



**Serological diagnosis of infections with *Helicobacter suis*,  
a zoonotic agent present in pork**

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## Abbreviation key

AGS	human gastric adenocarcinoma cell line
BabA	blood group antigen-binding adhesin
BHI	Brain Heart Infusion
bp	base pair
<i>cagA</i>	cytotoxin-associated gene A
CagA	cytotoxin-associated protein A
<i>cag</i> PAI	cytotoxin-associated gene pathogenicity island
CFU	colony forming units
CGC	corpus greater curve
CLC	corpus lesser curve
COX-2	cyclooxygenase 2
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
<i>dupA</i>	duodenal ulcer promoting gene
<i>E. coli</i>	<i>Escherichia coli</i>
EIA	enzyme immunoassay
EMA	ethidium monoazide
EMR	endoscopic mucosal resection
ELISA	enzyme-linked immunosorbent assay
FISH	fluorescent <i>in situ</i> hybridization
Fla	flagellin
GGT	$\gamma$ -glutamyl transpeptidase
HBSS	Hank's Balanced Salt Solution
HCl	hydrochloric acid
HE	haematoxylin-eosin
HP-NAP	<i>H. pylori</i> neutrophil-activating protein
HpaA	<i>H. pylori</i> adhesin A
HRP	horseradish-peroxidase
Hsp	heat-shock protein
ICA	immunochromatography
<i>iceA</i>	induced by contact with epithelium coding gene
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemical
IL	interleukin
IRMS	isotope ratio mass spectrometry
kDa	kilo Dalton
Le	Lewis
MALT	mucosa-associated lymphoid tissue
MLST	multilocus sequencing typing
mRNA	messenger RNA
min	minutes
<i>napA</i>	neutrophil-activating protein coding gene
NapA	neutrophil-activating protein
NF- $\kappa$ B	nuclear factor $\kappa$ B
NHPH	non- <i>H. pylori Helicobacter</i>
OipA	outer inflammatory protein A

OMP	outer membrane protein
ON	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMA	propidium monoazide
PG	pepsinogen
PPI	proton pump inhibitor
qPCR	quantitative real-time PCR
r	recombinant
RHG	rugal hyperplastic gastritis
RT	Real-time
ROS	reactive oxygen species
rRNA	ribosomal RNA
RUT	rapid urease test
SabA/B	sialic acid-binding adhesin A/B
SAT	stool antigen test
SDS	sodium dodecyl sulfate
STs	sequence types
Th	T helper
TNF	tumor necrosis factor
TLR	Toll-like receptor
Treg	regulatory T cell
UBT	urea breath test
UreA/B	urease subunit A/B
VacA	vacuolating cytotoxin
VBNC	viable-but-non-culturable
W	Watt
2D	two-dimensional

# **General introduction**



The present thesis deals with *Helicobacter suis*, a bacterium which is highly prevalent in pigs and can infect humans too, causing gastric disease. Research has been seriously hampered by the very fastidious nature of this microorganism and several fundamental questions remain open. In the general introduction, the nomenclature, clinical significance, epidemiology and diagnosis of gastric *Helicobacter* infections in humans and animals are described. Furthermore, a brief overview of the literature on the pathogenesis and the role of virulence factors is given. For a more comprehensive review regarding the virulence factors of gastric *Helicobacter* species we refer to Haesebrouck et al., 2009 and the general introduction of the thesis of Flahou, 2011 and Vermoote, 2013.

In 1984, it was first reported that both gastritis and gastric ulcer disease in humans can be caused by a typical curved bacterium (Marshall and Warren, 1984). The organism was originally considered to be a member of the genus *Campylobacter*, but was later included in a new genus, *Helicobacter*, with the curved bacterium *Helicobacter (H.) pylori* being its first member (Goodwin et al., 1989).

Meanwhile, this Gram-negative bacterium is known to play a key role in the development of even more severe upper gastrointestinal problems, eg. gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Stolte et al., 1993; Kusters et al., 2006). Although the prevalence of *H. pylori* in the Western world is decreasing, gastric colonization by this organism remains widespread with prevalence rates of more than 80% in developing countries (Kusters, 2006). The human gastric mucosa is, however, able to harbor other *Helicobacter* species, besides *H. pylori* (Heilmann and Borchard, 1991). These non-*H. pylori* helicobacters (NHPH), including *H. suis*, are characterized by a typical spiral-shaped morphology, and have been detected in 0.2 to 6% of human gastric biopsies (Heilmann and Borchard 1991; Stolte et al., 1994; Svec et al., 2000; Solnick et al., 2003). In a recent study, the prevalence of *H. suis* infections was examined in Parkinson's disease patients (Blaecher et al., 2013). *H. suis* DNA was found in gastric biopsies from 12% of Parkinson's disease patients, but only in 0.6% of controls. The significantly higher frequency of *H. suis* infections in patients with parkinsonism, might explain the higher prevalence of Parkinson's disease among farmers. To which extend *H. suis* infections can be associated with this disease is however still unclear, and remains the subject of future studies.

The spiral shaped NHPH were originally named "*Gastrospirillum hominis*" (Mc Nulty et al., 1989). After analysis of the 16S rRNA gene, this initial name was replaced by "*Helicobacter heilmannii*" (Heilmann and Borchard, 1991). Further research led to the

conclusion that these bacteria comprise more than one *Helicobacter* species, which are primarily associated with infections in animals (O'Rourke et al., 2004; De Groote et al., 2005; Van den Bulck et al., 2005; Haesebrouck et al., 2009).

Although *H. pylori* infections have occasionally been demonstrated in ruminants, cats and in an Australian marsupial, the stripefaced dunnart (*Sminthopsis macroura*) (Handt et al., 1994; Every et al., 2011; Momtaz et al., 2014), animals are not considered to play an important role in the transmission of this bacterium to humans. Demonstration of *H. pylori* in cats and dunnarts may be related to an anecdotic anthroponosis. Moreover, it can not be excluded that in some of the ruminant cases not *H. pylori* itself was involved, but rather a bacterium closely related to this micro-organism (Haesebrouck et al., 2009).

## 1. Gastric *Helicobacter* infections in humans and pigs

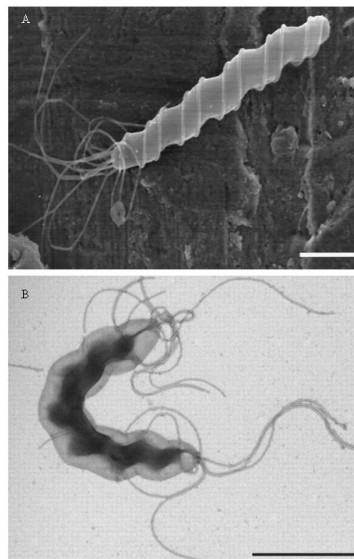
### 1.1. Nomenclature and characteristics of gastric non-*Helicobacter pylori* *Helicobacter* species

Since the identification of *H. pylori*, many other helicobacters have been described. Although many, if not all, of these “other” helicobacters, are enzootic in animals, there is now accumulating evidence that several of these species can also cause disease in humans (Andersen et al., 1999; Jalava et al., 2001; Kivistö et al., 2010; Joosten et al., 2013; Wüppenhorst, 2013). Since the onset of NHPH research, frequent changes in the nomenclature have been made, causing confusion among clinicians and bacteriologists. Subsequent to the proposal of the name *H. heilmannii*, further characterization of the 16S rRNA gene led to the subclassification into “*H. heilmannii*” type 1 and “*H. heilmannii*” type 2. “*H. heilmannii*” type 1 appeared to be, both morphologically and genetically, identical to *H. suis*, a bacterium colonizing the stomach of more than 60% of slaughter pigs (Mendes et al., 1991; Grasso et al., 1996; De Groote et al., 1999; Park et al., 2004; Hellemans et al., 2007; Baele et al., 2008). This large spiral-shaped microorganism was provisionally named “*Gastrospirillum suis*” (Mendes et al., 1990). However, based on 16S rRNA gene sequencing, fluorescent in-situ hybridization (FISH) and electron microscopy, nearly ten years later it was accepted that this bacterium belongs to the genus *Helicobacter* and is sufficiently different from other species to constitute a new taxon. Since at that time no pure *in vitro* isolates were available, this species could not be further characterized and the organism was named “*Candidatus Helicobacter suis*” (De Groote et al., 1999). Only in 2008, a successful *in vitro*

cultivation method was developed, which led to the description of *H. suis* as a formal species name (Baele et al., 2008).

“*H. heilmannii*” type 2, appeared not to represent one species, but actually comprises a group of species: *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis* and the “true” *H. heilmannii*, known as *H. heilmannii sensu stricto* (Haesebrouck et al., 2011). All of these species are known to primarily colonize the stomachs of cats and dogs, but humans can be infected as well.

All NHPH species share their spiral-shaped morphology, and although morphological differences between the different species have been described, microscopic investigation is not an accurate method for species identification. *H. felis*, *H. cynogastricus* and *H. bacculiformis* are characterized by the presence of periplasmic fibrils (Figure 1A) (Eaton et al., 1996; Van den Bulck et al., 2006; Baele et al., 2008). The first two species are both tightly coiled, whereas the latter is a large, slender, slightly spiral-shaped rod (Hänninen et al., 1996; Baele et al., 2008). *H. salomonis*, *H. bizzozeronii*, *H. heilmannii* and *H. suis* (Figure 1B) do not possess periplasmic fibrils and are all, except for *H. salomonis*, known to have very tight coils (Hänninen et al., 1996; Jalava et al., 1997; Baele et al., 2008; Smet et al., 2011).



