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HELICOBACTER – A VERSATILE PATHOGEN AND A VANISHING SPECIES IN THE STOMACH

(The evolution of stomach microbiology in the antibiotic era)

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SUMMARY

Helicobacter pylori is a highly versatile pathogen with a coevolution of the stomach in mammals, birds and other animals. *H. pylori* is closely related to enterohepatic, bile-tolerant species (EHS), colonizing the gut of most wild rodents, felines and several other species. This evolution is complex, and the primates seem to be the environment for *H. pylori* to develop before the exodus of man from Africa about 55 000 years ago. The cagA pathogenicity island (PAI) encoding for a series of surface proteins and virulence factors (cagA to E) is unique for *H. pylori* and strongly associated to its success as a pathogen, which in turn is strongly related to a number of human diseases, i.e. type B gastritis, duodenal and peptic ulcer disease (PUD), and gastric carcinoma. Aggressive treatment strategies and screening programmes have resulted in a decline of *H. pylori* in the Western world with a concomitant decline in PUD and gastric carcinoma. The unique human-to-human transmission explains the rapid associated decline in *H. pylori*-infections in children in the Western world but not in non-industrialized societies. There, gastric carcinoma is still the second most common cancer.

Possible "non-antibiotic" strategies, such as probiotic therapy to treat *H. pylori*, and "the empty stomach" after eradication of *H. pylori* will be discussed.

INTRODUCTION

In the whole Western world and some rapidly developing Asian societies we notice a rapid change of diseases with a decline of antibiotic-curable infections and a simultaneous increase in a number of allergic diseases including allergic childhood asthma, rhinitis, eczema and other skin disorders, often proposed to be related to a "too clean" lifestyle (Figure 1) (*Chen* and *Blaser*, 2008). An infection and antigen-rich environment may be essential for maturation of a normal immune system, preventing allergies, asthma and autoimmune diseases related to disturbances of a skin- and mucosa-associated microbiota in genetically prone individuals. An infection in the gastrointestinal tract (GIT) with the gut-associated lymphoid tissues, GALT, is related to

