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# The microbiological, histological, immunological and molecular determinants of *Helicobacter pylori* infection in guinea pigs as a convenient animal model to study pathogenicity of these bacteria and the infection dependent immune response of the host*

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***Helicobacter pylori* is an etiological agent of chronic gastritis, gastric and duodenal ulcers and gastric cancers. The use of an appropriate animal model for experimental studies on the pathogenesis of *H. pylori* infections is necessary due to the chronic character of such infections and difficulties in identifying their early stage in humans. The aim of this study was to develop a guinea pig model of *H. pylori* infection and identify its microbiological, histological, serological and molecular determinants. Guinea pigs were inoculated *per os* with *H. pylori* strains: CCUG 17874 or ATCC 700312, both producing vacuolating cytotoxin A (VacA) and cytotoxin associated gene A (CagA) protein, suspended in *Brucella* broth with fetal calf serum (FCS) and Skirrow supplement of antibiotics. To determine *H. pylori* colonization, 7 and 28 days after the challenge, a panel of diagnostic methods was used. It included culturing of microorganisms from the gastric tissue, histopathological analysis of gastric sections, stained by Mayer,s haematoxylin and eosin to assess inflammatory response, by Giemsa as well as Warthin-Starry silver staining to visualise *Helicobacter*-like organisms (HLO) and with anti-Ki-67 antigen to assess epithelial cell proliferation. *H. pylori* infection was also confirmed by polymerase chain reactions (PCR) for detection in gastric tissue of *ureC* and *cagA* genes and by serological assessment of *H. pylori* antigens in faeces. This study showed the usefulness of microbiological, histological, immunological and molecular methods for the detection of persistent *H. pylori* infections in guinea pigs, which could be an appropriate model for studying *H. pylori* pathogenesis and the related immune response against these microbes.**

**Key words:** *Helicobacter pylori*, guinea pigs, diagnostic procedures

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) are Gram-negative, microaerophilic, spiral-shaped, flagella rods with a length of 20 μm and width of 5 μm, which belongs to the *Epsilonproteobacteria* class. These bacteria were isolated for the first time by Marshall and Warren in 1984 from the stomachs of patients suffering from gastritis (Marshall & Warren, 1994). *H. pylori* can also be converted into coccoidal forms, which appear due to hostile conditions *in vivo* or *in vitro* in the course of cultivation of these bacteria. Spherical forms are considered to be degenerative *H. pylori* cells (Bode *et al*., 1993; Andersen & Rasmussen, 2009). The main reservoir of bacteria is human stomach epithelium. It is suggested that a potential habitat for *H. pylori* could be animals such as dogs, cats, pigs, monkeys (Vaira *et al*., 1991; Megraud & Brauter, 2000). Also, water as an environmental milieu for *H. pylori* seems to be a potential source of *H. pylori* transmission (Fujimura *et al*., 2008). *H. pylori* might be able to form biofilm on the surface of water pipes (Grande *et al*, 2012; Amirhooshang *et al*., 2014). However, these data still require confirmation. Infection occurs by oral-oral, faecal-oral or iatrogenic routes. These bacteria colonize mainly the gastric *antrum* and areas of gastric metaplasia in the duodenum. Infection occurs most often in childhood and if untreated, may persist throughout life. About 50% of people in the world are infected with *H. pylori*. However, there are geographical areas where the rate of infection reaches 80–90% (McColl *et al*., 2010). The risk of infection is related to the socioeconomic status and early life living conditions (Ahmed *et al*., 2007; Vale & Vitor, 2010). In 20% of individuals infected with *H. pylori,* the clinical symptoms of dyspepsia occur due to inflammatory response which is initiated by *H. pylori* as a result of gastric epithelial damage. The inflammatory response is initially acute and then becomes chronic. A consequence of the chronic colonization of the gastric epithelium by *H. pylori* may be the formation of epithelial erosions and ulcers in the stomach or duodenum as well as gastric

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**Abbreviations**: ATCC, American Type Culture Collection; CagA, cytotoxin associated gene A protein; CCUG, Culture Collection University of Gothenburg; CD, cluster of differentiation; CFU, colony forming unit; CXCR, chemokine receptor; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HLO, *Helicobacter*-like organisms; *H. pylori*, *Helicobacter pylori*; HRP, horseradish peroxidase; IFN-γ, interferon gamma; IL, interleukin; LPS, lipopolysaccharide; MALT, mucosa-associated lymphoid tissue; MHC, major histocompatibility complex; OD, optical density; PCR, polymerase chain reaction; PPI, proton-pomp inhibitor; RANTES, regulated on activation, normal T cell expressed and secreted (CCL5); RUT, rapid urease test; TBS, Tris-buffered saline; UBT, urea breath test; VacA, vacuolating cytotoxin A

carcinoma or gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Wroblewski *et al*., 2010). This is why the World Health Organization International Agency for Research in Cancer included *H. pylori* in class I carcinogens (Vogiatzi *et al*., 2007). *H. pylori* induce a cellular and humoral immune response of the host. However, the chronic character of *H. pylori* infections suggests that the immune mechanisms are not able to eradicate the infection. In addition, immune processes induced by *H. pylori* may be involved in the development of pathological symptoms of *H. pylori*-related inflammation (Chmiela & Michetti, 2006; Michalkiewicz *et al*., 2015). Some of *H. pylori* antigens, including urease, vacuolating cytotoxic (VacA) or cytotoxin associated gene A (CagA) protein intensify the inflammatory response, while others such as lipopolysaccharide (LPS) inhibit the activity of immune cells (Grebowska *et al*., 2010; Chmiela *et al*., 2014; Rudnicka *et al*.; 2015; Varbanova *et al*., 2014). It is believed that the domination of pro- or anti-inflammatory antigens may direct the elimination of these bacteria or, conversely, the maintenance of infection (Lina *et al*., 2014). A growing number of new data suggest the role of *H. pylori* in the development of systemic diseases including coronary heart disease, diabetes, obesity or anemia and growth disorders in children (Baudron *et al*., 2013; Kuo ChH *et al*., 2014; Chmiela *et al*., 2015).