**Figure 1:** (A) Scanning electron micrograph of a *H. felis* strain, isolated from the gastric mucosa of an adult cat, showing multiple bipolar flagella and the characteristic periplasmic fibers. Bar: 1  $\mu\text{m}$ . Adapted from Solnick and Schauer, 2001. Photo courtesy of Adrian Lee, Jani O'Rourke, and Lucinda Thompson. (B) Negatively stained cell of *H. suis* strain HS1, presenting tightly coiled spirals with up to six turns, with bipolar flagellae. Periplasmic fibrils are not observed. Bar: 2  $\mu\text{m}$ . Adapted from Baele et al., 2008.

## 1.2. Gastric non-*H. pylori* helicobacters in humans

### 1.2.1. Clinical significance of gastric non-*H. pylori* helicobacters in humans

In 1991, Heilmann and Borchard described the potential of other bacteria than *H. pylori* to colonize the human gastric mucosa. These organisms were found to be similar to the spiral shaped bacteria found in the stomachs of cats, dogs and non-human primates. Only several years later, the first human NHPH-strain was isolated *in vitro* from a gastric mucosa sample (Andersen et al. 1999). Analysis of the 16S rRNA gene sequence, DNA-DNA hybridization analysis and whole-cell protein profiling revealed that this isolate belonged to the species *H. bizzozeronii* (Jalava et al., 2001). Since then, only two other human NHPH-isolates were obtained, including another *H. bizzozeronii* strain, and a *H. felis* strain (Kivistö et al., 2010; Wüppenhorst, 2013).

In comparison with *H. pylori* infections, NHPH-infections are rather rare (Stolte et al., 1994; Boyanova et al, 2003; Yang et al., 1998; Yali et al., 1998; Ierardi et al., 2001; Yakoob et al., 2012). Therefore, *H. pylori* is still considered to be the major cause of chronic (atrophic) gastritis, peptic ulceration, gastric carcinoma and mucosa associated lymphoid tissue (MALT) lymphoma in humans. Furthermore, gastritis associated with NHPH infection is mostly less active than *H. pylori*-associated gastritis (Stolte et al., 1997; Joo et al., 2007). However, patients with NHPH gastritis develop more frequently MALT lymphoma than those with *H. pylori* gastritis (1.48% and 0.66% respectively) (Stolte et al., 1997; 2002).

Coinfections with both *H. pylori* and NHPH have been described in human patients (De Groote et al., 2005; Van den Bulck et al., 2005; Yakoob et al., 2012; Liu et al., 2014). These coinfections mainly result in aspecific gastro-intestinal symptoms, such as vomiting, epigastric pain and dyspepsia (Ojano et al., 2012; Yakoob et al., 2012). The significance of co-infections in terms of disease development and severity still needs to be determined.

### 1.2.2. Origin of infections and epidemiology

Human infections with NHPH organisms most likely originate from animals, and although the exact routes of transmission remain uncertain, living in close proximity to dogs, cats and especially pigs is a significant risk factor (Meining et al., 1998). To gain further insight into the diversity of both human and porcine *H. suis* strains, a multilocus sequence typing (MLST) technique, based on 7 housekeeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *yphC*, and *ureI*), was developed (Liang et al., 2013). Assessment of the allelic profiles for 10 available *H. suis* isolates led to 10 distinct sequence types (STs). In addition, by applying the technique on

gastric biopsy specimens from a human patient and 17 slaughter pigs originating from 4 different herds, 14 additional STs were distinguished and a close relationship was demonstrated between the human *H. suis* strain and porcine *H. suis* strains. This finding adds strength to the hypothesis that these infections indeed originate from animals. Since close contact with dogs and cats is more common than with pigs in Western society, it is remarkable for *H. suis* to be the most prevalent gastric NHPH species in humans (Trebesius et al., 2001; Van den Bulck et al., 2005). This higher prevalence might result from other routes of transmission, such as for example manipulation or consumption of raw or undercooked pork meat (as discussed in 1.2.3.). In addition, the ability of *H. pylori* to survive in common foods (Velázquez and Feirtag, 1999; Quaglia et al., 2007) supports the hypothesis that primary contamination of a food product or secondary contamination due to inappropriate handling can form a vector for the transmission of *Helicobacter* bacteria.

### **Waterborne transmission**

As epidemiological evidence supports the fecal-oral route for the transmission of *H. pylori*, fresh water might be the natural reservoir for this organism (Hopkins et al., 1993; Hultén et al., 1996; Hegarty et al., 1999; Moreno et al., 2003). Using molecular methods, *H. pylori* has been detected in various water systems, including rivers, surface and shallow ground water, and sewage systems (Enroth and Engstrand, 1995; Hultén et al., 1998; Lu et al., 2002). Isolation procedures, on the other hand, have rarely been successful starting from these sources. These findings implicate that detection of *H. pylori* DNA in water samples not necessarily confirms viability or infectivity of the microorganism. Moreover, the existence of a viable-but-not-culturable (VBNC)-state has frequently been the topic of debate (Shahamat et al., 1993). This state may correspond to the coccoid form, a state which the bacterium enters during *in vitro* experiments, when under stress and nutrient depletion (Adams et al., 2003). It has been demonstrated that the coccoid form of *H. pylori* remains viable, even though culturability is lost and no conversion from this state could be obtained in *in vitro* experiments. In the mouse model, reversion to an infective *H. pylori* form has been established when the coccoid form was inoculated intragastrically (Alejun et al., 1996). Adams and co-workers (2003) assessed the culturability and viability in cells incubated in both laboratory and a natural freshwater, using a LIVE/DEAD BacLight viability assay. During these studies, culturability of these cells was found to decline after ca. 10 days, although a large population of viable cells was still present. These results are consistent with data published for *H. pylori* and *Campylobacter coli* by other research groups (Benïssia et al., 1996; Boulos et al., 1999; Alonso et al., 2002).

Other studies report survival of *H. pylori* in sea and fresh water as culturable forms for several weeks and as non-culturable forms for up to a year (Shahamat et al., 1989; West et al., 1992; Konishi et al., 2007). Additional indirect evidence of the presence of *H. pylori* in water (used for irrigation) is the association of infections with the consumption of raw vegetables (Hopkins et al., 1993; Goodman et al., 1996; Chen et al., 2005; Mazari-Hiriart et al., 2008).

In drinking water systems, micro-organisms such as *Escherichia coli* and *Campylobacter spp.*, are predominantly associated with survival in the formed biofilms, rather than in the water itself (Mackerness et al., 1993; Buswell et al., 1998). Although several studies suggested that biofilms in water distribution systems may also harbor *H. pylori* organisms, culture from these biofilms has been unsuccessful and cell membranes did not remain intact (MacKay et al., 1999; Park et al., 2001; Azevedo et al. 2006; Gao et al., 2008).

### **Transmission through invertebrate species**

The role of insects as a vector for *H. pylori* has been investigated in studies on houseflies and cockroaches. Not only have researchers been able to amplify *H. pylori* DNA from the abdomens of wild flies, transmission of *H. pylori* to sterile petri dishes by experimentally infected house flies (*Musca domestica*) has been determined for up to 30 hours as well (Grubel et al., 1997; 1998). However, *H. pylori* could not be recovered from wild flies which had been exposed to human feces (naturally or experimentally) infected with *H. pylori* (Osato et al., 1998). In a study focusing on the eradication of flies, the control of flies did not influence the *H. pylori* infection rates in an area of high prevalence, suggesting flies are unimportant in *H. pylori* transmission (Allen et al., 2004). The presence of cockroaches has been suggested to represent a mode of transmission in poor socioeconomic environments (Imamura et al., 2003). Indeed, *H. pylori* has been successfully cultured from the excrements of cockroaches for 24 hours and the detection of *H. pylori* DNA could be established for up to 7 days after experimental exposure. The possible vector potential of insects for *H. suis*, or NHPH-infections in general, has not yet been investigated.

### **Transmission through milk**

Since *H. pylori* DNA was detected in raw sheep milk, milk has been considered a possible source of infection for humans (Dore et al., 1999; Dore et al., 2001; Fujimura et al., 2002). In a Japanese study, *H. pylori* DNA was detected in up to 72.2% of raw cow milk samples (Fujimura et al., 2002). In a more recent Italian study, detection of the *glmM* gene was established in 25.6% of all investigated goat milk samples, 33% of sheep milk samples, and 50% of cow milk samples (Quaglia et al., 2008). In this study, isolation procedures from the

samples were not successful, which might be attributed to the low number of bacteria present in the milk, to the presence of VBNC forms, or to the relatively long period of storage (up to 3 days at 4°C). Survival of *H. pylori* in milk has been demonstrated in artificially contaminated milk for a longer period (Quaglia et al., 2007). Successful isolation of *H. pylori* from raw milk samples, however, remains rare (Dore et al., 1999; Dore et al., 2001; Fujimura et al., 2002). It was shown that the percentage of detection of *H. pylori* DNA decreases when two genes are assayed (Dore et al., 1999, 2001; Fujimura et al., 2002; Quaglia et al., 2008). Moreover, caution should be taken on the use of 16S rRNA to identify *H. pylori*. The genetic relationship among strains isolated from milk and human patients has not been characterized during these studies, by for instance performing MLST or whole-genome sequencing. In conclusion, in the majority of studies describing the presence of *H. pylori* in milk, it is not clear whether it was really *H. pylori* that was demonstrated or rather *H. pylori*-like organisms.

### 1.2.3. The importance of contaminated pork carcasses and meat products

At the start of this thesis, nothing was known concerning the presence and persistence of *H. suis* bacteria on pork carcasses or in pork meat. As previously stated, research is hampered by the low success rate of isolation procedures, rendering detection of live *H. suis* bacteria by culture-based methods not feasible. As a consequence, the contribution of food of animal origin to human infection with *H. suis* remained unclear. Therefore, critical points for carcass contamination along the slaughterline, which are of significant value in the control of known foodborne pathogens, will be described in the following section.