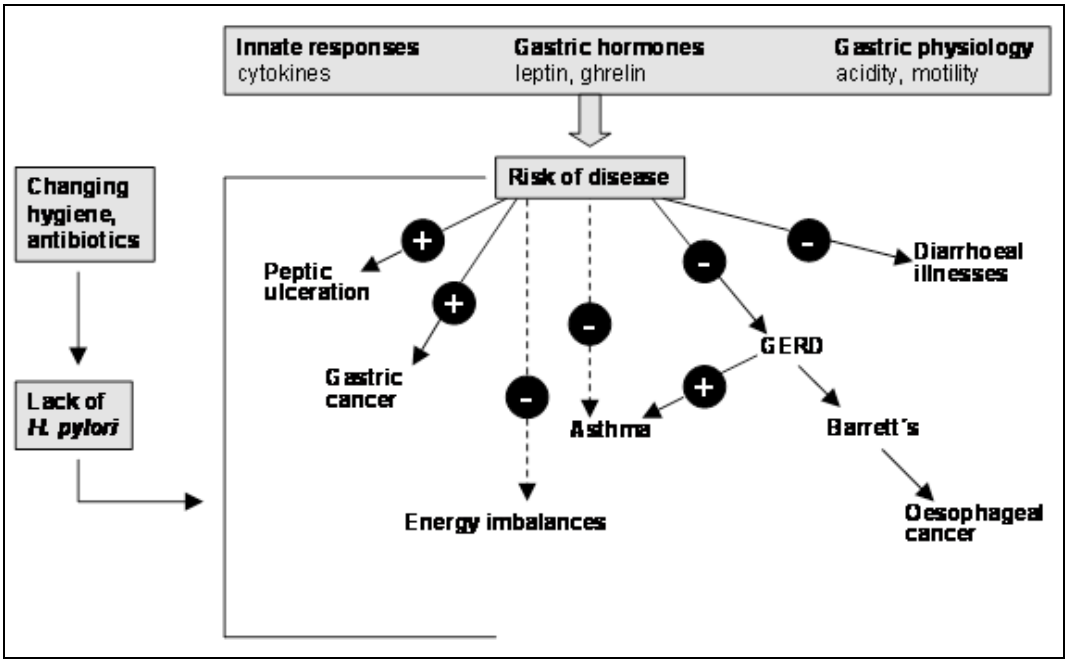

Figure 1: Who are we? Indigenous microbes and the ecology of human diseases.
[Adapted from: Blaser, M.J.: EMBO Rep. 7, 956-960 (2006)].

antibiotic use and overuse worldwide (*Vakil* and *Mégraud*, 2007).

Helicobacter pylori colonizes the human stomach shortly after weaning until recently, when modern living habits and common antibiotic use in early childhood prevent this colonization process. This fact was probably favoured by an early rotavirus and other enteric and respiratory infections. A transient pH rise in these infections may favour the gastric colonization to avoid the normal acid barrier. The impact of other factors such as diets after weaning as well as malnutrition and smoking with an alkaline stomach pH is still poorly understood (*Stenström* et al., 2007).

However, *H. pylori* gastritis studies in mouse and Mongolian gerbil models have been most useful to expand our understanding of the development of an acute, sub-chronic into chronic infection. *H. pylori* infections are always localized to the gastric mucosa in the stomach as well as to gastric tissues in other parts of the GIT, such as the Meckel's diverticle (*Haesebrouck* et al., 2009).

We know from extensive sero-epidemiological studies that *H. pylori* appeared in the human stomach in our ancestors in Africa > 58,000 years ago (*Linz* et al., 2007), is present in all human populations worldwide today, and acquired early after weaning. *Casswall* and co-workers (1999) analysed stool samples in young children in Bangladesh and showed that a transient colonization was followed by a later infection with a seroconversion in ELISA for urease and cell surface proteins (CSP). Early infections in some paediatric populations may affect appetite and food nutrient uptake, and induce a shunting syndrome in a subgroup, probably first reported by Oderda and colleagues in a South

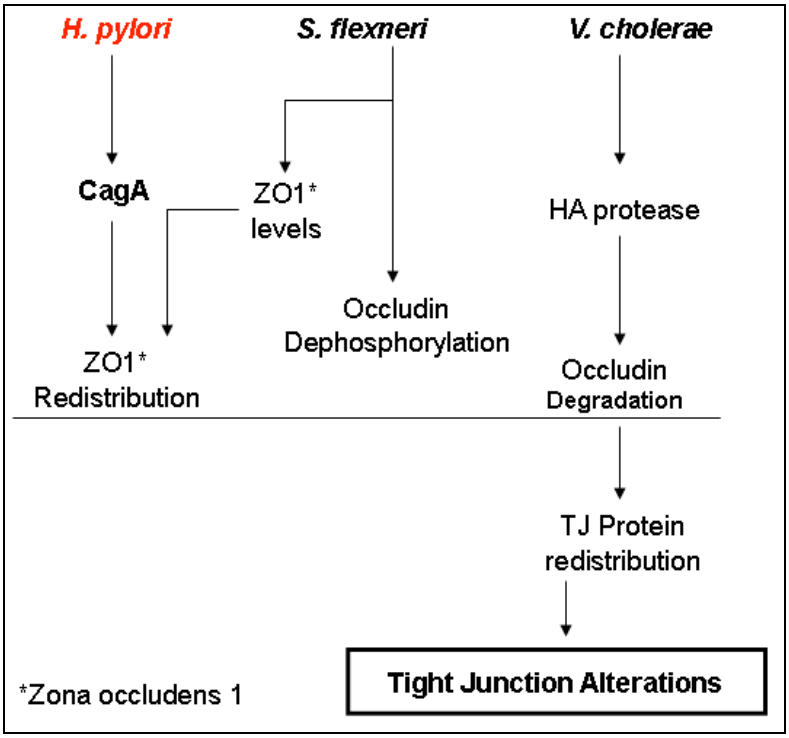

Figure 2: Disruption of tight junctions by microbes and microbial products.