The proton-pomp inhibitor (PPI), clarithromycin-based therapy has been a standard treatment approach recommended for *H. pylori* eradication for in recent years (Megraud, 2012; Wu *et al*., 2012). However, emergence of clarithromycin resistance often decreases the eradication rates of *H. pylori* infections. Several approaches are available, including testing of antibiotic resistance or using sequential treatment, beginning with amoxicillin and PPI treatment followed by clarithromycin and metronidazole with PPI. Another possibility is to use bismuth-based quadruple therapy with the application of PPI, bismuth salts, metronidazole and tetracycline. The main advantage of this alternative treatment is to avoid problematic antibiotics, such as clarithromycin and levofloxacin (Megraud, 2012). Maastricht III Consensus Report recommends invasive gastroscopic diagnostic tools only for patients suffering due to dyspeptic symptoms. In humans, biopsy specimens from the stomach obtained during gastroscopy are evaluated histologically for the presence of inflammation and neoplastic lesions, as well as *Helicobacter*-like organisms (HLO), according to the Sydney System qualification (Price 1991; Stolte & Mieining, 2001). Moreover, biopsy specimens are used for bacterial cultures. Alternatively, in asymptomatic patients, the $C^{13}$ Urea Breath Test ($C^{13}$ UBT) – related diagnostic procedure is recommended in combination with serological investigation for anti-*H. pylori* antibodies in the serum samples or *H. pylori* antigens in stool (Rechcinski *et al*., 1997; Malfertheiner P *et al*., 2012).

Due to complex interactions between *H. pylori* and the host cells, it is difficult to determine the pathogenesis of *H. pylori* infections and the immune processes induced by this pathogen. The natural history of infection is unknown. Therefore, we need animal models that will follow the natural history of *H. pylori* infection and related immune processes. So far, mice, Mongolian gerbils, guinea pigs, dogs, cats, pigs, and monkeys have been used (O'Rourke & Lee, 2003; Peek, 2008). Guinea pigs are considered as the optimal model because they share similarities with humans with regard to hormonal and immunologic responses, thymus and bone marrow physiology, innate immunity and the complement system, pulmonary physiology, and corticosteroid response (Claman *et al*., 1972; Bitter-Suermann *et al*., 1981; D'Erchia *et al*., 1996; Wicher *et al*., 1998; Hiromatsu *et al*., 2002). They need an exogenous source of vitamin C (Ganguly *et al*., 1976), and demonstrate a delayed-type hypersensitivity reaction (McMurray 2001). Guinea pig leukocyte antigen (that is, the major histocompatibility complex (MHC)) is homologous to the human leukocyte antigen complex (Antczak 1982). These animals have several homologues of the human group 1 CD1 proteins expressed in lymphoid and non-lymphoid tissues (Hiromatsu *et al*., 2002). These proteins serve as antigen presenting molecules for non-peptide antigens to T-cells during infections. Humans and guinea pigs have a similar pattern of interferon gamma (IFN-γ) expression and inducible nitric oxide synthase during infection (Jeevan *et al*., 2006; Padilla-Carlin *et al*., 2008). The guinea pig has been chosen as a model for the study of infections related to secretion of interleukin (IL)-8 due to the presence of the CXCR1 tissue receptor (Takahashi *et al*., 2007). Also, IL-12 is similar in humans and guinea pigs (Shiratori *et al*., 2001). The sequence of the CD8 co-receptor of cytotoxic T lymphocytes demonstrates amino acid sequence similarity. Guinea pigs and humans produce the RANTES (regulated on activation, normal T cell expressed and secreted (CCL5) (Campbell *et al*., 1997)). An important advantage is that guinea pigs are not naturally infected with different species belonging to the *Helicobacter* genus (Whary *et al*., 2004).

There is a substantial need to fully characterize the route of *H. pylori* infection in guinea pigs in order to understand the pathogenesis and immunology of human *H. pylori* infections, and to accelerate the development of new treatments and vaccines. The value of an experimental animal model depends on the ability to confirm the infection and related markers of the immune response. Therefore, the aim of this study was to develop conditions of *per os* inoculation of guinea pigs with *H. pylori*, and select a set of diagnostic methods to confirm gastric colonization and *H. pylori*-related inflammation. A set of microbiological methods based on bacterial culture as well as histological, immunological and molecular approaches was selected. In humans, *H. pylori* can be found throughout the entire gastric mucosa from the pylorus up to the cardia. However, the main habitat of these bacteria is antrum, a pyloric part of the stomach (Stolte & Meining, 2001). It is also the area preferentially colonized by *H. pylori* in most of laboratory animals (Sturegard *et al*., 1998; Sjunesson *et al*.,2003; O'Rourke & Lee, 2003; Peek, 2008). Therefore, our present research has been focusing on pyloric part of the stomachs of *H. pylori* infected and control guinea pigs.

## MATERIALS AND METHODS

**Animals**. Adult, three-month-old, 400–600 g of weight male Himalayan guinea pigs certified as free from pathogenic microorganisms were used in the experiments. Animals were bred in the Animal House at the Faculty of Biology and Environmental Protection, University of Lodz (Poland), kept in cages with free access to drinking water and fed with standard chow. All animal experiments were approved by the Local Ethics Committee LKE9 (Decision ŁB 646/2012).

***H. pylori* strains and culture conditions**. *Helicobacter pylori* reference strain CCUG 17874 (Culture Collection, University of Gothenburg, Gothenburg, Sweden) and ATCC 700312 (American Type Cell Culture Collection, Manassas, USA), Vac and CagA positive were used in