#### **Carcass contamination**

Critical points for pork carcass contamination are scalding, polishing, evisceration and meat inspection procedures (Borch et al., 1996). These steps, during which the carcasses, the slaughter equipment and environment can become contaminated, will be discussed in chronological order. After stunning, the animals are immediately stuck by cutting the main blood vessels in the chest and then bled over a trench. Each animal is then scalded for 4 min. (at 63°C) followed by immediate mechanical dehairing. Hald and co-workers (2003), showed a higher risk of carcass contamination with *Salmonella* if scalding water was *Salmonella* positive. After scalding, pigs are secured to the overhead conveyor by hooks behind the Achilles' tendons of both legs, after which the carcasses are singed and dry polished. Based on a literature review, Berends et al (1997) suggested that 5-15% of *Salmonella* carcass contamination occurs during polishing. Moreover, a positive correlation between the

contamination level of the carcass immediately after polishing and the contamination level of the carcasses after splitting and forced chilling was observed (De Busser et al., 2011). Wringing of the carcasses at this step and the difficulty of cleaning of equipment, might allow bacteria to establish on the surface of the brushes and scrapes (Borch et al., 1996). Adding a second singeing device, after polishing, is helpful in avoiding contaminated carcasses to enter the clean part of the slaughterhouse (Delhalle et al., 2008; De Sadeleer et al., 2009; De Busser et al., 2011). After this step, carcasses are given a pre-evisceration wash with potable warm water. Evisceration is performed by first detaching and bagging the anus and rectum, followed by splitting the belly and manually removing the diaphragm, heart, lungs, trachea and tongue along with the digestive tract. Studies show between 55 and 90% of all carcass contamination with *Salmonella* occurs during the evisceration step (Berends et al., 1997). Fasting of the pigs, correct evisceration techniques and proper training of slaughterhouse personnel are effective measures in diminishing the risk of accidental cutting through the intestines. Subsequently, carcasses are cut along the midline using an automatic splitting saw. The splitting saw can also cause cross-contamination of carcasses (Swanenburg et al., 2001; Bertrand et al., 2010; Smid et al., 2012). Cleaning and disinfection of the saw, several times a day, was shown to be beneficial in reducing *Salmonella* contamination (Delhalle et al., 2008). Meat inspection procedures concerning the carcass head (eg. inspection of the submaxillary lymph nodes) also seem to represent a risk for contamination, as well as splitting of the head (Andersen, 1988; Nesbakken et al., 2003). It seems plausible that organisms colonizing the stomach (such as *H. suis*) might contaminate the oral cavity, and generate cross-contamination by the splitting saw after reaching the head. Finally, the carcasses are (most often) first shock-chilled for 75 min by forced ventilation, and then stored in cooling rooms at 3°C for at least 24h. Van Driessche and co-workers (2007) reported that, although cooling combined with drying of the surface of the carcasses reduced the number of positive areas on the carcasses, no effective elimination was established. Similar observations have been made in *Campylobacter* research in pig slaughterhouses (Oosterom et al., 1985; Epling et al., 1993).

Other factors that should be taken into account are contamination through the hands of the slaughterhouse personnel and meat inspectors and the processing water (Houf et al., 2002; Gude et al., 2005; Vieira-Pinto et al., 2006; Bertrand et al., 2010; Duggan et al., 2010).

### **Detection of foodborne pathogens in processed meat samples**

In order to determine whether processed pork meat constitutes a possible source of *H. suis* infections to humans, detection methods need to be developed. Although culture methods are labour-intensive and time-consuming, which is not desirable in cases when urgent diagnosis is necessary or when many samples have to be tested, these methods are still recommended for pathogens such as *Salmonella*. As previously stated, culture-based methods for the detection of *H. suis* bacteria in food are not feasible. Therefore, more convenient, and reliable alternatives could be the goal of future research.

Real-time (RT-)PCR for instance, is the most common approach in rapid testing, characterized by an even higher sensitivity and specificity than regular PCR methods (Hoorfar, 2011). A major disadvantage of PCR-methods (classic or Real-time), is that no distinction can be made between DNA derived from live or dead bacterial cells. This limitation has been overcome by the development of an ethidium monoazide (EMA) or propidium monoazide (PMA)-PCR method (Nogva et al., 2003; Nocker et al., 2006). By treating bacterial samples with EMA, prior to PCR, only DNA from viable cells is amplified, since EMA selectively binds to DNA of dead cells. Since the first description of this detection method by Nogva and co-workers (2003), multiple optimizations have been described for various microorganisms (Rueckert et al., 2005; Nam et al., 2011; Qin et al., 2012).

#### 1.3. Gastric non-*H. pylori* helicobacters in pigs

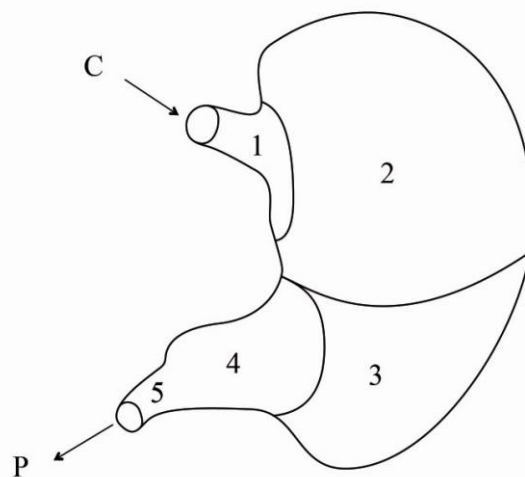
##### 1.3.1. Prevalence and epidemiology of *H. suis* infections in pigs

Back in 1990, the role of pigs as a possible reservoir for (*H.*) *pylori*-like organisms was studied (Queiroz et al., 1990). However, instead of *H. pylori*-like organisms, not previously described Gram negative, large spiral-shaped bacteria were found in the mucus layer of the lumen and antral pits of porcine stomachs. These bacteria are nowadays known as *H. suis*. *H. suis* is a worldwide spread bacterium, often colonizing the stomach of pigs. Prevalence rates range from 8% to 95%, depending on the study, though most reports state a prevalence of 60% or higher at slaughter age (Barbosa et al., 1995; Grasso et al., 1996; Cantet et al., 1999; Roosendaal et al., 2000; Choi et al., 2001; Hellemans et al., 2007; Kopta et al., 2010). Prevalence rates not only depend on the geographical region, but also on the age of the animals (Hellemans et al., 2007; Kopta et al., 2010). The high prevalence of infection in adult pigs suggests that *H. suis* persists in adult pigs, indicating that the natural immune response against *H. suis* does not lead to its clearance from the stomach.

Opposed to a very high prevalence in adult animals, the frequency of *H. suis* infection in young pigs is much lower: only 2% in suckling piglets (Hellemans et al., 2007). A very rapid increase in the prevalence was observed after weaning, suggesting a possible maternal protection of the sow milk. The protective effect of (human and mouse) mother milk has also been shown for *H. pylori* (Corthésy-Theulaz et al., 2003; Bhuiyan et al., 2010).

### 1.3.2. *H. suis* infections and porcine gastric pathology

The stomach mucosa of pigs can be divided in a glandular and a non-glandular part, with the glandular part consisting of the cardia, fundus and antrum, and the non-glandular part the pars oesophagea (Figure 2). Together with the cardiac gland zone, the pars oesophagea represents almost 50% of the stomach, and both have a pH range between 5 and 7 due to the presence of saliva and bicarbonate from the cardiac glands (Höller, 1970). The pars oesophagea is a small rectangular region surrounding the oesophageal opening, which is covered with stratified squamous epithelium and shows no mucus secretion (Friendship, 2006). As a consequence, only limited protection against low pH and gastric enzymes is ensured in this region. The distal, or glandular, compartment ensures postprandial digestive enzymatic activity due to acid secretion, maintaining a low pH in this region, which is necessary for pepsin activity.



**Figure 2:** Schematic overview of the stomach mucosa of pigs, with C. the cardia; P. the pylorus; 1. the pars non-glandularis; 2. the cardiac gland region; 3. the fundic gland region; 4. the pyloric region; 5. the gastro-duodenal transition.

Both in naturally and experimentally infected pigs, *H. suis* infections have been associated with chronic gastritis, mainly located in the antrum (Mendes et al., 1991; Queiroz et al., 1996; Park et al., 2000; Hellemans et al., 2007; De Bruyne et al., 2012). Gastric ulcerations, however, typically appear in the pars oesophagea. Certain predisposing factors would lie at the basis of ulcer development. Their presence would lead to a disruption of the segregation of the proximal and distal compartment, with loss of the distinct pH and enzymatic conditions. As a result, the mucosa of the pars oesophagea is exposed to increasingly acidic conditions, which can lead to epithelial insults, followed by hyperkeratosis, erosion, and finally, ulceration (Friendship, 2006). Possible predisposing factors are: particle size of feed (pelleting and fine grinding of feed), the presence of short chain fatty acids in the stomach, concurrent diseases leading to decreased food intake and stress (Hessing et al., 1992; Argenzio et al., 1996; Ayles et al., 1996; Robertson et al., 2002; Amory et al., 2006; Millet et al., 2012). Some studies also report an association of *H. suis* with gastric ulcers (Barbosa et al., 1995; Queiroz et al., 1996; Roosendaal et al., 2000; Choi et al., 2001; Appino et al., 2006; Krakowka and Ellis, 2006; Proietti et al., 2010). In contrast, other research groups did not find this association, leaving the exact role of *H. suis* in the development of porcine gastric ulcers unclear (Grasso et al., 1996; Melnichouk et al., 1999; Park et al., 2000; Szeredi et al., 2005).

