. felis* CCUG 37471 was isolated in Sweden from the stomach of an adult dog (CCUG database [www.ccug.gu.se](http://www.ccug.gu.se)). Despite the different sources
of these isolates, and the finding that *H. felis* strains can be genetically very diverse (Jalava *et al.*, 1999), we only found a significant difference in the level of inflammation at an early stage (3w) p.i. and only in the SJL mouse strain. This again stresses the influence of the experimental host on the degree of inflammation but also the importance of the timepoint of euthanasia p.i. when comparing *Helicobacter*-induced inflammation.

*H. bizzozeronii* has been shown to be of less pathogenic importance in dogs than *H. felis* (Peyrol *et al.*, 1998). In our mouse study, this was partly confirmed after experimental infection of SJL mice with *H. bizzozeronii*. In these mice, in the early stages p.i., inflammation induced by *H. bizzozeronii* was less severe than after infection with *H. felis*. The severity of inflammation due to *H. bizzozeronii* infection increased in time. At the final timepoint, this bacterial species induced the same degree of inflammation in SJL mice as that seen after *H. felis* infection. This might indicate that *H. bizzozeronii*, in contrast to *H. felis*, needs a longer time period to adjust to this host. In BALB/c mice, the *H. bizzozeronii* induced inflammation equal to the (low) degree of *H. felis* inflammation at most of the timepoints investigated. Therefore, as suggested above for *H. felis*, the host seems to be an important factor in the degree of *H. bizzozeronii* induced inflammation.

Although in our mouse model *H. felis* and *H. bizzozeronii* were mainly seen in the antrum, they were also present in lower numbers at the transition zone cardia-fundus. An interesting finding was that in that area both bacterial species were sometimes found in close association with parietal cells without visible pathological changes to these cells. The latter is in contrast to our studies conducted in gerbils (see below). In carnivores, helicobacters are often associated with parietal cells, in contrast to *H. pylori* in man (Lee *et al.*, 1988; Geyer *et al.*, 1993; Hermanns *et al.*, 1995; Happonen *et al.*, 1996; Scanzani *et al.*, 2001).

Differences in virulence have not only been reported between different gastric *Helicobacter* species but also strains belonging to one species may differ in virulence. This has clearly been shown for *H. pylori* (Wang *et al.*, 2003). *H. pylori* strains from different individuals are extremely diverse due to point mutations, gene insertions, and/or deletions (Akopyanz *et al.*, 1992; Jiang *et al.*, 1996), and genetically unique derivatives of a single strain are present simultaneously within an individual human host. Moreover, the genome of *H. pylori* may change continuously during chronic colonization of an individual host by importing small pieces of foreign DNA from other *H. pylori* strains during mixed infections, allowing the gradual adaptation of the bacteria to the host (Kersulyte *et al.*, 1999; Falush *et al.*, 2001). This extraordinary diversity can hinder characterization of bacterial factors associated with different disease outcomes.
Colonization of Mongolian gerbils with *H. pylori* was first described in the early 1990’s (Yokota et al., 1991) and since then this animal model has been used extensively in *Helicobacter* research (Hirayama et al., 1996; Takahashi et al., 1998; Ikeno et al., 1999; Nakagawa et al., 2005; Sun et al., 2005). There are several advantages and disadvantages of using Mongolian gerbils as a model for the study of *Helicobacter* infections. Gastric mucosal changes in *H. pylori* infected gerbils are similar to those seen in humans and inflammatory changes progress more rapidly and aggressively than in other laboratory animal models (Matsumoto et al., 1997; Ikeno et al., 1999). Moreover, gerbils appear to be more susceptible to *H. pylori*-induced gastric carcinogenesis than other rodents (Zheng et al., 2004). Crabtree et al. (2004) determined the mucosal immune response to *H. pylori* infection in gerbils and found, as in humans, non-human primates and mice, a Th1 polarized response. This Th1 response (and concurrent absence of a down-regulatory response) was more pronounced in the female than in the male gerbil, making it more interesting for us to use female animals with the prospect of rapid development of severe gastritis after *Helicobacter* infection. Mongolian gerbils, however, are outbred, with undefined genetic backgrounds, which tends to increase the variability of responses to any stimulus. Compared with mice, gerbils are relatively poorly characterized and few reagents, including antibodies and immune markers, are currently available. Nevertheless, most antibodies developed for mouse tissue antigens crossreact with the gerbil antigens. At the onset of our research only one study reported *H. felis* infection in Mongolian gerbils, with the emphasis on epithelial cell proliferation (Court et al., 2002).