Italian study (*Mégraud* and *Malfertheiner*, 2010). The majority of these infections are symptom-free (i.e. silent) but induce a local and **severe** immune response unlike a number of transient gastric colonizing microbes from food or the oral cavity. In brief, *H. pylori* is the single and dominant member of the human stomach microbiota (*Del Prete* et al., 2008). It penetrates the mucus layer, colonizes the epithelium, and pass cellular tight junctions into the sub-epithelial extracellular matrix (ECM), and binds to specific components by cell surface proteins (CSP:s) and LPS such as laminin and collagen type IV (Figure 2) (*McGuckin* et al., 2010).

HELICOBACTER AND MAN: A CO-EVOLUTION STORY

Mucus layer colonization and immune evasion

Gastric and enterohepatic Helicobacter species (EHS) colonize the mucus layer like other micro-aerophilic microorganisms such as *C. jejuni*. However, *McGuckin* and co-authors (2011) emphasize that microbes such as the indigenous microflora but not pathogens, rarely penetrate to the mucin deep layer. However, a chronic colonization and immune evasion is most characteristic for *H. pylori* unlike many animal Helicobacter species, such as *H. felis* (Table 1), rarely causing a chronic infection similar to *H. pylori* gastritis. *H. pylori* has developed a strategy to minimize stimulation of Toll-like receptors (TLRs), such as TLR-5 recognizing flagellae of *Salmo-*

Table 1. Enteric *Helicobacter* species isolated or detected in humans

Bacterial cultivation and/or PCR detection species	Cause/association in man	Animal host
H. canadensis	Gastroenteritis	Goose
H. winghamensis	Gastroenteritis	?
H. pullorum	Gastroenteritis, bacteraemia	Poultry
H. cinaedi	Diarrhoea, bacteraemia	Macaque, dog, hamster
H. sp flexispira	Bacteraemia	Pig, sheep, dog, cat
H. fennelliae	Bacteraemia, septic shock	?
H. bilis	Cholecystitis	Mouse, rat, dog, cat
H. hepaticus	Chronic liver disease	Mouse, hamster
H. rappini	Cholecystitis	Sheep
H. canis	Chronic liver disease	Dog, cat?

nellae but not of *H. pylori* (*Blaser* and *Atherton*, 2004). TLR-9 recognizes unmethylated DNA of most bacteria but not the highly methylated *H. pylori* DNA which minimizes recognition. *H. pylori* LPS is anergic, compared to enteric pathogens, related to lipid modifications and with poor reaction in classical LPS bioassays, such as the limulus test (*Moran* et al., 1992; *Hynes* and *Wadström*, 2004). *H. pylori* LPS is camouflaged from innate immune responses on cell surfaces. However, cagA positive strains stimulate NF-κB activation in the epithelium and phagocytes with subsequent induction of pro-inflammatory cytokine production (*Yanagisawa* et al., 2005). The relative scarcity of *H. pylori*-associated disease in non-industrialized countries despite a high *H. pylori* prevalence may be due to predominant Th_2 responses among black Africans, often chronically infected by intestinal parasites, helminths and malaria (the African enigma) (*Linz* et al., 2007; *Del Prete* et al., 2005)

Effects of *H. pylori* on leptin and ghrelin.

Gastric leptin levels are higher in *H. pylori*-infected adults than in uninfected individuals (*Konturek* et al., 2006). In an animal model, *H. pylori* infection is associated with up-regulation of ghrelin and several adipocyte genes (i.e. adipsin, resistin, adiponectin etc.), and likewise in a study from Poland (*Plonka* et al., 2006). Weight gain is common after *H. pylori* eradication, maybe related to the obesity epidemic in many Western societies and to a rapid decline in *H. pylori* infections (*Chen* and *Blaser*, 2008).

Chronic gastritis and carcinogenesis.