It has been suggested that *H. suis* can increase the number of endocrine gastrin-producing cells, and decrease the number of somatostatin-producing cells (Sapierzyński et al., 2007). Additionally, *H. suis* was found in close contact with parietal cells of the fundic region of experimentally *H. suis* infected pigs, which might indicate that the bacterium may have an impact on these HCl-producing cells (Hellemans et al., 2007). As a result, a higher production of hydrochloric acid (HCl) might occur, which in turn may lead to increased contact of the non-glandular part with HCl and may result in the development of ulcers. Another possibility is that *H. suis* does not act as a primary cause of ulcers, but instead causes a delay in healing (De Bruyne et al., 2012).

## **2. Virulence factors of gastric *Helicobacter* species**

Until recently, very little was known about virulence factors of non-*H. pylori* helicobacters. Most research concerning virulence mechanisms has been realized with *H. pylori*. Therefore, the main focus in the next section will lie on this microorganism, mentioning possible similarities and differences for the NHPH virulence factors. In table 2, *H. pylori* virulence-associated genes are presented for which homologues were detected in the genomes of *H. heilmannii*, *H. suis*, *H. felis* and *H. bizzozeronii*.

## 2.1. Virulence factors involved in gastric colonization and persistence

Several virulence factors are considered to play a role in colonization and persistence of *H. pylori* in the stomach. These enzymes and proteins include the urease cluster protein, alpha carbonic anhydrase, sheathed flagella, the pH taxis *tlpB* gene, arginase and several adhesins and outer membrane proteins.

All gastric helicobacters possess a family of genes encoding the **urease enzyme** (Burne and Chen, 2000). This enzyme consists of two subunits, UreA and UreB, and forms an essential component of *H. pylori* acid resistance, by converting urea into ammonia and carbon dioxide. The ammonia neutralizes the hydrochloric acid of the stomach, which results in an increase of the pH. Urease activity is regulated by different parameters, including the pH itself, as well as the availability of urea and nickel (Eaton et al., 1991; Tsuda et al., 1994; Karita et al., 1995). The urease gene cluster includes a second operon, downstream of the *ureAB* genes, comprising *ureI* and the urease accessory *ureEFGH* genes (Akada et al., 2000). The UreEFGH proteins probably function in the subunit assembly and the regulation of urease activity by the incorporation of nickel (Cussac et al., 1992; Mobley et al., 1995). The UreI protein functions as an acid-activated urea channel, which controls the urea transport into the cell (Scott et al., 2000). Apart from genes of the urease complex, the hydrogenase accessory proteins HypA and HypB have been shown to be important for a good urease activity (Olson et al., 2001).

Next to the acidic environment, gastric helicobacters have to counteract **oxidative stress**, produced by the active immune response. *H. pylori* expresses several key components such as catalase, (KatA) superoxide dismutase (SodB) and alkyl hydroperoxide reductase (AhpC) (Seyler et al., 2001; Harris et al., 2002; Olczak et al., 2003; Barnard et al., 2004; Ernst et al., 2005). In addition, antioxidant proteins were described, including the *H. pylori* neutrophil-activating protein, HP-NAP (Wang et al., 2006). HP-NAP has a role in both inducing and combating oxidative stress resistance. The protein is thought to protect DNA from the detrimental effects of reactive oxygen species (Olczak et al., 2003; Barnard et al., 2004). On the other hand, HP-NAP is also implicated in the activation of neutrophils, leading to the formation of reactive oxygen species (Evans et al., 1995).

**Motility** is another essential characteristic for stomach colonization, which in this case, allows *Helicobacter* species to move toward a more neutral pH (Evans and Evans, 1995). *Helicobacter* species are motile by the presence of flagellae, which consist of a body, hook

and flagellar filament. Each filament, in turn, is composed of two flagellins, flagellin A (FlaA) (encoded by the *flaA* gene) and flagellin B (FlaB) (encoded by the *flaB* gene). Other genes, known to be essential in flagellar structure and motility include *flgE* and *fliD*, encoding the flagellar hook and flagellar cap proteins respectively (O'Toole et al., 1994; Kim et al., 1999). Experiments in different animal models with *H. pylori*, *H. felis* and *H. mustelae* illustrated the need for flagellar motility to colonize the stomach (Eaton et al., 1992; Josenhans et al., 1999; Ottemann and Lowenthal, 2002). *H. mustelae* mutants defective in hook production, *H. mustelae flaA* and *flaB* double mutants and *flgE* and *fliD* mutants are nonmotile, whereas single *flaA* and *flaB* mutants show a decreased motility, still enabling the microorganism to colonize and persist in the ferret's stomach (O'Toole et al., 1994; Josenhans et al., 1995; Andrutis et al., 1997; Kim et al., 1999).

In addition to motility, **chemotactic behavior** of *H. pylori* has been indicated by several studies (Foyne et al., 2000; McGee et al., 2005; Terry et al., 2005). The microorganism shows a directed motility towards urea, bicarbonate, cholesterol, arginine and other amino acids, but moves away from hydrochloric acid (O'Toole et al., 1994; Kim et al., 1999; Lertsethtakarn et al., 2011). For the latter, the pH taxis, the chemotaxis receptor TlpB is required. Moreover, *tlpB* mutants are defective for mouse colonization.

Several **adhesins and outer membrane proteins** are involved in the adhesion of *H. pylori* to the gastric epithelium. There are five families of outer membrane proteins (OMPs) for *H. pylori*: the major OMP family consisting of Hop and Hor proteins, the Hof protein family, the Hom protein family, the iron-regulated OMPs and the efflux pump OMPs. The large number of *H. pylori* adhesins probably reflects the importance of these proteins for the microorganism, but makes it difficult to examine the true contribution of each adhesin. Therefore, a summary of the most important adhesins and their primary role in virulence is given (Table 1), rather than a complete overview.

**Table 1.** HP-Adhesins and their association with *H. pylori*-related disease

Protein - <b>abbreviation</b>	Function	Clinical association	Reference(s)
Blood group antigen-binding adhesin- <b>BabA</b>	Binds on Lewis b (Le <sup>b</sup> ) ABO blood group antigen on the surface of epithelial cells	Correlation <i>babA2</i> allele and development of gastric diseases	Ilver et al., 1998 Gerhard et al, 1999 Olfat et al., 2005 Yamaoka, 2008
Sialic acid binding adhesin- <b>SabA</b>	Adheres to sialyl-Le <sup>x</sup> and sialyl-Le <sup>a</sup> antigens and is involved in activation of neutrophils	None	Mahdavi et al., 2002 De Jonge et al., 2004 Unemo et al., 2005
Sialic acid binding adhesin- <b>SabB</b>	Binding specificity is unknown	A nonfunctional <i>sabB</i> gene is a predictor of duodenal ulcer disease	De Jonge et al, 2004
Outer inflammatory protein A- <b>OipA</b>	Associated with increased interleukin (IL)-8 secretion	Increases gastric inflammation <i>in vivo</i>	Yamaoka et al., 2000
<i>Helicobacter pylori</i> outer-membrane protein <b>B-HomB</b>	Associated with increased interleukin (IL)-8 secretion	<i>homB</i> status is a putative marker for the risk of development of gastric cancer	Oleastro et al., 2008 Jung et al., 2009
<i>H. pylori</i> adhesin A- <b>HpaA</b>	Results of adhesion studies are not conclusive	Unknown	O'Toole et al., 1995 Jones et al., 1997 Carlsohn et al., 2006
<b>AlpA</b> and <b>AlpB</b>	Inactivation of the respective genes results in decreased adherence to gastric epithelial cells in a guinea pig model	Unknown	Odenbreit et al., 1999 De Jonge et al., 2004
<b>HorB</b>	Disruption of the <i>horB</i> gene reduces <i>H. pylori</i> adhesion to gastric	Unknown	Snelling et al., 2007

epithelial cells by more than twofold.

<b>HP-NAP</b>	This neutrophil-activating protein is reported to be a possible adhesin to mucin	Unknown	Evans et al., 1995 Namavar et al., 1998 Tonello et al., 1999 Montemurro et al., 2001 Montemurro et al., 2002
Duodenal ulcer promoting gene- <b>DupA</b>	The <i>dupA</i> gene encodes for a VirB4 ATPase homolog. There are two alleles: <i>dupA1</i> and <i>dupA2</i>	Associated with gastric and duodenal ulcers. The association with gastric cancer is not universal.	Lu et al., 2005 Hussein, 2010a Hussein et al., 2010b
“Induced by contact with epithelium” coding gene- <b>IceA</b>	The <i>iceA1</i> allele encodes a CATG-recognizing restriction endonuclease.	Association with peptic ulcers is not universal.	Peek et al., 1998 Yamaoka et al., 1999 Donahue et al., 2000 Kidd et al., 2001 Xu et al., 2002