**Table 1. Conditions of *H. pylori cagA* and *ureC* gene amplification.**

| Target | Sequences (5′-3′) (F: forward, R: reverse) | Annealing temperature & number of PCR cycles | Product length |
|---|---|---|---|
| cagA | F 5′-ATAATGCTAAATTAGACAACTTGAGCGA-3′<br>R 5′-TTAGAATAATCAACAAACATCACGCCAT-3′ | 60°C<br>40 | 298 bp |
| ureC | F 5′-AAAGCTTTTAGGGGTGTTAGGGGTTT-3′<br>R 5′-AAGCTTACTTTCTAACACTAACGC-3′ | 55°C<br>40 | 294 bp |

this study. *H. pylori* bacteria were stored at –80°C in Tris-buffered saline (TBS) containing 10% glycerol. Before being used in the experiments, *H. pylori* bacteria were grown for 5 days on modified *Helicobacter* agar (Becton Dickinson, Heidelberg, Germany) under microaerophilic conditions (Gas Pak, Becton Dickinson, Heidelberg, Germany), at 37°C. Bacteria were harvested by scraping from agar plates, suspended in *Brucella* broth containing 10% fetal calf serum (FCS) and Skirrow supplement of antibiotics: polymyxin B — 0.2 mg, vancomycin — 5 mg, trimetroprim — 2.5 mg (Becton-Dickinson GmbH, Heindelberg, Germany), pelleted by centrifugation (4000 × *g*, for 15 min), and then washed twice under the same conditions. The bacterial pellet a was suspended in complete *Brucella* broth to obtain an inoculum containing $1\times10^{10}$ colony forming units – CFU/ml according to the McFarland scale, centrifuged as above and resuspended in complete *Brucella* broth.

***H. pylori* infection in guinea pigs**. For the experiments, adult, three-month-old, 400–600 g of weight male Himalayan guinea pigs were used. The animal study groups consisted of guinea pigs (16), which were inoculated *per os* three times (at two-day intervals) with 1 ml of sterile complete *Brucella* broth (4), using a feeding needle (control group) or with 1 ml of freshly prepared suspension of *H. pylori* ($10^{10}$ CFU/ml) (6 per each *H. pylori* strain). Before administration of complete *Brucella* broth or *H. pylori*, the animals obtained orally 1 ml of 0.2 M $NaHCO_3$ to quickly neutralize the acidic pH of the stomach. Two guinea pigs inoculated with *H. pylori* were euthanized after 7 days and the rest of *H. pylori* infected guinea pigs as well as control animals 28 days after the final *H. pylori* challenge, on the basis of the Local Ethics Committee consent. Gastric tissue was collected from a pylorus part of the stomach and used for culturing of bacteria, isolation of DNA for polymerase chain reaction (PCR) and for histopathological investigation. Also, stool samples were collected to assess the presence of *H. pylori* antigens. All animals subjected to experiments were monitored for the loss of body weight. The consumption of water and food was also under control.

**Assessment of the *H. pylori* status by culture**. The gastric tissue was washed 3x with complete *Brucella* broth. Tissue sections from pyloric region were homogenized and plated on the agar plates containing modified *Helicobacter* agar (Becton Dickinson, Heidelberg, Germany) and grown for 5 days in under microaerophilic conditions (Gas Pak, Becton Dickinson, Heidelberg, Germany), at 37°C. *H. pylori* bacteria were identified on the basis of colony morphology, negative Gram staining, as well as positive urease, catalase and oxidase activity. Typical isolates were passaged once under the above conditions and re-evaluated again. In addition, mucus covering the gastric tissue was collected and checked for urease activity.

***ureC* and *cagA* PCR**. Gastric tissue sections from *H. pylori* infected and uninfected guinea pigs were washed and homogenized in complete *Brucella* broth and then centrifuged at 2600 × *g*, for 15 min at 20°C. The pellet was used for DNA isolation using the Genomic Mini Kit (A&A Biotechnology, Gdansk, Poland) including a proteinase K digestion step and columns for the purification of genomic DNA and removal of DNA-damaging substances. In addition, DNA was isolated from the stomach tissues, which were supplemented *in vitro* with a suspension of *H. pylori* in the range of $10^1$–$10^8$ bacterial cells. Purified DNA was used for detection of specific *H. pylori ureC* and *cagA* genes by PCR, utilizing primers for *cagA* sequence derived from *H. pylori cagA* gene (298 bp product, nucleotides 1751-2048 of the *cagA* gene) as well as primers for *ureC* sequence derived from *H. pylori ureC* gene (294 bp product, 1-294 of the *ureC* gene) (Oligo.pl Sequencing and DNA Synthesis Service, IBB PAS, Warsaw, Poland) (Table 1). Taq DNA polymerase (2.5 U/µl), $MgCl_2$ solution, chelating buffer (Thermo Scientific, Waltham, USA), deoxyribonucleoside triphosphates – dNTP (25 mM) (Promega, Madison, USA), and primers were used for the amplification step. Amplification conditions are shown in Table 1. PCR amplified products were detected by ethidium-bromide agarose gel electrophoresis utilizing DNA isolated from the reference *H. pylori* strains CCUG 17874 and ATCC 700312, and visualized by UV illumination. The samples were amplified through 40 consecutive cycles (Covacci *et al.*, 1993; Bickley *et al.* 1993). The assay was calibrated by the addition of a known number of bacterial cells (the reference strains) to gastric tissue sections isolated from *H. pylori* uninfected guinea pigs. The PCR detection limit for *ureC* and *cagA* genes was $1\times10^1$ bacteria for DNA isolated from *H. pylori* ATCC 700312. The PCR detection limit for *ureC* gene was $1\times10^3$ and for *cagA* gene — $1\times10^4$ CFU for DNA isolated from *H. pylori* CCUG 17874.

**Histological examination of tissue sections**. The *H. pylori* infection in guinea pigs was confirmed by the detection of HLO in the thin layer sections of the stomach tissue from pyloric region. Guinea pig stomachs were fixed in 10% formaldehyde and embedded in paraffin. Thick sections (4 µm) were stained using a routine histological procedure with the Giemsa stain solution or Mayer's haematoxylin, and analysed according to the selected criteria of the Sydney scale for the detection of HLO and categorization of inflammatory response using a light microscope. Moreover, tissue sections from gastric *antrum* were stained by a silver staining procedure of Warthin-Starry (Bio-Optica, Milan, Italy), to visualize bacteria in the tissue. For HLO the visual grading system used was as follows: 0, no bacteria detected in gastric crypts; 1, mild level of colonization and bacteria not detected in every gastric crypt; 2, moderate level of colonization with bacteria detected in the majority of crypts present; 3, severe level of colonization, bacteria present in all gastric crypts (Price 1991; Lee *et al.*, 1997). Inflammation in the stomach was evaluated on the basis of granulocyte (activity) and lymphocyte (chronic inflammatory cells) infiltration and graded 0 when no inflammatory cells were observed; 1, mild when few inflammatory cells were detected; 2, moderate level when more than few immune cells were detected; 3, severe, when infiltration with the immune cells was intense (Price 1991,