Carcinomas arise in stomachs with pangastritis (intestinal type, metaplasia, dysplasia). *H. pylori*-infected neutrophils release oxygen free radicals (ROS), and ascorbic acid levels are low in *H. pylori*-infected stomachs. Gastric cancer (GC) is number two of all cancers worldwide, increasing in African countries with an ageing population but declining in Western Europe related to an efficient eradication program (*Vakil* and *Mégraud*, 2007). The importance of dietary carcinogens in these malignancies is not well understood. However, food and snuff carcinogens act in synergy in *H. pylori*-infected mouse stomachs and tumours develop

regularly (*Stenström* et al., 2007). Well-developed mouse models are now most relevant for further studies of microbes, food and genetic background interactions in gastric cancer, similar to the more complex colon microflora-food interaction and colon cancer.

Probably all mammalian species including whales and dolphins seem to be colonized in the stomach by Helicobacter or Helicobacter-like organisms (*Haesebrouck* et al., 2009). However, several species studied in the early "Helicobacter history", such as *H. felis* in cats and dogs, migrate freely in the mucus layer but cause no or transient mucosal penetration in contrast to *H. pylori* in man (*Marshall,* 2002). However, *H. mustelae* in ferrets and *H. suis* in piglets colonize the gastric mucosa and cause mucosal ulcers much like *H. pylori* with the cagA pathogenicity island (PAI) and vacuolating toxin. *Candidatus Helicobacter suis* is a spiral bacterium in the stomach of pigs. Experimental infections in pigs have fulfilled the Koch's postulate with lymphoid follicle proliferation and a mild inflammation mainly in the antrum close to the parietal cells, as described for *H. pylori* in humans (*Haesebrouck* et al., 2009). However, the mild infection is making this model less likely to be developed for further studies of Helicobacter/gastrospirillum pathogenesis. Some epidemiological studies in China indicate that an early *H. suis* infection may protect against a *H. pylori* infection by unknown mechanisms of interference with a lower GC incidence in the population (*Haesebrouck* et al., 2009).

Several outer membrane proteins (OMPs) have been identified, like mucus-binding molecules including a neutrophophil binding and activating protein, or HPNAP (*Parker* et al., 2010). In this *H. pylori* OMP gene family (*Oleastro* et al., 2010) single or two copies of the homA/homA, homA/homB, or homB/homA and homB were found to be associated with gastro-duodenal disease with gastric inflammation and stomach atrophy, and identified as tissue adherence factors and implicated in IL-8 secretion. On the opposite, the homA gene is not associated with gastric inflammation.

H. pylori displays the highest level of genomic variability in bacteria with a high frequency of recombination contributing to the great diversity (*Linz* et al., 2007). Intragenomic recombination in the OMP genes occurs during a chronic *H. pylori* infection, probably reflecting a selective pressure for adhesins to adapt to the individual host and variations in the immune responses. *H. pylori* polymorphism reflects human phylogeography and human migrations, a most reliable marker of host pathogen co-evolution, facilitated by the long-term contact between the infecting strain and the host. Resulting genetic combinations seem to be critical for the ecological success of *H. pylori* strains (*Oleastro* et al., 2010). This great complexity in antigenic variations is a great challenge for *H. pylori* vaccine research.

H. PYLORI AND ANTIBIOTIC RESISTANCE

Suboptimal antibiotic eradication of *H. pylori* with up to a new quadruple therapy to treat patients to eradicate *H. pylori* upon several therapy failures confirmed by urea breath testing (UBT), gastroscopy or serology is a new challenge to develop alternative therapies (*Vakil* and *Mégraud*, 2007).

Cazzato and co-authors (2004) proposed to combine an antibiotic regime with a probiotic, i.e. Lactic Acid Bacteria (LAB) treatment to prevent side ef-

fects such as gastric reflux disease. *Sakamoto* and co-authors (2001) characterized a probiotic strain of *Lactobacillus gasseri,* and developed a Japanese yoghurt at the Meiji company to suppress *H. pylori* stomach infection as shown by UBT. Michetti and co-workers (1999) showed by UBT that another probiotic strain of LAB, *L. acidophilus* La1, inhibits the infection. Interestingly, LAB in the murine fore stomach inhibits the *H. pylori* infection in C57 Black and BALB/c mice (*Wang* et al., 1997), while germfree mice are more easily infected by a cagA positive strain of *H. pylori* devoid of LAB microflora in the upper stomach.