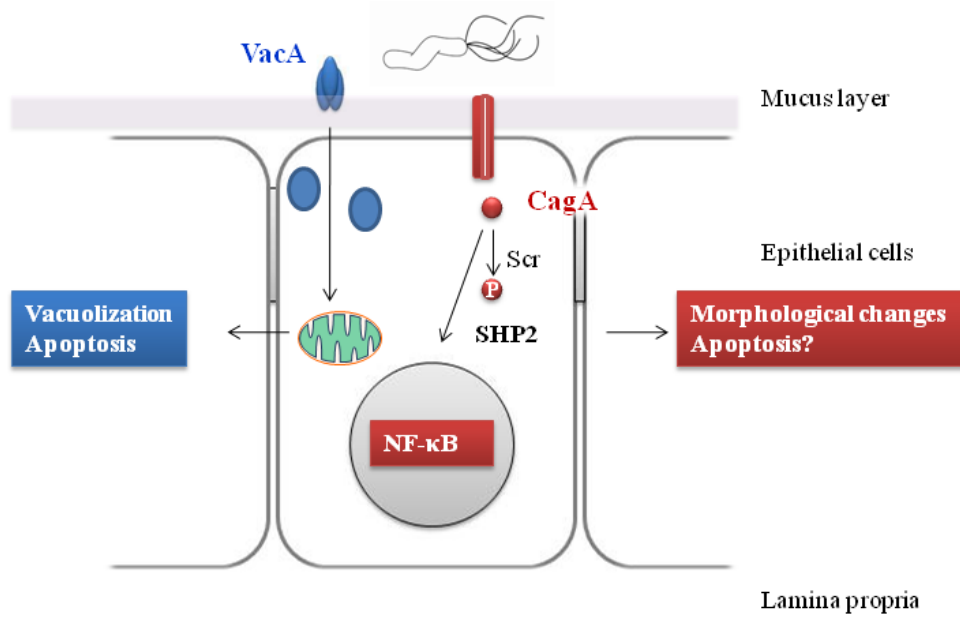
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## 2.2. Virulence factors involved in the induction of gastric lesions

**Urease** is not only essential in the *H. pylori* acid resistance (2.1.), the urease-mediated production of ammonia has also been shown to reduce the viability of gastric epithelial cells (Smoot et al., 1990). Ammonia or urease molecules are probably unable to directly induce apoptosis, but ammonia would accelerate cytokine-induced apoptosis in gastric epithelial cells (Igarashi et al., 2001). After damaging the mucosal barrier, nutrients for the microorganism are released, which in turn sustains the inflammation process (Smoot et al., 1990).

The **Cytotoxin-associated gene pathogenicity island** or *cagPAI*, is the most widely examined virulence factor of *H. pylori* (Cover et al., 1990). No homologs have been reported in any other *Helicobacter* species. Approximately 50 to 70% of *H. pylori* strains represent the *cagPAI*, rendering these strains more virulent (Tummuru et al., 1993; Cover et al., 1995; Ching et al., 1996; Akopyants et al., 1998). These so-called *cagA*<sup>+</sup> strains do not only induce significant antibody titers after infection, they also induce a higher inflammatory response and carry a significantly higher risk for the development of peptic ulcer disease or gastric cancer in Western populations, than avirulent strains, lacking the *cagPAI* (Kuipers et al., 1995;

Blaser and Crabtree, 1996; van Doorn et al., 1999). In Asian populations, this outcome was not observed (Peek et al., 1995; Beales et al., 1996; Go and Graham, 1996; Hamlet et al., 1999). The *cagPAI* consists of about 30 genes, and encodes a type IV secretion system (Akopyants et al., 1998) which mediates the formation of a syringe-like structure, that penetrates the epithelial cells in the stomach, delivering the CagA protein and possibly other bacterial factors into the host cells (Covacci et al., 1993; Segal et al., 1999; Asahi et al., 2000; Christie and Vogel, 2000; Odenbreit et al., 2000; Fischer et al., 2001; Backert et al., 2008). Once inside the cytoplasm of the host cell, the CagA protein is phosphorylated at tyrosine residues in EPIYA motifs (Segal et al., 1999; Asahi et al., 2000; Odenbreit et al., 2000; Stein et al., 2000; Higashi et al., 2002) by Scr family kinases (Figure 3) (Selbach et al., 2002; Stein et al., 2002). Phosphorylated CagA then interacts with tyrosine phosphatase SHP-2 (Higashi et al., 2002), which results in morphological changes in the epithelial cells, including elongation and spreading of the cells, which has been called “hummingbird” phenotype (Segal et al., 1999; Yamazaki et al., 2003). CagA also interacts with Grb2, which in turn indirectly leads to the activation of pro-inflammatory nuclear factor (NF)- $\kappa$ B, cytoskeletal rearrangements and proliferation of cells *in vitro* (Brandt et al., 2005; Mimuro et al., 2007). Both overexpression of pro-apoptotic proteins and (to a lesser extent) overexpression of anti-apoptotic proteins have been described after human infections with *cagA*<sup>+</sup> strains (Cabral et al., 2006). Mimuro et al. (2007) suggested that the anti-apoptotic effects of CagA may aid persistence, by maintaining the turnover of the epithelial cells to which *H. pylori* bacteria are attached.



**Figure 3:** The secreted vacuolating cytotoxin, **VacA**, induces the formation of large vacuoles in epithelial cells and mitochondrial-mediated apoptosis. Cytotoxin-associated protein, **CagA** is injected into host cells by a specialized type IV secretion system and causes alterations of the cytoskeleton, can induce apoptosis *in vitro* and signals the nucleus to induce nuclear factor (NF)- $\kappa$ B.

Another major virulence factor is the **vacuolating cytotoxin** (VacA), which is secreted by approximately 50% of all *H. pylori* strains (Cover and Blaser, 1992). VacA induces massive vacuolization in epithelial cells (Figure 3), which depends on the formation of membrane channels by this very same toxin (Cover and Blaser, 1992; Szabó et al., 1999). There is considerable variation in vacuolating activities among strains (Leunk, 1991; Cover and Blaser, 1992; Cover, 1996; de Bernard et al., 1997). Namely, the toxicity of VacA depends on variations of the signal region (s) and the middle region (m) of the encoding gene (Atherton et al., 1995; van Doorn et al., 1999). The s region of the gene, occurs as either an s1 or s2 type, whereas the m region, exists as an m1 or m2 type (van Doorn et al., 1999). The s1/m1 genotypes show the highest vacuolating activity and in line with this, these genotypes are more frequently associated with peptic ulcers and gastric carcinoma (Atherton et al., 1995). Vacuolating activity is intermediate in s1/m2 genotypes and absent in s2/m2 alleles. Atherton's group recently described an intermediate (i)-allele in *vacA* that determines the vacuolating activity among the s1/m2 strains (Rhead et al., 2007). They observed that s1/m2

strains that have an i1 allele are vacuolating, whereas s1/m2 strains that have an i2 allele are nonvacuolating.

Through the formation of pores in epithelial cell membranes, VacA induces the release of the urea, nutrients, cations and anions from host cells (Montecucco and de Bernard, 2003). Moreover, VacA is able to enter the cytosol, and accumulate in the mitochondrial inner membrane, causing a reduction of the mitochondrial transmembrane potential, thereby inducing apoptosis by release of the mitochondrial cytochrome c (Kimura et al., 1999; Willhite and Blanke, 2004). Interestingly, secreted VacA seems to be able to penetrate into tissues deeper than the epithelium, where it can interact with granulocytes, monocytes and B and T cells (Molinari et al., 1998). VacA has been shown to inhibit antigen presentation and T cell proliferation, but, unlike CagA, VacA does not seem to induce the apoptosis of T cells (Molinari et al., 1998; Wang et al., 2001; Gebert et al., 2003). As to the presence of VacA in NHPH-species, a *vacA* homolog has not been detected in *H. suis* strain 5, but the presence of a paralog could be confirmed for *H. suis* strain 1 (Vermoote et al., 2011). Up till now, the presence of VacA in other gastric non-*Helicobacter pylori* species could not be demonstrated.

It has been suggested that the *cagPAI* and *vacA* genes are able to reduce each other's effects on epithelial cells, which could lead to avoiding excessive cellular damage (Argent et al., 2008). Two complementary mechanisms, in which CagA inhibits VacA-induced apoptosis, were identified (Oldani et al., 2009). One depends on the prevention of pinocytosis of VacA by tyrosine-phosphorylated CagA, the other one on blocking of VacA-induced apoptosis at the mitochondrial level, which doesn't require phosphorylation of CagA.

**$\gamma$ - glutamyl transpeptidase** (GGT) activity is found in all gastric *Helicobacter* species including *H. suis*, and has a conserved function in the genus *Helicobacter* (Haesebrouck et al., 2009; Wachino et al., 2010; Rossi et al., 2012). The role of GGT as a virulence factor of gastric *Helicobacter* species, however, has only been investigated for *H. pylori* and, more recently, for *H. suis* as well. Membrane-associated *H. pylori* GGT activity plays an important role in the metabolism of glutathione (Orlowski and Meister, 1970), a free thiol maintaining an optimal intracellular redox environment (Circu and Aw, 2010). Moreover, *H. pylori* GGT is very efficient in using both glutamine and glutathione from epithelial cells as a source of glutamate (Shibayama et al., 2003). As glutamine and glutathione are important nutrients for maintenance of healthy gastrointestinal tissue, their depletion by the GGT enzyme probably accounts for the damage of gastric epithelial cells (Shibayama et al., 2007). Other studies demonstrated that the *H. pylori* GGT induces apoptosis of AGS cells via a

mitochondrial-mediated pathway and inhibits growth of these cells by inducing cell cycle arrest in the G1-S transition phase (Kim et al., 2007; 2010).

*H. pylori* GGT has also been shown to play a pivotal role in the inhibition of T-cell proliferation and in the upregulation of COX-2 and EGF-related peptide expression, suggesting a possible role in gastric carcinogenesis (Busiello et al., 2004; Schmees et al., 2007). Significantly higher GGT activity has been observed in *H. pylori* isolates obtained from patients with gastric ulcer disease than isolates from patients with non-ulcer dyspepsia (Gong et al., 2010).