Lee *et al.*, 1997, Sturegard *et al.*, 1998, Sjunnesson *et al.*, 2003). Atrophy and metaplasia were not evaluated. Two independent pathologists analyzed thin layer preparations for the presence of HLO and inflammation. In guinea pigs inoculated with *H. pylori* CCUG 17874, we evaluated gastric tissue proliferation. To evaluate epithelial cell proliferation index, Ki-67 antigen was unmasked using 1 x Target Retrieval Solution (Dako, Glostrup, Denmark), boiling at 95–99°C for 20 min. The gastric epithelial cell turnover was assessed by using Ki-67 monoclonal antibody (MIB-1 clone; diluted 1:150, Dako), which recognizes the nuclear antigen Ki-67 present in all active phases of the cell cycle, but is absent in the $G_0$ phase. The antibody was identified by incubation with EnVision/HRP complex (Dako). Haematoxylin was used as a counterstain. Sections which were not treated with the monoclonal mouse anti-human Ki-67 antibody were used as a negative control. They were treated with EnVision/HRP complex in order to evaluate the level of unspecific staining. We defined the proliferation (labelling) index (LI %) as the proportion of Ki-67 positive cells (brown labelled-cells) among foveolar epithelial cells. For cell counting, we used ImageJ software program.

**Stool antigen immunoenzymatic test**. The stool antigen immunoenzymatic test (Immundiagnostik AG, Benshei, Germany) utilizing about 200 mg of faeces was performed according to the instructions of the manufacturer. Diluted stool samples were incubated in microwells coated with antibodies recognizing *H. pylori* antigens and then with an anti-*H. pylori* antibody conjugate in the presence of a colour developing solution. OD values were determined at 450 nm wave length. The stool antigen test results were positive if the values for test samples exceeded the *cut-off* value equal to 0.150 by 0.020.

## RESULTS

### Culture

*H. pylori* was recovered from the stomachs of 2/2 guinea pigs challenged with the *H. pylori* ATCC 700312 strain and 1/2 animals inoculated with the *H. pylori* CCUG 17874 strain, 7 days post infection. In one animal challenged with *H. pylori* CCUG 17874 the isolated bacteria did not display urease activity, although they produced catalase and oxidase and had a shape of spiral rods (Table 2, Fig. 1 A). By comparison, *H. pylori* was recovered in 8/8 guinea pigs inoculated with both *H. pylori* strains, 28 days after the final challenge (Table 3). *H. pylori* was not cultured from any uninfected animals (0/4) (Tables 2 and 3).

### Histopathology

In tissue sections of all culture-positive animals, characteristic HLO were detected by Warthin-Starry silver staining, as well as by Giemsa staining, as shown in Table 2, 3. The typical localization of the bacteria is shown in the representative figures (Fig. 1C, H, I). HLO were not detected using these staining procedures in the guinea pigs inoculated with complete *Brucella* broth (Fig. 1B, G). The level of colonization in guinea pigs 7 days post infection was at grade 1, whereas after 28 days post infection at grade 2. All culture positive animals displayed gastritis after 7 (Table 2) and 28 days (Table 3) post infection, with inflammatory cell response involving both, granulocytes and lymphocytes, infiltrating the whole mu-

**Table 2. The recovery of *H. pylori* by culture, grading HLO and gastritis in the stomachs of guinea pigs inoculated with the reference *H. pylori* strains, 7 days post infection.**

| Sample | Diagnostic assay | Inoculation with the reference *H. pylori* strain: | | | | | |
|---|---|---|---|---|---|---|---|
| | | CCUG 17874 | | ATTC 700312 | | No challenge | |
| | | 7 days post infection | | | | | |
| | | Guinea pig tested | | | | | |
| | | 1a | 1b | 2a | 2b | 3a | 3b |
| Gastric tissue | Culture | | | | | | |
| | Gram (–) | + | + | + | + | No isolate | No isolate |
| | spiral shape | + | + | + | + | | |
| | urease | – | + | + | + | | |
| | oxidase | + | + | + | + | | |
| | catalase | + | + | + | + | | |
| | Histology | | | | | | |
| | HLO grading: | | | | | | |
| | Giemsa | 1 | 1 | 1 | 1 | 0 | 0 |
| | silver staining | 1 | 1 | 1 | 1 | 0 | 0 |
| | Immune cell grading (H&E) | | | | | | |
| | granulocytes | 1 | 1 | 1 | 1 | 0 | 0 |
| | lymphocytes | 1 | 1 | 1 | 1 | 0 | 0 |
| | PCR | | | | | | |
| | *cagA* | + | + | + | + | – | – |
| | *ureC* | + | + | + | + | – | – |
| Stool | ELISA | | | | | | |
| | | – | + low | – | + low | – | – |

HLO, *Helicobacter* like organisms; H&E, hematoxilin and eosin staining; PCR, polymerase chain reaction; *cagA*, cytotoxin associated gene A; *ureC*, gene encoding *ureC* subunit of H. pylori urease

**Table 3. The recovery of *H. pylori* by culture, grading HLO and gastritis in the stomachs of guinea pigs inoculated with the reference *H. pylori* strains, 28 days post infection.**

| Sample | Diagnostic assay | Inoculation with the reference *H. pylori* strain: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CCUG 17874 | | | | ATTC 700312 | | | | No challenge | |
| | | 28 days post infection | | | | | | | | | |
| | | Guinea pig tested | | | | | | | | | |
| | | 4a | 4b | 4c | 4d | 5a | 5b | 5c | 5d | 6a | 6b |
| Gastric tissue | Culture | | | | | | | | | | |
| | Gram (–) | + | + | + | + | + | + | + | + | No isolate | No isolate |
| | spiral shape | + | + | + | + | + | + | + | + | | |
| | urease | + | + | + | + | + | + | + | + | | |
| | oxidase | + | + | + | + | + | + | + | + | | |
| | catalase | + | + | + | + | + | + | + | + | | |
| | Histology | | | | | | | | | | |
| | HLO grading: | | | | | | | | | | |
| | Giemsa | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 |
| | silver staining | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 |
| | Immune cell grading (H&E) | | | | | | | | | | |
| | granulocytes | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| | lymphocytes | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 |
| | PCR | | | | | | | | | | |
| | *cagA* | + | + | + | + | + | + | + | + | – | – |
| | *ureC* | + | + | + | + | + | + | + | + | – | – |
| Stool | | $+^{low}$ | $+^{low}$ | $+^{low}$ | $+^{low}$ | – | $+^{high}$ | $+^{low}$ | $+^{high}$ | – | – |