LAB and Bifidobacteria produce a number of antimicrobial activities (AMA), including peptides, which may inhibit *H. pylori* as well as a number of other pathogens such as *Clostridium difficile* (*Ljungh* and *Wadström*, 2009). This may be important in the future with a rapid increase in antibiotic-associated diarrhoea (AAD), sometimes as a severe complication upon a *H. pylori* antibiotic eradication therapy including clarithromycin or a quinolone (*Szajewska* et al., 2009). Thus, probiotics may be a useful adjunct with increased rates of eradication with an increase of 77 % to 80 % in one study (*O'Connor* et al., 2010).

THE STOMACH – AN EMPTY MICROECOLOGY NICHE AFTER *H. PYLORI* ERADICATION

An increase of gastro-duodenal reflux disease, or GERD, occurs in all Western societies today, associated with a rapid decline *H. pylori* infections in children varying from 33% to 7% in some epidemiological studies (Figure 1) (*Chen* and *Blaser*, 2008). One study demonstrated that *H. pylori* is 600 times more abundant in vomit samples compared with stool samples, and that vomiting is an important factor for transmission among children, e.g. in day care centres.

THE EVOLUTION OF *H. PYLORI*, OTHER GASTRIC AND ENTERIC HELICOBACTER SPECIES

A few years before Marshall and Warren published the Nobel Prize winning original reports in 1983 on *H. pylori* as the cause of type B ("bacterial") gastritis (Table 2) while *H. felis* and other related spiral-shaped organisms colonize rodents, cats and dogs (Table 1) (*Haesebrouck* et al., 2009). Studies in monkeys and other animals have confirmed that *H. pylori* is a unique human pathogen. The closest species to *H. pylori* may be a gastric species reported in beluga whales and dolphins (*Haesebrouck* et al., 2009). Genetic analyses of *H. pylori* in various populations confirmed a close relation to the human exodus from Africa 58.000 years ago (*Linz* et al., 2007). The great number of newly discovered species and new species "in the pipeline" of enteric Helicobacter in wild and laboratory mouse colonies suggest a complex evolution from unknown ancestor(s) closely related to *Wollinella* (Table 1).

Eradication of *H. pylori* from the human stomach may be in close analogy to another strictly human pathogen, i.e. the smallpox virus. However, the drastic fall in colonization rates in

Table 2: The discovery of *Helicobacter pylori*

Year	Author(s)	Discovery
1893	Bizzozero	Spirochetes in dog stomach
1906	Krienitz	Spirochetes in the stomach with gastric cancer
1917	Dragstedt	Bacteria do not induce gastric ulcer
1938	Doenges	Spirochetes induce gastritis in monkeys and humans
1979	Warren	Spiral bacteria in the human stomach
1983	Marshall	*H. pylori* isolated and cultured
1985-1987	Marshall/Morris	Oral feeding with *H. pylori* proved Koch's 3rd postulate by Marshall and Morris

young children in US, EU and other highly developed human societies today will never reach zero, as for smallpox. Moreover, we never experienced an "empty" sterile human mucosal surface. We know from studies in obesity patients before and after gastric bypass operations that oral microbes as alpha streptococci and enteric organisms (as various enterococcal species) can cause overgrowth in the stomach in chronic type A and B gastritis. A few new *Lactobacillus* species, like *L. kalixanda* (*Roos* et al., 2005) in gastroscoped human volunteers, and a few acid-tolerant *L. salivarium* and *L. reuteri* were reported recently.