In the first study (chapter 3.1), we established a Mongolian gerbil model for *H. bizzozeronii* infections and compared the resulting inflammation with that seen in *H. felis* infected gerbils. The most striking feature with both infectious agents was the loss of parietal cells, extending from the limiting ridge into the fundic mucosa. Bacteria were found in the area where parietal and chief cells were lost and where apoptosis and proliferation were seen. Parietal and chief cells were replaced by abundant mucus producing cells. AB (alcian blue)/PAS staining was performed to distinguish gastric-type metaplasia (mucous metaplasia) from intestinal-type metaplasia (Van de Bovenkamp et al., 2003). The metaplastic areas were stained intensely by PAS and not by AB, indicating gastric-type metaplasia (unpublished results). Mucous metaplasia (pyloric gland metaplasia or “antralization”) is associated with an increased risk of gastric mucosal atrophy and intestinal metaplasia in humans (Xia et al., 2000). Gastric mucosal atrophy and intestinal metaplasia are associated with hypochlorhydria,
but whether these lesions are the cause or the consequence of this is still controversial (McGowan et al., 1996; Kuipers et al., 1995).

In the subsequent study (chapter 3.2), the evolution of gastric epithelial cells loss was examined at different timepoints starting early after inoculation until 70 days p.i.. Moreover, the effect of the bacteria on the gastric epithelial cells was examined more in detail by means of TEM. This revealed \textit{H. felis} in close proximity to necrotic parietal cells and showed close association of the bacteria with parietal cells making contact through their periplasmic fibrils and flagella.

\textit{H. felis} adhesion to gastric epithelial cells is generally believed to be rare (Schreiber et al., 1999). Nevertheless some authors have reported the ability of this bacterium to attach to host cells (Haziroglu et al., 1995; Peyrol et al., 1998; Lecoindre et al., 2000). Haziroglu et al. (1995) described adhesion to the surface of canine parietal cells. We recently carried out an \textit{in vitro} adherence assay of fluorescent \textit{H. pylori} ATCC 43504, \textit{H. felis} ATCC 49179 and \textit{H. bizzozeronii} CCUG 35545 bacteria to a human gastric adenocarcinoma cell line (AGS), assessed by flow cytometry (Logan et al., 1998). The assay revealed no and very weak adherence of \textit{H. bizzozeronii} and \textit{H. felis}, respectively, while \textit{H. pylori} strongly adhered to these cells. \textit{H. pylori} is, indeed, known to adhere to AGS cells (Nilius et al., 1994). To approximate the \textit{in vivo} situation, we isolated gerbil parietal cells and used these cells in a similar experimental setup. No positive control was available as there are no bacteria known to adhere to gastric parietal cells. Therefore, as \textit{H. pylori} is believed not to adhere to parietal cells (negative control) (Testerman et al., 2001), \textit{H. felis} was compared to \textit{H. pylori}. \textit{H. bizzozeronii} was not investigated, as only a limited number of parietal cells was available. In this second assay, no adherence of both \textit{H. felis} and \textit{H. pylori} was observed (unpublished results). We conclude that \textit{H. felis} does not adhere to gerbil parietal cells.