HLO, *Helicobacter* like organisms; H&E, hematoxilin and eosin staining; PCR, polymerase chain reaction; *cagA*, cytotoxin associated gene A; *ureC*, gene encoding *ureC* subunit of *H. pylori* urease

cosa (Fig. 1E, F). The granulocyte and lymphocyte infiltration in gastric tissue from guinea pigs 7 days post infection was at grade 1 (Table 2). After 28 days since the final challenge, the granulocyte infiltration was still at grade 1, whereas the lymphocyte infiltration increased up to grade 2 (Table 3). Lymphoid follicles were detected in the gastric tissue in 7 animals infected with *H. pylori*: 3 with *H. pylori* CCUG 17874 and 4 with *H. pylori* ATCC 700312. Four control guinea pigs did not show signs of gastritis (Fig. 1D). Only guinea pigs inoculated with *H. pylori* had an increased number of epithelial cells stained by anti-Ki67 antibody (Fig. 1K), as compared to non-infected animals (Fig. 1J). The number of Ki-67 antigen positive cells per gland was increased more than twofold in *H. pylori* CCUG 17874 infected, than uninfected guinea pigs (n=13, $p<0.00004$, Mann-Whitney U). The mean Ki-67 proliferation index (% labelled cells) for infected guinea pigs was $32.6 \pm 2.4$ and that for uninfected animals was $16.1 \pm 3.2$.

### PCR

Colonization of the gastric mucosa of all animals challenged with *H. pylori* strains, CCUG 17874 and ATCC 700312, was confirmed by *ureC* and *cagA* PCR (Fig. 2). Neither of 2 control animals was positive in regard to *H. pylori* infection as shown by the absence of these two DNA sequences.

### Detection of *H. pylori* antigens in stool samples

In this study, the stool antigen test was considered as a diagnostic procedure for establishing the *H. pylori* status of the guinea pigs challenged with two reference *H. pylori* strains. Anti-*H. pylori* specific antibodies used in the stool antigen test did not react with the faecal samples of guinea pigs inoculated with complete *Brucella* broth (Fig. 3). By comparison, *H. pylori* antigens were detected in 9/12 stool samples of the guinea pigs infected with *H. pylori*: 7 samples were classified as weakly positive, and 2 as highly positive, according to the manufacturer's protocol. Detecting *H. pylori* antibodies reacted with both *H. pylori* reference strains (CCUG 17874 and ATTC 700312), which were added to faecal samples.

## DISCUSSION

The gold standard for detection of a *H. pylori* infection in humans consists of invasive endoscopy based tests: rapid urease test (RUT) with histological examination for HLO and/or culture (Malfertheiner *et al.*, 2012). Among non-invasive methods, the $^{13}C$ UBT is commonly accepted as sufficiently specific and sensitive for primary diagnosis and confirming the effectiveness of eradication therapy (Bielanski *et al.*, 1996). Many ELISA tests are used for detection of specific anti-*H. pylori* antibodies. Detection of specific *H. pylori* DNA sequences in the gastric tissue and *H. pylori* antigens in stool samples was considered as a diagnostic tool (Chisholm *et al.*; 2001; Wisniewska *et al.*, 2002). Humans possess a simple stomach with a glandular epithelium throughout and indigenous microflora is limited due to acidic pH. A series of studies have revealed the existence of a distinct stomach-associated microflora besides *H. pylori*. At the phyla level, members of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Bacteroides* and *Gemmatimonadetes* have been identified. The proximal gastrointestinal tract can be viewed as a likely point of entry (Wu *et al.*, 2014). However, under-