It seems likely that we can develop a new probiotic concept to encourage use of stomach-adaptable non-virulent microorganisms to fill this empty niche. Since the borderline plastic pathogen and *H. pylori* probably now continues to decline and disappear it seems likely that a new concept discussed by B Marshall to develop a non-virulent attenuated *H. pylori* strain as a vaccine delivery system may also become a future probiotic candidate in analogy with *E. coli* strain Nisslé in ulcerative colitis (*Ljungh* and *Wadström*, 2009).

Finally, studies on the stomach microbiota should continue to explore the pathogenic properties in pigs (*H. suis*) and the complex gastric microflora in dogs and cats. The fore-stomach of these animals as well as of horses and rodents are colonized with LAB (*Haesebrouck* et al., 2009). These should be eradicated to permit a successful Helicobacter experimental infection (*Wang* et al., 1997).

HELICOBACTER AND CANCER

Marshall and Warren already in 1983 proposed that *H. pylori* was causing chronic type B (bacterial) gastritis and was an important vector in the development to atrophy and gastric metaplasia to dysplasia and gastric cancer, or GC (*Marshall*, 2002). The development of combined proton pump inhibitors´ (PPI) and antibiotic therapies now rapidly changed the GC epidemiology in societies all over the world. With a rapid decline in *H. pylori* infections in early childhood the person-to-person spread is rapidly declining. Without a chronic *H. pylori* gastritis at least 80-90 % of GC will be prevented, previously calculated to occur in about 11 % of *H. pylori* type B gastritis, thus a stronger

cancer risk factor than smoking in lung cancer (*Stenström* et al., 2007). Since most *H. pylori* infections are silent, a strategy to screen for GC with serology and gastroscopy with histopathology and culture, and PCR diagnosis of *H. pylori* infections are discussed, most recently at the European *H. pylori* conference in Florence, November 2010 (see: www.helicobacter.org).

In brief, it seems most appropriate to develop a pan-European screening program to diagnose *H. pylori* infections in our aging population with special attention to high *H. pylori* infection rates in South and East Europe and in immigrants from Turkey and other countries in the Middle East and in Africa. GC is still number four of all cancers worldwide and increasing in Indonesia and some other Asian countries with a rapidly growing young population, living in poor sanitation conditions. Interestingly, Canada decided already a decade ago to perform *H. pylori* serology on all immigrants applying for a Canadian citizenship (*R. Hunt*, McMaster University, personal communication). Thus, a *H. pylori* vaccine and other prevention methods seem to be a most attractive strategy to fight peptic ulcer disease and stomach cancer in Asia and Africa. B Marshall's laboratory in Perth is developing a new vaccine concept based on an attenuated *H. pylori* strain as a candidate to carry other vaccine candidates for an oral to stomach vaccine delivery.

HELICOBACTER, ANIMAL MODELS AND CANCER

The development of models to study *H. pylori* infection in mice and Mongolian gerbils are important tools to evaluate vaccine candidates and other strategies to prevent and treat *H. pylori* and other Helicobacter infections (see below). A number of dietary components such as antioxidants suppress these infections, and a low antioxidant diet enhances them. Recently, also some EHS, such as *H. bilis*, *H. pullorum* and *H. hepaticus* were shown to cause a chronic gut, biliary tree and liver infection in specific mouse models as the IL-10-/- mouse. We isolated *H. ganmani* in a spontaneous outbreak of IBD-like disease in IL-10 -/- mice with a histopathology similar to ulcerative colitis and sclerosing cholangitis in humans (*Nilsson* et al., 2008). These findings are also supported as new disease syndromes in a mouse model to study bile stone chronic cholecystitis in hyperlipemic mice (Maurer *et al.*, 2005). Likewise, they support early observations of chronic cholecystitis related to a *Salmonella* and *C. jejuni* biliary tract infection, probably very common in many non-Western societies**.** A possible link between Helicobacter infections and chronic cholangitis and bile stone disease is under investigation in several laboratories (*Nilsson* et al., 2005; *Pandey* et al., 2009; *Karagin* et al., 2010).

More research on a relation between EHS, biliary disease and IBD is certainly needed!

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