The contacts observed between the periplasmic fibrils and the flagella with the microvilli of Mongolian gerbil parietal cells observed in the \textit{in vivo} study were not accompanied by pedestal formation. The sheaths of the flagella and the outer membrane covering the periplasmic fibrils were in contact with the parietal cells only at small contact points. These contacts were, however, frequently observed and therefore cannot be considered as irrelevant accidental findings. It may be that these constitute only weak focal interactions. These small focal interactions may, however, be sufficient for signal transduction between bacteria and host cells. Flagella-mediated signal transduction through interaction with TLR5 has been described for many flagellated bacteria including \textit{Escherichia coli}, \textit{Salmonella} and \textit{Listeria}.
species (Andersen-Nissen et al., 2005). TLRs are a conserved family of receptors that play a role in innate immunity by recognizing microbial ligands (Akira et al., 2001), such as flagellin. Conflicting reports have been published on the recognition of \textit{H. pylori} flagellin by TLR5, but TLR5 evasion of \textit{H. pylori} and \textit{H. felis} is most likely (Andersen-Nissen et al., 2005). TLR11 is another recently described receptor, which recognizes flagellin (Tsujimoto et al., 2005) or profilin-like molecules involved in motility (Lauw et al., 2005; Yarovinsky et al., 2005). As periplasmic fibrils are believed to be involved in motility, it is possible that interactions between specific ligands expressed on the outer plasma membrane overlaying these structures and specific cell receptors (such as TLR11) could play a role in the observed \textit{H. felis} inflammation. TLR11 is only present in mice and as a pseudogene in humans, while its presence in the gerbil has yet to be determined. In the mouse, it is found in the liver, kidney and gallbladder (Zhang et al., 2004). The expression of TLR11 in the stomach has not yet been described. Therefore, we recently tried to determine TLR11 expression in the stomach by using a polyclonal rabbit anti-TLR11 antibody on mouse and gerbil stomach tissue. Preliminary results indicate that immunohistochemistry is not an adequate tool to examine this (unpublished results). Other techniques, such as Northern blot, are needed to determine the expression pattern of TLR11 mRNA in stomach tissue.

The signalling pathways activated by TLRs all eventuate in NF-κB activation. NF-κB activation was also found in our gerbil study after \textit{H. felis} infection. Numerous pathways, however, can lead to the activation of NF-κB. In addition to Toll-like receptors, other signal transduction pathways emanating from TNF receptors and T-cell receptors play an important role in its activation (Hayden and Gosh, 2004). Hynes et al. (2004) described the NF-κB activation ability of LPS from various gastric \textit{Helicobacter} species in the AGS cell line. \textit{H. bizzozeronii} LPS induces NF-κB activation in AGS cells. \textit{H. felis} LPS was not tested. Of the \textit{H. bizzozeronii} infected gerbils in our study, that were sacrificed at day 21 (5 animals) and day 35 (5 animals) p.i., only one gerbil showed gastric NF-κB activation. In our \textit{H. felis} infected gerbils however, 7 out of 10 animals showed NF-κB activation. The discrepancy between the ability of \textit{H. bizzozeronii} to induce NF-κB activation in our study compared to the study conducted by Hynes et al. (2004) may be explained by differences between the \textit{in vitro} versus \textit{in vivo} situation. The NF-κB activated cells in our study were most likely parietal cells. They were only seen in the fundic region where bacteria were found. We cannot exclude that the observed NF-κB activation was due to the (T-cell mediated) inflammation,
although inflammation was also seen in other parts of the stomach while NF-κB activation was only seen in cells in close proximity to the bacteria.

Apoptosis of gastric epithelial cells was seen on the electron micrographs, but clearly not as frequently as necrosis. In chronic _H. pylori_ infections, there is a notable lack of epithelial necrosis (Robert and Weinstein; 1993) and cellular demise is mainly due to apoptosis (Moss _et al._, 1996). It is not clear if a direct effect of _H. felis_ is responsible for the observed necrosis and apoptosis in our study. Neu _et al._ (2002) described the apoptosis of rat parietal cells after incubation with _H. pylori_ and this was in most cases accompanied by NF-κB activation. NF-κB has been shown to be either detrimental or protective, depending on the cell type in which it is expressed (Van Antwerp _et al._, 1996; Shishodia and Aggarwal, 2002). In gastric epithelial cells, NF-κB activation may promote apoptosis (Lim _et al._, 2001; Gupta _et al._, 2001; Neu _et al._, 2002).