Whole-genome screening of *H. suis* strain 5 has revealed the presence of a gene homologous to the *H. pylori* GGT (Vermoote et al., 2011). Further research showed that the enzyme catalyses the degradation of extracellular glutathione (Flahou et al., 2011). The degradation products, in turn, cause a cell-independent extracellular generation of H<sub>2</sub>O<sub>2</sub>, leading to lipid peroxidation and finally, necrosis. During this process, cellular contents, including inflammation-promoting molecules, are released from the gastric epithelial cells (Fink and Cookson, 2005; Vanlangenakker et al., 2008; Flahou et al., 2010; 2011).

**Neutrophil-activating protein** (HP-NAP), another *H. pylori* factor with pro-inflammatory capacities, was originally designated to stimulate the production of toxic reactive oxygen species (ROS) in neutrophils and promote adhesion of neutrophils to endothelial cells (Evans et al., 1995). HP-NAP was found to be chemotactic for human neutrophils and monocytes *in vitro* and to promote rapid neutrophil adhesion *in vivo* in rats (Satin et al., 2000; Polenghi et al., 2007). Furthermore, after crossing the epithelial monolayer, HP-NAP is able to activate underlying mast cells, monocytes and neutrophils, which in turn release pro-inflammatory cytokines (Montemurro et al., 2001; 2002; Amedei et al., 2006; Polenghi et al., 2007). A recent study showed that *in vitro* exposure of monocytes to HP-NAP, induced anti-apoptotic proteins, and reported that HP-NAP promotes survival of neutrophils in a monocyte-dependent manner (Capon et al., 2010). As a result, the lifespan of monocytes and neutrophils is increased, which further contributes to inflammation.

HP-NAP induces the expression of IL-12 and IL-23 by human neutrophils and monocytes, and has the potential to shift antigen-specific T cell responses from a predominant T helper (Th)2 to a Th1 cytotoxic phenotype (Amedei et al., 2006; D'Elis et al., 2007). As a result, high levels of interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$  are produced.

Homologs of the *napA* gene, have been found in the genomes of *H. suis*, *H. felis*, *H. bizzozeronii*, *H. heilmannii* and *H. mustelae* (O’Toole et al., 2010; Arnold et al., 2011; Schott et al., 2011; Vermoote et al., 2011; Smet et al., 2013).

**Table 2.** *H. pylori* virulence-associated genes for which homologues were detected in the genomes of *H. heilmannii*, *H. suis*, *H. felis* and *H. bizzozeronii*. The proteins they encode and the associated function are also presented. References: Arnold et al., 2011; Schott et al. 2011; Vermoote et al., 2011; Smet et al., 2013.

<i>H. pylori</i> virulence-associated genes	Protein	Associated function
urease gene cluster <i>ureABIEFGH</i>	Urease subunit alfa Urease subunit beta Urease transporter Urease accessory proteins	pH homeostasis
<i>tlpA</i> , <i>tlpB</i>	Methyl-accepting chemotaxis proteins <i>tlpA</i> , <i>tlpB</i>	Chemotaxis
<i>horB</i> , <i>hop</i> , <i>hof</i> , <i>hom</i>	Outer membrane proteins	Adhesion
<i>ggt</i> <i>napA</i>	$\gamma$ -glutamyl transpeptidase Neutrophil-activating protein A	Induction of gastric lesions

### 3. Recent developments in the diagnosis of gastric *Helicobacter* infections

At present, various tests for the diagnosis of *H. pylori* infections are readily available, and from these, tests were derived for the detection of other gastric *Helicobacter* species. These diagnostic methods include both non- or less-invasive and invasive procedures, which often need to be combined in order to ascertain a correct diagnosis. It should be stressed that the diagnosis of *Helicobacter* infections is often hampered by the focal distribution of the bacteria in the stomach and the very fastidious nature of the gastric *Helicobacter* species, with the NHPH-species in particular.

### 3.1. Non- or Less-invasive tests

The  $^{13}\text{C}$ -urea breath test ( $^{13}\text{C}$ -UBT) is considered to be a simple and safe non-invasive test, which is based on the marked urease activity present in all gastric *Helicobacter* species. Orally administrated  $^{13}\text{C}$ -labelled urea is broken down in the stomach by urease into ammonia and soluble  $^{13}\text{CO}_2$ . The latter is absorbed across the mucus layer to the gastric mucosa and hence, via the systemic circulation, expired in the exhaled breath. The amount of  $^{13}\text{C}$  can then be measured as a ratio of  $^{13}\text{C}$  to  $^{12}\text{C}$  in the patient's breath, or, in an adapted tool, in the serum (Logan, 1998; Graham and Klein, 2000; Gisbert and Pajares, 2004). The  $^{13}\text{C}$ -UBT protocol may be performed with relatively low doses (<100 mg) of urea, with the most common dose of urea at 75 mg. After breath collection, as early as 10-15 minutes after urea ingestion, analysis can be performed by nondispersive, isotope-selective infrared spectroscopy, laser-assisted ratio or isotope ratio mass spectrometry (IRMS). Since  $^{13}\text{C}$ -UBT combines a high sensitivity (88%-95%) and specificity (95%-100%) in *H. pylori*-infected adults, the test is used for both initial diagnosis and for the confirmation of eradication after treatment (Malfertheiner et al., 2012). In young children, however, a heterogenous accuracy was reported, with sensitivity and specificity levels between 75% and 100% before and after treatment of a *H. pylori* infection (Guarner et al., 2010).  $^{13}\text{C}$ -UBT performance variability in children might be due to (1) variation in urease activity from oral bacteria; (2) differences in the time at which the samples were obtained (eg. a remarkable decrease in specificity is found if samples are obtained at 15 minutes instead of 30 minutes); (3) variable cut-off values in different studies; (4) the tracer dose (for which a body weight adjustment has been suggested by some authors); and (5) pretest meal (Mégraud, 2005; Bik et al., 2006; Vaira et al., 2009, Leal et al., 2011). Since accurate, non-invasive tests for the diagnosis of *H. pylori* infections in younger children are highly preferred, several modifications of the protocol have been investigated. However, at present, performance criteria have not yet been sufficiently established (Machado et al., 2004). Likewise, the administration of  $^{13}\text{C}$ -urea capsules, which was proved effective in adults, cannot bring the desired solution, since this is not feasible in infants or toddlers (Vaira et al., 2009).

Assumptions should be taken with care, when  $^{13}\text{C}$ -UBT is carried out immediately after emergency endoscopy in peptic ulcer bleeding, since a relatively low sensitivity and specificity has been reported in these cases (Barkun et al., 2010; Velayos et al., 2012). Another specific group of patients, in which the identification of *H. pylori* infection is of great importance, are patients that have been subjected to a partial gastrectomy. In contrast with

what one could expect after partial surgical removal of the stomach, a positive and negative predictive value of 0.64 and 0.92 respectively, were observed for UBT (Wardi et al., 2012).

To the author's knowledge, no information is yet available about the sensitivity and specificity levels of the application of  $^{13}\text{C}$ -UBT in patients infected with gastric NHPH. Since a more focal distribution of these microorganisms has been described in the human stomach, accuracy of  $^{13}\text{C}$ -UBT might be less than is the case for *H. pylori*-infected patients (Haesebrouck et al., 2009).

UBT has been applied experimentally in cats and dogs too, in order to detect gastric infection with *Helicobacter spp.* (Cornetta et al., 1998). Next to a difficult usage in animals, a lower sensitivity was noted for  $^{13}\text{C}$ -UBT in comparison with cytological evaluation of impression smears or histological examination of biopsy specimens. The result of the test in dogs is not only influenced by the speed of gastric emptying and any recent antimicrobial therapy, but depends on the colonization level of *Helicobacter spp.* in the stomach as well (Cornetta et al., 1998). False negative results may therefore be obtained when there is only a low amount of helicobacters present in the gastric mucosa, while false positive results have been described when other urease positive bacteria are present. A more recent study, however, reported a sensitivity and specificity of 89% for  $^{13}\text{C}$ -UBT in dogs (Kubota et al., 2013).

Another non-invasive test developed for the detection of *H. pylori* antigens is the **stool antigen test** (SAT). This test forms a fast, safe, and reliable tool for the detection of *H. pylori* antigens in human fecal samples (Gisbert and Pajares, 2004). For this diagnostic method, which is usually recommended in children when  $^{13}\text{C}$ -UBT is not possible, no expensive equipment and medical personnel is needed (Cirak et al., 2007; Choi et al., 2011; Chehter et al., 2013). Two types of stool antigen tests are available: the enzyme immunoassay or EIA and an assay based on immunochromatography (ICA). In the EIA test (eg. The Testmate pylori antigen EIA), a drop of the suspended stool sample is mixed with a peroxidase-conjugated *H. pylori* antibody and, after incubation and washing steps, the optical density is measured (Suzuki et al., 2002). Early EIAs were based on polyclonal antibodies, and although they provided reliable results for the diagnosis of *H. pylori* infection, controversial results were sometimes observed in the post-eradication assessment (Odaka et al., 2002; Veijola et al., 2005). The more recently developed monoclonal antibody-based techniques generally have a higher specificity (Domínguez et al., 2006; Deguchi et al., 2009). Tests based on immunochromatography (ICA) are often presented as a strip (eg. Testmate rapid pylori antigen) to which a drop of suspended stool sample is applied (Suzuki et al., 2002). When *H.*

*pylori* antigens are present in the stool sample, they form immune complexes with the color-labeled monoclonal antibodies in the strip, which then migrate by capillary action. Next, these immune complexes are captured by secondary antigens and a visible line is formed on the strip. Although both types of tests are highly sensitive and specific, a recent study showed that currently available ICA-tests provide less reliable results than EIA-tests (Gisbert et al., 2006; Sato et al., 2012; Korkmaz et al., 2013). ICA-tests are, on the other hand, more easy to perform and can be used for on spot rapid diagnosis, since they don't require any specialized equipment (Shimoyama et al., 2011).