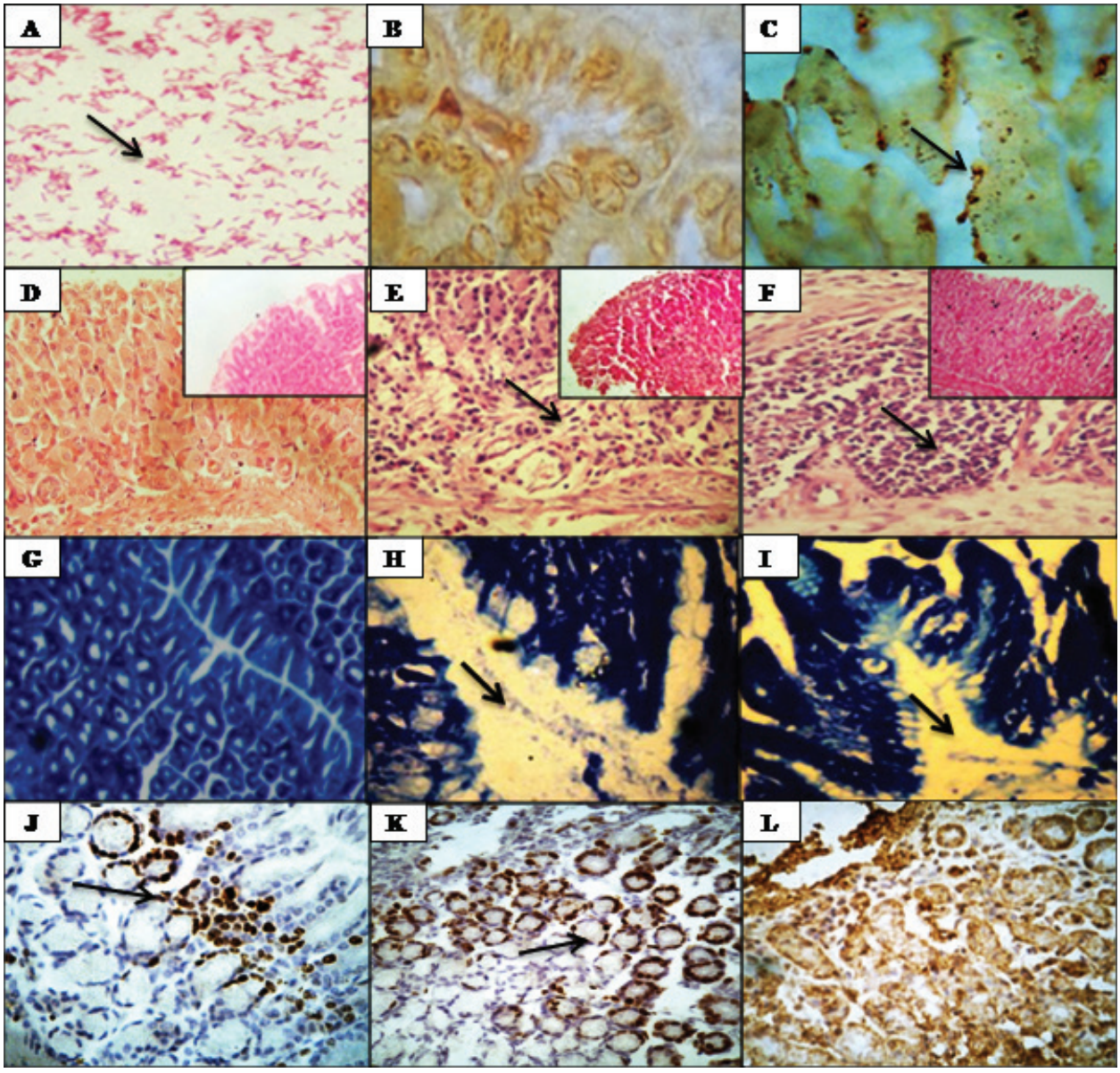


**Figure 1. Microscopic image of the gastric mucosa of guinea pigs inoculated or not inoculated with *H. pylori*.**
Gram-staining of ***H. pylori*** isolated from the gastric mucosa of a guinea pig challenged with ***H. pylori*** bacteria (**A**, 1000x); representative Warthin-Starry silver staining of gastric tissue of control (**B**, 1000x) and *H. pylori* infected (**C**, 1000x) animals, the arrow shows the location of bacteria. Representative haematoxylin-eosin staining of gastric tissue for evaluation of inflammatory cell infiltration of control (**D**, 200x) and *H. pylori* infected animals, *H. pylori* CCUG 17874 (**E**, 200x), *H. pylori* ATCC 700312 (**F**, 200x), 28 days post infection; arrows show the placement of inflammatory cells. Small fields (100x) show representative haematoxylin-eosin staining of gastric tissue for evaluation of inflammatory cell infiltration of control (**D**) and *H. pylori* infected animals, *H. pylori* CCUG 17874 (**E**), *H. pylori* ATCC 700312 (**F**), 7 days post infection. Representative Giemsa staining of gastric tissue of control (**G**, 1000x) and *H. pylori* challenged guinea pigs, *H. pylori* CCUG 17874 (**H**, 1000x), *H. pylori* ATCC 700312 (**I**, 1000x), arrows show the location of bacteria. Anti-Ki-67 staining of gastric tissue of control (**J**, 400x), *H. pylori* CCUG 17874 infected animals (**K**, 400x), and unspecific staining control (**L**, 400x), arrows show Ki-67 positive cells.

standing of this microbiota is still at the early stages, including its composition and influencing factors. In recent years, due to the use of animal models, a rapid progress has been made in the research on pathogenesis of *H. pylori*. However, a fully satisfactory model has not been designed so far, due to difficulties in the development of chronic colonization of the stomachs of laboratory animals with *H. pylori*. A lot of effort is also required to develop proper diagnostic procedures. The guinea pig is the only small laboratory animal with the stomach structure similar to the human stomach, and which is prone to the development of the inflammatory response especially due to the secretion of IL-8 by gastric epithelial cells. The present study has shown that guinea pigs were successfully colonized by one of the two *H. pylori* strains and that the infection sustained for 28 days after the challenge. We focused on two time points, 7 and 28 days after the final challenge of animals with *H. pylori* to identify the nature of the inflammatory response occurring as a result of infection. Since *H. pylori* adhesion was found to be pH dependent, we were feeding the animals with bicarbonate to raise the pH of the stomach (Obenbreit, 2005). The culture of *H. pylori* from the stomach tissue is considered as the most important proof of colonization. For this purpose, it is necessary to create optimal conditions for the growth of bacteria, taking into ac-