Another mechanism by which _H. pylori_ is known to induce apoptosis is through the Fas-FasL system (Jones _et al._, 1999). Ishihara _et al._ (2001) showed that Fas is abundantly expressed in human fundic gland epithelium and that the Fas-L expressing lymphoid cells are also located in the fundus of the _H. pylori_-infected stomach. They suggested that this _H. pylori_-related apoptosis-inducing system was mainly associated with the induction of apoptosis in the fundus. In our study with _H. felis_ the apoptotic cells were also mainly found in the fundus, indicating that _H. felis_-related Fas-FasL induced apoptosis could be one of the possible mechanisms of the observed apoptotic cell death. The Fas-FasL system has been shown to play a role in increased apoptosis and also increased proliferation in _H. felis_ infected mice. Fas knock-out mice did not show altered levels of apoptosis nor proliferation after _H. felis_ infection (Houghton _et al._, 2000). The Fas-FasL system is present in all mammals including gerbils (Kawano _et al._, 2002). Further investigations are warranted regarding the presence of increased Fas and FasL expression in our _H. felis_ and _H. bizzozeronii_ infected gerbils by means of immunohistochemistry or RNA extraction followed by RT-PCR.

The marked parietal cells loss could be the consequence of an autoimmune process as described for _H. pylori_. In humans, the gastric H⁺,K⁺-ATPase has been identified as the single major autoantigen in chronic _H. pylori_ gastritis with fundus atrophy (Claeys _et al._, 1998). In _H. felis_ infected mice, however, no anti-H⁺,K⁺-ATPase autoantibodies were detected by use of indirect immunofluorescence (Sakagami _et al._, 1996). We have recently assayed sera from uninfected control, _H. felis_ and _H. bizzozeronii_ infected gerbils for parietal cell autoantibodies
by using the same technique as Sakagami et al. (1996). As described for *H. felis* infected mice (Sakagami et al., 1996), no anti-H\(^+\),K\(^+\)-ATPase autoantibodies were detected in our Helicobacter infected gerbils (unpublished results).

In conclusion, the results described in this thesis clearly demonstrate the pathogenicity of *H. felis*. In the gerbil model, a direct interaction of *H. felis* with the acid producing cells of the stomach was found. *H. bizzozeronii* was clearly less pathogenic in these experimental models. There is still no animal model available to study *H. salomonis* infections.

Many questions, however, remain unanswered and warrant further research into the pathogenic mechanisms of these gastric non-*H. pylori* Helicobacter species.

Research should emphasize on the determination of the actual function of the periplasmic fibrils and their role in *H. felis* virulence. Determination of the gene(s) encoding these structures using transposon mutagenesis followed by construction of mutants and subsequent *in vivo* and *in vitro* experiments could help answering these questions. Transposon mutagenesis has been described for *H. pylori* (Salama et al., 2004).

Moreover, differentiation between the direct effect of the bacteria on the gastric epithelial cells and the indirect effect mediated through cells of the immune system is needed. Primary gastric epithelial cells and the recently obtained GSM06 gastric cell line could be used to assess the direct pathogenic effects of pet carnivore helicobacters on gastric epithelial cells such as apoptosis, necrosis, proliferation and NF-κB activation. GSM06 is a mouse cell line derived from gastric fundic mucus producing cells and is so far the only gastric cell line which responds to coculture with *H. felis* through the production of cytokines (personal communication R. Ferrero, 2006).

*H. felis*, *H. bizzozeronii* and *H. salomonis* LPS composition should be examined more in detail. At the moment, these studies are ongoing in collaboration with Prof. Dr. A. Moran from the University of Galway, Ireland.
References