Several factors influence the accuracy of SATs. For example, the accuracy is lower when the stool samples are unformed, or watery, because *H. pylori*-specific antigens are diluted (Saez et al., 2012). Moreover, the sensitivity of SATs is lower in patients with gastrointestinal bleeding. Other factors that can affect the result of SATs are temperature, the interval between stool sample collection and measurement and the antigenicity of *H. pylori* strains (Ritchie et al., 2009; Queiroz et al., 2013).

Monoclonal antibody-based SAT was proved to be a more reliable non-invasive test than UBT in children, both in populations with high and low prevalences of *H. pylori* infection (Cardenas et al., 2008; Queiroz et al., 2013). Similarly, the accuracy of SAT was higher than UBT in patients who underwent distal gastrectomy, with a specificity of 90.5%, while that of UBT was only 59.1% (Adamopoulos et al., 2009; Yan et al., 2010).

Stool antigen tests, developed for the use in human medicine, are also useful to detect *H. pylori* in infected animal models, such as C57BL/6 mice (Moon et al., 2013). However, antigen assays for application in animal patients have not been developed yet.

Shinozaki et al. (2002) investigated whether *Helicobacter* infections in animals could be detected by using **stool samples in a PCR**, based on relatively conserved regions of the 16S rRNA gene. Sequences derived from fecal material were shown to reflect intestinal *Helicobacter* species, and hold no information on the infection status of the stomach. Moreover, the presence of numerous bacterial species, food degradation products and PCR inhibiting factors may hamper the PCR, rendering false negative results. In conclusion, stool specimens have been shown valuable for the detection of *H. pylori* infection in humans, but are so far not representative for the diagnosis of *Helicobacter* infections in animals. To date, no information is available about the value of stool-antigen tests in the detection of other gastric *Helicobacter* infections in humans.

**Serology** was one of the first diagnostic methods to be developed after the successful cultivation of *H. pylori* (Jones et al., 1984; Kaldor et al., 1985). Various antigen preparations have been examined, eg. preparations from whole cells, sonicated cell extracts, non-ionic detergent extracts, highly purified individual antigens and recombinant antigens (Herbrink and van Doorn, 2000). The highest sensitivity was shown for the use of complex antigen mixtures of various strains. Most commercial tests are based on IgG detection through enzyme immunoassay (EIA). Additionally, IgA antibody presence can be tested as well, only in some cases rendering a slight increase in sensitivity. One of the limitations of serology, is the failure to distinguish between past and present *H. pylori* infections, even after a successful eradication therapy (Hirschl and Rotter, 1996; Choi et al., 2011). Moreover, individual genetic differences have been shown to contribute substantially to the formation of *H. pylori* antibodies. The success of a serological test also depends on the use of antigens that are present in *H. pylori* strains in a given population, since considerable heterogeneity of *H. pylori* strains has been described for different geographical areas (Vale et al., 2008; 2009; Vitoriano et al., 2011). Therefore, a local validation of serological tests has been considered essential (Malfertheiner et al., 2007). The use of high-molecular-weight, well conserved antigens should overcome this limitation as well (Marchildon et al., 2003).

In a recent study, the performance of 29 different serological test kits was investigated, demonstrating sensitivities ranging from 55.6% to 100% and specificities ranging from 59.6% to 97.9% (Burucoa et al., 2013). Based on these figures, both the urea breath test (UBT) and the monoclonal stool antigen test are assumed to be superior methods than serology for the detection of acute *H. pylori* infections and for therapeutic follow-up (Herbrink and van Doorn, 2000; Malfertheiner et al., 2007). There are, however, several indications for which serology is believed to be the method of choice. Serology is not only recommended for initial screening, in which case further confirmation by histology and/or culture is necessary, but is also highly relevant in patients receiving antimicrobial treatment or presenting acute bleeding (Rubicz et al., 2011; Mégraud, 2012). Serologic tests have also been appreciated in the evaluation of the association between *H. pylori* and gastric carcinoma or extraintestinal diseases, and in the context of epidemiologic use and vaccination studies.

At present, no test for the identification of NHPH-antigens in blood samples of humans or animals are available. Obviously, there is a need for a simple and reliable tool to achieve diagnosis with identification up to the species level.

Several studies investigated the presence of *H. pylori* in **human dental plaque and saliva** by conducting PCR, culture, determining urease activity, and quantifying specific oral antibodies (Marshall et al., 1999; Bode et al., 2002; Cellini et al., 2010). Studies performed with saliva of adult patients show an overall sensitivity of 81 to 94% and a specificity of 70 to 90% compared with endoscopy-based methods (Luzza et al., 1997; De Pascalis et al., 1999; Sunnerstam et al., 1999; Malaty et al., 2000). Bode and co-workers evaluated saliva IgG against *H. pylori* in children (Bode et al., 2002). Results were compared in parallel with <sup>13</sup>C-UBT results, leading to a sensitivity and specificity of the salivary antibody ELISA of 80.9% and 95.3% respectively. These findings add strength to the assumption that salivary ELISA is a reliable, convenient, and easy-to use tool, which may be highly valuable in the design of diagnostic and epidemiological studies. However, the test should be further evaluated in young children, in whom immune responses might not be fully developed (Raymond et al., 1996).

Several methods have been assessed in the detection of *H. pylori* DNA in the oral cavity, concluding PCR as the most suitable (Teoman et al., 2007). Using PCR, *H. pylori* has been detected in the mouth of both patients with and without a history of gastric upset (Teoman et al., 2007; Souto and Colombo, 2008). Furthermore, the prevalence of *H. pylori* in the oral cavity may be independent of the prevalence of this agent in the stomach after successful treatment of infection, indicating the possibility of gastric reinfection through the oral cavity as a reservoir (Gebara et al., 2006).

### 3.2. Invasive tests

#### **Endoscopy guided gastric biopsy**

Endoscopic evaluation, in the diagnosis of *H. pylori* infections, has received increased attention as endoscopic imaging can improve early detection of gastric cancer.

Recently, the diagnostic yield of endoscopy for *H. pylori* infections was examined (Watanabe et al., 2013). Interestingly, intra-observer agreement for *H. pylori* infection status was good for all endoscopists, while inter-observer agreement was lower for physicians at beginning level. Evenso, for all physicians, good inter-observer agreement in endoscopic findings was shown for atrophic change of the mucosa, but a lower accuracy was found for beginning endoscopists.

Several studies emphasize the high risk of gastric cancer development in patients with *H. pylori*-associated highly-active non-atrophic gastritis. This form of inflammation can be

assessed by a combination of (1) serum levels of pepsinogen (PG) I and II, (2) *H. pylori* antibodies and (3) a specific endoscopic feature: endoscopic rugal hyperplastic gastritis (RHG) (Watanabe et al., 2012). Rugal hyperplastic gastritis presents large and tortuous gastric folds, with a fold width  $\geq 5$  mm (Oguri et al., 2007). In patients with RHG, high *H. pylori* antibody titers and a low PG I/II ratio, a significantly higher cancer incidence was described. After *H. pylori* eradication, however, no cancer development was observed in these high-risk subjects.

The optimal biopsy site for *H. pylori* in patients undergoing endoscopic mucosectomy (EMR) has repeatedly been the topic of research (Calvet et al., 2010; El-Zimaity et al., 2013). Considering the antrum, corpus lesser curve (CLC), and greater curve (CGC), the authors designated the CGC as the optimal biopsy site for *H. pylori* diagnosis in EMR patients with extensive atrophy.

Attempts have been made to categorize the endoscopic findings characteristic of an *H. pylori*-infected stomach. Diffuse redness, spotted redness, and mucosal swelling have been indicated as useful for diagnosing *H. pylori*-infections (Kato et al., 2013). Most of these findings, however, have a rather low sensitivity and specificity, and their presence therefore form no reliable diagnostic method.

In the future, multi-center trials, in which standard and magnifying endoscopy are compared, as well as the different levels of experience of the endoscopists, are necessary to confirm previously stated data. At this moment, endoscopy is necessarily combined with gastroscopic biopsy-based tests, such as the rapid urease test (RUT), histological examination, and culture for diagnosis of *H. pylori* infections.

### **Histological examination of gastric biopsies**

Spiral-shaped bacteria, present in patients with active gastritis, were first described by Warren through histological examination, and formed the true onset to culture *H. pylori* (Warren and Marshall, 1983). Next to the Warthin-Starry silver stain, used by Warren, numerous other stains were found suitable for the detection of *H. pylori* in gastric biopsy specimens, brush cytology and imprint cytology specimens: modified Giemsa, haematoxylin eosine, Gram, Genta, toluidine blue, Romanouski and immunohistochemical methods (Warren and Marshall, 1983; Mendoza et al., 1993; Misra et al., 1993). A recent study confirmed that in most cases, immunohistochemical (IHC) stains are not cost-effective or necessary and that HE staining remains the most expedient test for the identification of *H. pylori* (Wang et al. 2010). The use of special stains should, therefore, be reserved for biopsy specimens with moderate to severe chronic active or inactive gastritis, post-treatment biopsy specimens and for cases in which

suspicious structures are observed by HE staining, without leading to a definite diagnosis (Aggarwal et al., 2011).