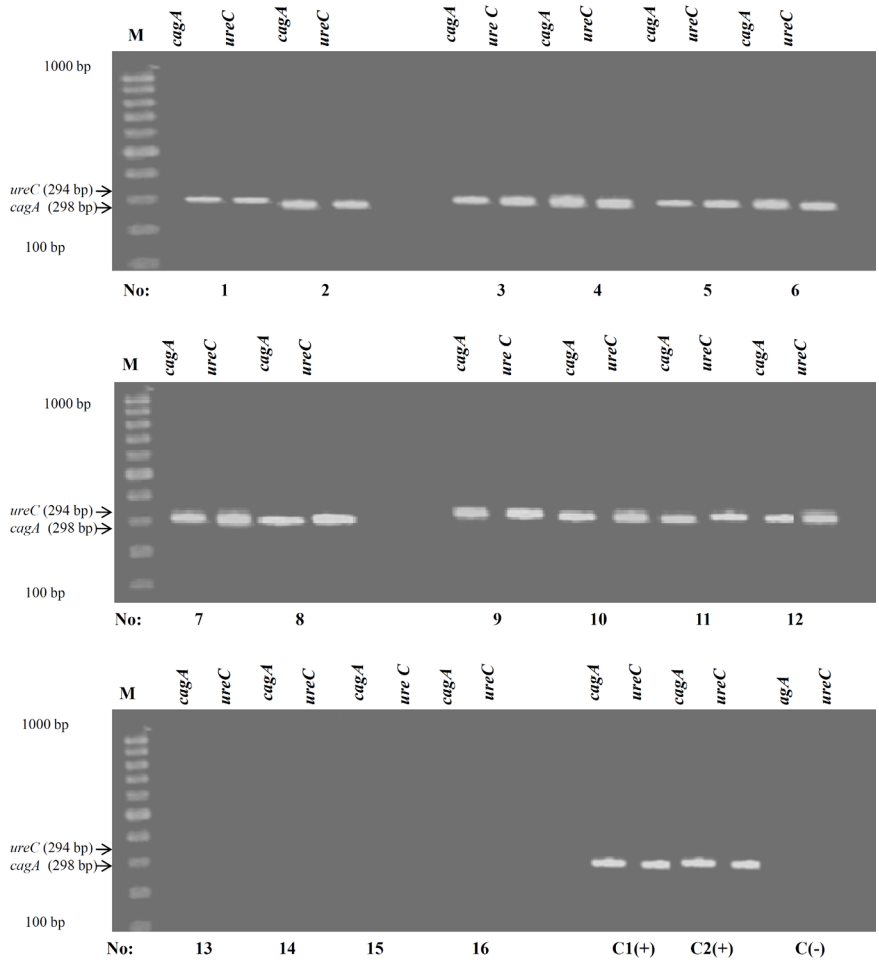


**Figure 2. Amplified PCR products loaded onto 1.4% agarose gel stained with ethidium bromide.**
M — 1000 base pair (bp) ladder molecular weight marker. Amplification products: *cagA* (298 bp), *ureC* (294 bp). No: number of samples, gastric tissue DNA of guinea pigs challenged with *H. pylori* CCUG 17874 (1, 2) or *H. pylori* ATCC 700312 (3, 4), 7 days post-infection; gastric tissue DNA of guinea pigs challenged with *H. pylori* CCUG 17874 (5–8) or with *H. pylori* ATCC 700312 (9–12), 28 days post-infection; gastric tissue DNA of guinea pigs inoculated with complete *Brucella* broth (13–16); C1 (+), positive control containing DNA of *H. pylori* ATCC 700312, $1\times10^8$ colony forming units (CFU); C2 (+), positive control containing DNA of *H. pylori* ATCC 700312, $1\times10^8$ CFU; C9(–), negative control (no *H. pylori* DNA content).

count their nutritional requirements, the composition of the atmosphere, the humidity and temperature, as well as the elimination of foreign microflora (Stevenson *et al.*, 2000). Regarding these factors, we prepared bacterial suspension for the inoculation of guinea pigs in complete *Brucella* broth supplemented with 10% FCS, and a Skirrow complex of antibiotics limiting the growth of bacteria other than *Helicobacter*. Using commercial *Helicobacter* blood agar, microaerophilic conditions and temperature of 37°C, we recovered *H. pylori* isolates with characteristic morphology of Gram-negative spiral shaped rods and enzymatic features, including the urease, catalase and oxidase activity from all *H. pylori* inoculated animals 28 days after the final challenge. One of 4 *H. pylori* isolates obtained 7 days post infection did not show urease activity despite catalase and oxidase production, as well as Gram-negative staining and the spiral shape of bacterial cells. According to standard diagnostic procedure, detection of *H. pylori* should be based on the production of urease, catalase and oxidase. Lack of urease activity in one of the isolates is difficult to explain. This could be due to abnormal growth conditions. The growth of bacteria was negligible and further passage has failed. However, it has been also suggested that production of urease could be regulated. However, the regulatory signals for controlling the levels of urease are not fully understood (McGee *et al.*, 1999; Stingl & Reuse 2005). Colonization of gastric epithelium of tested guinea pig by spiral shaped bacteria was confirmed by histology and by specific *cagA* PCR. However, detection of the gene is not synonymous with the presence of live microorganisms. In this particular animal, *H. pylori* antigens were not detected in the faecal sample. This indicates that various markers are necessary to confirm a *H. pylori* infection. Several elements of the histological classification according to Sydney System have been suggested for evaluation of the *H. pylori* colonization and gastric pathologies in laboratory animals (Lee *et al.*, 1997). For this purpose, in this study thin layer preparations were analyzed under light microscopy in terms of location of HLO in the gastric crypts and gastric glands, after staining the gastric tissue with Giemsa stain solution and by silver staining according to Warthin-Starry. Giemsa stain is easy to use, inexpensive, and provides consistent results; with human tissues it is the preferred method in many laboratories. Warthin-Starry silver staining was cru-