GENERAL DISCUSSION


SUMMARY
Since the discovery of *Helicobacter (H.) pylori* in the early eighties and its association with gastric pathology, research on the *Helicobacter* genus has increased tremendously. In addition to *H. pylori*, other helicobacters, commonly denominated “*H. heilmannii*”, are found to colonize the human stomach. These organisms have been associated with gastritis, peptic ulcers and the development of MALT lymphoma in humans. Nowadays it is generally accepted that “*H. heilmannii*” does not represent a single species. Genetic analysis has revealed two different kinds of spiral-shaped organisms in the human stomach, “*H. heilmannii*” type 1 and “*H. heilmannii*” type 2. The first type, both morphologically and genetically, reflects “*Candidatus* H. suis”, a hitherto non-culturable bacterium occurring in the stomach of pigs. “*H. heilmannii*” type 2 is closely related to the *Helicobacter* species naturally occurring in the gastric mucosa of cats and dogs, such as *H. felis*, *H. bizzozeronii*, *H. salomonis*, and the recently described *H. cynogastricus* and “*Candidatus* H. heilmannii”. Identification of “*H. heilmannii*” to the species level has now become possible in histological specimens by the use of PCR, allowing the determination of the prevalence of these various species in human, canine and feline stomach biopsies.

The gastric colonization, inflammation and pathology observed in natural and experimental hosts after infection with *Helicobacter* species is the result of a complex interplay between the pathogen and the host immune response, with the gastric epithelial cell getting caught in the crossfire. Most of the research concerning *Helicobacter* virulence factors and the evoked host response has been focussed on *H. pylori*. In contrast, few attempts have been made to unravel the pathogenic mechanisms of the canine and feline helicobacters.

The use of animal models in *Helicobacter* research allows more detailed investigation of the mechanisms by which these bacteria cause gastric disease and provides us with an *in vivo* equivalent of *in vitro* results.

The general aim of this thesis was to investigate the differential pathological effects induced by pet carnivore *Helicobacter* species and strains on the gastric mucosa of experimental hosts under standardized conditions.

Chapter one highlights the importance of research on *H. felis* virulence mechanisms by describing a case in which *H. felis* was associated with human gastric ulcer disease. An elderly woman was referred to the hospital for gastroscopy because of right hypochondrial pain, nausea, anorexia and vomiting. Antral gastric ulcers were found and biopsies were taken for rapid urease testing, histology and PCR. The urease test was positive and histology
revealed the presence of multiple “H. heilmannii”-like bacteria. A PCR test which enables to discriminate five different *Helicobacter* species (*H. felis, H. bizzozeronii, H. salomonis, “Candidatus H. suis” and “Candidatus H. heilmannii”) was used and identified the bacteria as *H. felis*. Treatment with pantoprazole, amoxicillin and clarithromycin cleared the symptoms and the infection. Her asymptomatic wirehaired Dachshund, with which she lived in close contact, was brought to the Faculty of Veterinary Medicine (Ghent University, Belgium) for endoscopic examination. No severe gastric lesions were found but the urease test was strongly positive and histology showed the presence of “H. heilmannii”-like bacteria. The above-mentioned PCR identified *H. felis* as well as *H. bizzozeronii* and “Candidatus H. heilmannii”.

The dog received a 10-day triple therapy consisting of amoxicillin, metronidazole and omeprazole, which cleared the *H. felis* and *H. bizzozeronii* infection but not the “Candidatus H. heilmannii” infection. Repeated treatment with azithromycin instead of amoxicillin eradicated the latter bacteria. This report associates *H. felis* infection in humans with severe gastric pathology. Contact with dogs, cats and pigs has in earlier times been identified as a significant risk factor in humans for contracting non-*H. pylori* *Helicobacter* organisms. Therefore, the suggestion can be made that the patient contracted the *H. felis* infection from her dog.

In the second chapter, the pathological response to infection with two *H. felis* strains, *H. bizzozeronii* and *H. salomonis* was studied in a mouse model. Six-week-old SPF male mice of four different strains (BALB/c, SJL, C57BL/6 and CFW) were used, and sacrificed at three different timepoints post-infection (p.i.) (3, 9 and 16 weeks). For each mouse strain and timepoint, six mice were infected with either *H. felis* ATCC 49179, *H. felis* CCUG 37471, *H. bizzozeronii* CCUG 35545 or *H. salomonis* CCUG 37845. The mice were inoculated three times every other day. Three mice served as uninfected controls. Gastric colonisation by the bacteria was assessed using histology (Giemsa staining) and PCR. Inflammatory scores were determined on haematoxylin and eosin (H&E) stained slides using a previously described scoring system. *H. salomonis* was not detected by histology nor by PCR, but the samples of the *H. felis* and *H. bizzozeronii* inoculated animals were positive at all timepoints after inoculation. SJL mice consistently showed the most severe inflammation with all three *Helicobacter* strains. Lymphocytes, eosinophils and occasionally neutrophils were seen infiltrating the submucosa and lamina propria. BALB/c mice showed the mildest pathological changes. Regarding differences between bacterial strains, *H. felis* ATCC 49179 induced more inflammation than the other *H. felis* strain in the SJL mice at three weeks p.i.. *H. bizzozeronii*
differed from the two \textit{H. felis} strains in producing less striking pathological changes in the mice, particularly in the early stages of infection. This study highlights the importance of the host in \textit{Helicobacter} pathology. Moreover, these non-\textit{H. pylori} \textit{Helicobacter} species were shown to display obvious virulence differences that need further investigation.

In the third chapter, Mongolian gerbils were used for the study of the non-\textit{H. pylori} \textit{Helicobacter} infections. Nowadays, Mongolian gerbils are often used and universally accepted as a model for the study of \textit{H. pylori} pathogenicity. This infection model was the first to provide conclusive evidence that \textit{H. pylori} alone can induce gastric cancer. Moreover, inflammation develops more rapidly and is more severe than seen in other rodent models.

In the first part of chapter three, the short-term effects of \textit{H. felis}, \textit{H. bizzozeronii} and \textit{H. salomonis} on the gastric mucosa of gerbils were investigated, with a special attention given to parietal cells. Ten six-week-old SPF female gerbils were inoculated intragastrically with \textit{H. felis} ATCC 49179, five gerbils with \textit{H. bizzozeronii} CCUG 35545 and five gerbils with \textit{H. salomonis} CCUG 37845. The inoculation was done three times with 48h intervals. Three gerbils were inoculated with sterile culture medium and served as uninfected controls. The animals were sacrificed at three weeks p.i.. A longitudinal strip covering the full length of the stomach was taken for histology as well as fundus and antrum samples for PCR. Gastric colonisation by the bacteria was assessed using Giemsa staining, immunohistochemistry (anti-\textit{H. pylori} antibody) and a species specific PCR. The stomach was analyzed histologically for inflammation (H&E stain). Apoptosis and cell proliferation were visualised using immunohistochemical staining for caspase-3 and Ki67, respectively. Parietal cells were identified using an anti-hydrogen potassium ATPase antibody. All gastric samples of the \textit{Helicobacter} inoculated gerbils were positive for \textit{H. felis} or \textit{H. bizzozeronii} in PCR. The \textit{H. salomonis} inoculated gerbils, however, yielded negative results by PCR. The control animals were negative by histology and PCR. In the \textit{H. felis} and \textit{H. bizzozeronii} inoculated animals, the gastric antrum was mainly colonised. Inflammation consisted primarily of lymphocytic and neutrophilic infiltrates in the propria mucosae of the antrum, and to a much lesser extent in the fundus. All animals showed a remarkable loss of parietal cells extending from the limiting ridge into the fundus. This was more pronounced in the \textit{H. felis} inoculated gerbils compared to the \textit{H. bizzozeronii} inoculated animals. A high cell proliferation rate was observed in the mucosal area devoid of parietal cells. A dense band of apoptotic cells and large numbers of \textit{Helicobacter} bacteria were seen at the transition zone between affected and normal parietal cells. At three weeks p.i., focal apoptotic loss of gastric epithelial cells was
spatially associated with the presence of bacteria; this was especially true in the case of *H. felis* and, to a lesser extent, *H. bizzozeronii* infected gerbils. This loss of cells may lead to intestinal metaplasia, a condition that predisposes to malignancy. The gerbil model thus seems to be more suitable than the mouse model for the study of *H. felis*-parietal cell interaction.