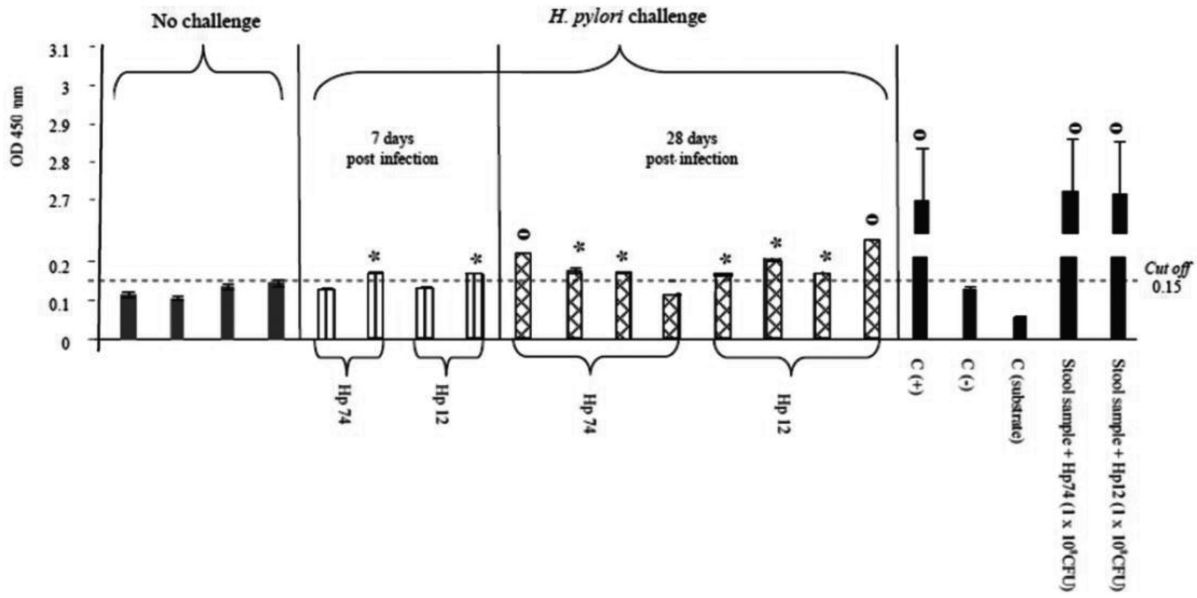


**Figure 3. Detection of *H. pylori* antigens by the immunoenzymatic stool antigen assay.**
Stool samples collected from guinea pigs 7 and 28 days after the final inoculation were analyzed for the presence of *H. pylori* antigens using the enzyme-linked immunosorbent assay (ELISA) using specific anti-*H. pylori* antibodies. Hp74 (*H. pylori* CCUG 17874), Hp12 (*H. pylori* ATCC 700312); C(+), positive assay control; C(–), negative assay control; CFU, colony forming unit; OD, optical density; O, highly positive results; *weakly positive results.

cial to the original demonstration of *H. pylori*. Due to sharp contrast between stained tissue and bacterial cells, silver staining allows better localization of bacteria in the tissue (Lee & Kim, 2015). Since the identification of *H. pylori* in gastric tissue of infected animals could pose problems, in this work we used both methods. By using them ,we were able to confirm the presence of HLO in locations typical for these bacteria in the stomachs from all *H. pylori* infected guinea pigs. However, evaluation of colonization was rather qualitative, although with some grading. It should be followed by more sensitive, specific, quantitative, molecular PCR methods. The histopathological picture of the gastric tissue of guinea pigs inoculated with *H. pylori* was substantially similar to that observed in humans infected with *H. pylori*. However, in this study we selected a simplified measurement of the inflammatory response, on the basis of granulocyte and lymphocyte infiltration and grading in the range of 0, 1, 2, 3, without taking into account atrophy and metaplasia. Gastritis was observed in all infected animals and control guinea pigs showed no or very low inflammatory response. In all infected animals, both 7 and 28 days after the challenge, a neutrophil infiltration of grade 1 severity and infiltration of lymphocytes were detected. However, lymphocyte infiltration 28 days after the final inoculation of the guinea pigs with *H. pylori* was stronger than in the animals examined after 7 days post infection. Stronger inflow of lymphocytes is a characteristic feature of chronic inflammatory reaction (Dixon, 1991). Also, Sturegard *et al.* had shown a transmucosal inflammation with crypt abscess, erosions and formation of lymphoid follicles in the guinea pigs after 3 and 7 weeks after the *H. pylori* challenge (Sturegard *et al.*, 1998). The persistence of *H. pylori* infection in the guinea pigs for 5 months, accompanied by severe gastritis was also demonstrated by Sjunnesson (Sjunnesson *et al.*, 2003). Rijpkema *et al.*, followed the *H. pylori* infection in guinea pigs up to 13 weeks after the challenge and showed that early infection was characterized by infiltration of mononuclear cells and eosinophils near the parietal glands, whereas as infection progressed, inflammation and tissue damage became more variable between individual animals (Rijpkema *et al.*, 2001). Gastric tissue erosions initiated by *H. pylori* colonization in humans are followed by increased epithelial cell proliferation, which is necessary for wound healing. However, epithelial cell expansion due to upregulation of cell proliferation can be associated with accumulation of harmful mutations and development of gastric cancer (Vogiatzi *et al.*, 2007; Wroblewski *et al.*, 2010). In this study, we showed that *H. pylori* infection in guinea pigs was associated with the increased rate of epithelial cell proliferation.

Due to a constant turnover of the gastric mucosa, *H. pylori* bacteria should be shed into the gastric lumen and then to faeces. In humans, a stool antigen test was recommended as a diagnostic procedure for establishing the *H. pylori* status in symptomatic patients and for the monitoring of eradication results (Deguchi *et al.*, 2009). A similar system was used in this study. However, a high concentration of *H. pylori* antigens expressed as OD values was detected using a similar immunoenzymatic assay in 2/8 guinea pigs after 28 days post infection. In 5/8 animals in this group, a low concentration of *H. pylori* antigens was detected in stool samples, as well as in the faeces of two of four guinea pigs 7 days after infection. The low OD ELISA values in stool antigen test might be due to weak or mild *H. pylori* colonization, which was not followed by sufficient shedding of *H. pylori* antigens into the gastric lumen or their enzymatic degradation. Despite low intensity of the reaction in the stool ELISA test, it may be helpful in confirming the *H. pylori* infection in guinea pigs because in the control animals the *H. pylori* antigens were not detected in faces, indicating a high specificity of the test.

Several studies were performed to detect specific *H. pylori* genes in gastric biopsy specimens in humans. The *ureA*, *ureC*, *cagA* and *vacA* genes were used as molecular markers of *H. pylori* infection (Li *et al.*, 1997; Chisholm *et*

*al*., 2001). In this study, usefulness of the PCR method for the detection of specific *H. pylori ureC* and *cagA* sequences in the gastric tissue of *H. pylori* infected guinea pigs was estimated. *H. pylori ureC*-PCR and *cagA*-PCR-positive results were obtained for all *H. pylori* inoculated animals. Since the PCR technique may detect *H. pylori* DNA in tissue samples containing non growing *H. pylori* coccoidal forms, it is necessary to evaluate the PCR results together with culture results, as well as with histopathological assessment.

Based on the obtained results, it can be suggested that diagnostic tests should involve a combination of diagnostic tools. The proposed panel of tests is as follows: culture of *H. pylori* from the stomach tissue, histopathology of thin layer sections of the gastric tissue stained with Mayer's haematoxylin and eosin, Giemsa staining solution as well as silver staining according to the Warthin Starry protocol, detection of *ureC* and *cagA* DNA sequences by PCR and detection of specific *H. pylori* antigens in a faecal samples. Additionally, staining of tissue sections using peroxidase labeled anti-Ki-67 antibody facilitates the detection of proliferating cells. The ability to confirm the infection and colonization of the stomach tissue of guinea pigs by *H. pylori* makes these animals suitable for studying the pathogenesis of infection and the course of related immune response.

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