The second part of chapter three was undertaken to obtain a greater insight into bacteria-host cell interactions in *H. felis* and *H. bizzozeronii* inoculated Mongolian gerbils. The infected animals were sacrificed at five different timepoints (7, 11, 21, 35 and 70 days p.i.). For each timepoint, five animals were inoculated with either *H. felis* ATCC 49179, or *H. bizzozeronii* CCUG 35545, while three animals served as uninfected controls. After euthanasia, stomach tissue was taken for light and transmission electron microscopy (TEM) and for PCR. Inflammation scores were determined on H&E stained slides using the updated Sydney system. Parietal cells, apoptosis, cell proliferation and NF-κB activation were visualised immunohistochemically. From day 11 p.i. onwards, *H. felis* inoculated animals showed inflammation consisting of neutrophilic granulocytes mainly in the antrum and lymphocytic infiltrates around the limiting ridge and throughout the stomach mucosa and submucosa. Moderate to severe loss of parietal cells extending from the limiting ridge into the fundus was seen. A front of apoptotic cells, bacteria, cell proliferation, and NF-κB activation were seen in the transition zone between affected and normal parietal cells. TEM revealed interactions between *H. felis* flagella and periplasmic fibrils with parietal cells and chief cells. Moreover, *H. felis* was seen close to and inside necrotic cells. At 10 weeks p.i., some *H. felis* infected gerbils had completely lost their fundic glands and showed mucous metaplasia of the epithelium. No contact was observed between *H. bizzozeronii* flagella and epithelial cells. This infection was associated with only mild parietal cell loss. The mechanism by which *H. felis* induces parietal cell necrosis and apoptosis remains unclear. The observed flagellar contact and NF-κB activation could play an important role in *H. felis*-associated inflammation.

In conclusion, the results described in this thesis clearly demonstrate the pathogenicity of *H. felis*. In the gerbil model, a direct interaction of *H. felis* with the acid producing cells of the stomach was found. *H. bizzozeronii* was clearly less pathogenic in these experimental models. There is still no animal model available to study *H. salomonis* infections.
Sinds de ontdekking van *H. pylori* in de vroege jaren tachtig en de associatie van deze kiem met maag pathologie, is de interesse in het Helicobacter onderzoek enorm toegenomen. Naast *H. pylori* zijn er nog andere Helicobacter bacteriën die de maag van de mens kunnen koloniseren. Ze worden gezamenlijk aangeduid als “*H. heilmannii*”. Deze micro-organismen kunnen bij de mens gastritis, maagzweren en MALT lymfomen veroorzaken. Tegenwoordig wordt algemeen aangenomen dat “*H. heilmannii*” niet één enkele kiemsoort is. Genetische analyse heeft uitgewezen dat twee verschillende types van spiraalvormige organismen voorkomen in de maag van de mens, “*H. heilmannii*” type 1 en “*H. heilmannii*” type 2. Het eerste type lijkt zowel morfologisch als genetisch sterk op “*Candidatus H. suis*”, een bacterie die tot nu toe niet in vitro geïsoleerd kan worden en die voorkomt in de maag van het varken. “*H. heilmannii*” type 2 is dan weer zeer nauw verwant met de Helicobacter soorten die voorkomen bij hond en kat, zoals *H. felis*, *H. bizzozeronii*, *H. salomonis* en de recent beschreven *H. cynogastricus* en “*Candidatus H. heilmannii*”. Sinds kort is er een PCR beschikbaar waarmee kan bepaald worden welke Helicobacter species aanwezig is/zijn in een maagbiopt waarin men morfologisch “*H. heilmannii*” vindt. Dankzij deze techniek is onlangs de prevalentie van *H. felis*, *H. bizzozeronii*, *H. salomonis* en “*Candidatus H. suis*” in humane maagbiopten bepaald.

De gastrale kolonisatie, inflammatie en pathologie waargenomen in de natuurlijke en experimentele gastheer na infectie met helicobacters is het resultaat van een complexe interactie tussen de pathogene kiem en de immuunrespons van de gastheer. Onderzoek betreffende Helicobacter virulentiefactoren en de uitgelokte gastheer immuunrespons werd tot nu toe voornamelijk uitgevoerd met *H. pylori*. Slechts weinig onderzoek is verricht naar eventuele virulentiemechanismen van helicobacters die voorkomen bij honden en katten.

In diermodellen kan men meer in detail de mechanismen bestuderen die verantwoordelijk zijn voor het pathogene karakter van deze kiemen. Diermodellen laten ook toe om in vitro bekomen resultaten te toetsen in vivo.

Het algemeen doel van deze thesis was nagaan of verschillen in pathogeniciteit bestaan tussen Helicobacter soorten en stammen die voorkomen bij hond en kat. Hierbij was het noodzakelijk diermodellen op punt te stellen zodat deze studies onder gestandaardiseerde omstandigheden konden uitgevoerd worden.
SAMENVATTING


Het tweede deel van hoofdstuk drie werd uitgevoerd om meer inzicht te krijgen in de kinetiek van de bacterie-gastheer interacties bij gerbils na infectie met *H. felis* en *H. bizzozeronii*. De geïnfecteerde dieren werden geëuthanaseerd op vijf verschillende tijdstippen (7, 11, 21, 35 en 70 dagen p.i.). Voor elk tijdstip werden telkens vijf dieren geïnoculeerd met *H. felis* ATCC 49179, vijf met *H. bizzozeronii* CCUG 35545 en drie dieren dienden als controles. Na euthanasie werd maagweefsel genomen voor lichtmicroscopie, transmissie-elektronenmicroscopie (TEM) en PCR. Aanwezigheid van pariëtale cellen, apoptosis, celproliferatie en NF-κB activatie werd immunohistochemisch aangetoond. Vanaf dag elf vertoonden de dieren, die geïnoculeerd waren met *H. felis*, inflammatie bestaande uit neutrofielen, vooral in het antrum, en lymfocyten ter hoogte van de limiting ridge en door heel de maagmucosa en submucosa. Matig tot ernstig verlies van pariëtale cellen werd opgemerkt, gaande van de limiting ridge tot ver in de fundus,. Een zone van apoptotische cellen, bacteriën, celproliferatie en NF-κB activatie was aanwezig op de overgang van beschadigde pariëtale cellen naar normale pariëtale cellen. Op dag 21 toonde TEM een interactie aan van *H. felis* flagella en periplasmatische fibrillen met pariëtale en zymogene cellen. Bovendien was *H. felis* aanwezig in de nabijheid van en zelfs in necrotische cellen. Op tien weken na infectie vertoonden sommige *H. felis* geïnfec
de fundusklieren. Dit ging gepaard met mucus metaplasie van het epitheel. *H. bizzozeronii* maakte geen flagellair contact met de epitheliale cellen. Bij deze bacterie was er slechts mild verlies van pariëtale cellen. De manier waarop *H. felis* necrose en apoptose van pariëtale cellen induceert, blijft onduidelijk. Het waargenomen flagellair contact en de NF-κB activatie zouden een belangrijke rol kunnen spelen in de inductie van inflammatie door *H. felis*.


Onmiddellijk na het afstuderen, trad ze in dienst als doctoraatsstudent bij de vakgroep Pathologie, Bacteriologie and Pluimveeziekten van de Faculteit Diergeneeskunde van de Universiteit Gent. In het kader van een Geconcerteerde Onderzoeksactie (GOA), verrichtte zij onderzoek naar de pathogenese van *Helicobacter* infecties afkomstig van kat en hond door gebruik te maken van verscheidene experimentele knaagdier modellen. In 2006 voltooide zij haar doctoraatsopleiding en werd ze houder van het diploma van “Master of Laboratory Animal Science” categorie D volgens FELASA (Federation of European Laboratory Animal Science Associations) uitgereikt door de Universiteit Gent.

Manuelle De Bock is auteur of mede-auteur van meerdere publicaties in (inter)nationale tijdschriften en nam deel aan internationale congressen met actieve inbreng.
PUBLICATIONS IN NATIONAL AND INTERNATIONAL JOURNALS


ABSTRACTS ON NATIONAL AND INTERNATIONAL MEETINGS


**ORAL PRESENTATIONS ON INTERNATIONAL MEETINGS**


6th International Workshop on Pathogenesis and Host Response in *Helicobacter* infections, Helsingør, Denmark. Presentation awarded.
DANKWOORD
“It ain’t over until the fat lady sings...”

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Manuelle