A major disadvantage, is the contribution of the frequently-used proton pump inhibitors (PPIs) in creating false negative diagnosis. Because the number and distribution of *H. pylori* organisms in patients using PPIs is variable, it has been recommended to discontinue PPIs two weeks before endoscopy (El-Zimaity et al., 2013). Nevertheless, histology is considered as the standard method for the diagnosis of *H. pylori* infection, additionally providing information on the status of the mucosa, eg. the presence of acute or chronic inflammation, lymphoid aggregates, intestinal metaplasia, and glandular atrophy (Lash and Genta, 2013). The diagnostic reliability of histological examinations largely depends on the number and localization of biopsies collected, which is of special importance in patients with atrophy (El-Zimaity et al., 2013). As indicated in the previous paragraph, specimens obtained from the greater curve (CGC), appear to be best suited for *H. pylori* detection in these patients. Interestingly, the sensitivity of biopsy-based tests seems to decrease as the degree of atrophic gastritis increases, regardless the biopsy location (Lan et al., 2012).

It should be stressed that histology and cytology are the only routinely available methods for the detection of other gastric *Helicobacter* species in human biopsy-specimens (Debonnie et al., 1995). However, microscopic investigation of biopsy samples is not an accurate method for species identification, since morphology can slightly alter. In fact, electron microscopy (both scanning and transmission), is the only effective tool for the differentiation of NHPH species by morphology. Since specific fixation procedures are time-consuming, and the necessary equipment is expensive, electron microscopy is only sporadically applied for identification (Happonen et al., 1998; Stoffel et al., 2000). Furthermore, visualization of NHPH-microorganisms is hampered by the focal distribution of these bacteria and the morphological differences among these species are not always obvious (Happonen et al., 1998). Therefore, identification up to species level, based on *in situ* electronal microscopical images remains very difficult.

### **Rapid Urease Test on gastric biopsies**

The rapid urease test (RUT) is based on the urease activity of *Helicobacter* species, which results in the formation of ammonia, as was previously elucidated for the <sup>13</sup>C-urea breath test (3.1.). Many commercial RUTs are available, including gel-based, paper-based and liquid-based tests. Depending on bacterial load in the biopsy specimen and the format of these tests,

they provide a result in 1-24 hours (Tseng et al., 2005). A major advantage of these tests is their relative low cost, in comparison with histology and culture (Ozaslan et al., 2010; Choi, 2012). Overall, commercial RUTs show a high specificity of 95% to 100%; the sensitivity is less, ranging from 85% to 95%. In patients with bleeding peptic ulcers or partial gastrectomy, however, the sensitivity can decrease down to 67%-85%, and 79% respectively (Tseng et al., 2005; Gisbert et al., 2006; Choi et al., 2012; Tian et al., 2012). A reduced sensitivity has also been reported in case of formalin contamination of the forceps, used for collection of the biopsies (Ozaslan et al., 2010; Choi et al., 2012). A higher sensibility of RUT can be obtained by increasing the number of biopsy specimens and including various regions from the stomach (Hsu et al., 2010). In addition, combining different specimens prior testing, improves the detection rate and speed, compared to testing with separate specimens (Moon et al., 2012).

Measuring the urease activity in a certain time period, might allow a semi-quantitative determination of the colonization density (Happonen et al., 1998). False negative results, however, are more frequently encountered in the case of low grade colonization, than for example with histology or molecular techniques.

### **Culture of gastric biopsies**

Culture is being considered the gold standard for *H. pylori* diagnosis, since it can be performed directly from biopsy samples and has a specificity of up to 100% (Ndip et al., 2003). Although sensitivity was reported > 90%, significant variation have been shown. These mainly depend on the quality of the specimens and transportation conditions. Moreover, immediate attention is necessary for specimens obtained for culture, since *Helicobacter* organisms are very fastidious and fragile outside their native environment (Ricci et al., 2007).

Despite the common presence of spiral bacteria in the gastric mucosa of pigs, dogs and cats, isolation and cultivation of NHPH form a very laborious process with low success rate (Happonen et al., 1998; Jalava et al., 1998). Jalava and co-workers conducted one of the more successful isolation procedures, obtaining an isolation rate of up to 51% from canine stomachs (Jalava et al., 1998). The majority of the isolated strains were identified as *H. bizzozeronii* (55.6%), while *H. felis* and *H. salomonis* were both identified in 22.2% of the cases. In cats, however, the isolation rate was only 13.6%, and all isolated strains were designated as *H. felis*.

Only a few years ago, a successful *in vitro* isolation method for *H. suis* was described, using a new biphasic culture method (Baele et al., 2008). After genomic fingerprinting of the *in vitro* isolates HS1, HS2, and HS3 by amplified fragment length polymorphism (AFLP),

distinct AFLP patterns were revealed for these 3 isolates. Since the description of this isolation method, 20 *H. suis* strains have been isolated from the gastric mucosa of pigs from different herds. These *in vitro* isolates are, to the author's knowledge, the only available isolates worldwide. Until now, no *H. suis* isolates, originating from infected humans, have been obtained. In fact, only few studies report successful isolation of NHPH species from human gastric biopsies. A German study described the first isolation of *Helicobacter felis* from an infected human (Wüppenhorst et al., 2012). The authors succeeded by using their routine *H. pylori* isolation protocol, on gastric biopsy specimens from a 14-year-old girl, presenting with persistent epigastric pain and vomiting episodes. The only other NHPH-species that has been successfully cultivated from gastric biopsy tissues of human patients is *H. bizzozeronii* (Andersen et al., 1999; Kivistö et al., 2010; Schott et al. 2011).

In conclusion, to date, culture-based methods for routine diagnosis of NHPH-infections in animals and humans are currently not feasible.

### **Polymerase chain reaction analysis on gastric biopsies**

Molecular tests are frequently applied in the diagnosis of infections, due to their speed, accuracy, and sensitivity. In addition, these tests not only allow diagnosis, but also the detection of virulence factors, and in case of quantitative (q)PCR bacterial quantification. Moreover, molecular detection of microorganisms is also possible in a number of materials obtained by non-invasive methods (eg. stool and saliva specimens) (Fontana et al., 2003; Schabereiter-gurtner et al., 2004). PCR, and real-time (RT) PCR in particular, not only show good diagnostic reliability, they are also valuable for the detection of clarithromycin resistance in *H. pylori* (Schabereiter-Gurtner et al., 2004). Since clarithromycin forms one of the key agents in the treatment of *H. pylori* infections, questions were raised regarding the usefulness of the classical “test and treat” strategy. A culture-independent molecular test, in recognition of clarithromycin resistance, would allow the prudent use of this antimicrobial drug and would probably dramatically reduce treatment failures.

PCR-analysis of the *VacA* gene in gastric biopsies, has shown to be very valuable in patients with severe precancerous lesions (intestinal metaplasia type III and dysplasia) (de Martel et al., 2010). In this setting, PCR displayed a significantly elevated sensitivity for the detection of *H. pylori* infection in comparison with histopathology.

Since the recognition of “other” gastric helicobacters, several PCR protocols have been developed, mostly based on a part of the 16S rRNA or the urease gene (De Groote et al., 2001; Norris et al., 1999; Hwang et al., 2002; Baele et al., 2004; O'Rourke et al., 2004). In a

study by Ekman et al (2013), two multiplex PCR reactions with three, species-specific primer pairs in each reaction, were assessed on saliva, stomach, duodenum and faeces samples of dogs. All dogs harboured more than two different *Helicobacter spp.* in their gastric mucosa, and in two dogs four different species were found. A high prevalence for *H. bizzozeronii*, and *H. salomonis* was reported in the stomach of clinically healthy dogs, while DNA of these species was only occasionally detected in saliva.

#### 4. Host response to gastric NHPH infections

In contrast with the scarce information on host immune responses against NHPH, several studies evaluated the innate and acquired immune responses against *H. pylori*. Instead of reviewing the available literature on host immune responses against *H. pylori*, the following section is focused on the recent insights on the acquired immune response elicited by *H. suis* and other gastric NHPH species, reflecting on similar or opposite observations made for *H. pylori*.

One study reported an increased messenger (m)RNA expression of **IFN- $\gamma$**  and **IL-10** in the gastric mucosa of *H. suis* infected mice, indicating both **T-helper 1 (Th1)** and **T-helper 2 (Th2)** responses are associated with infection (Park et al., 2008). Other studies reported an upregulation of mRNA expression levels of IFN- $\gamma$  in the murine gastric mucosa, indicating a Th1 response (Nobutani et al., 2010; Mimura et al., 2011; Vermoote et al. 2012; 2013). In a more recent study, pure *H. suis* cultures were used for experimental infection of both BALB/c and C57BL/6 mice, which led to a mild up-regulation of the Th2 signature cytokine, **IL-4**, only (Flahou et al., 2012). In comparison, a Th2 response which is associated with a suppression of the inflammatory response and with increased *H. pylori* colonization, has been observed in the *H. pylori*-infected gastric mucosa too (Serrano et al., 2007; Robinson and Atherton, 2010). The predominant T cell response to *H. pylori* in humans, however is formed by a CD4<sup>+</sup> T cell or Th1 response (Fan et al., 1994; D'Elis et al., 1997; Bamford et al., 1998). These Th1 cells are associated with reduced bacterial colonization in the human gastric mucosa as well as in mouse models (Holck et al., 2003; Lucas et al., 2001; Stoicov et al., 2004). For *H. pylori*, the Th1 cell-mediated responses and cytokines are known to be important in the development of infection-induced gastric pathology (Mohammadi et al., 1996; Smythies et al., 2000; Sommer et al., 2001). In addition, IFN- $\gamma$  itself appears to be a key mediator in the development of pre-cancerous gastric atrophy, metaplasia and dysplasia. *In vitro* studies have suggested a number of *H. pylori* virulence factors to promote Th1 responses. HP-NAP promotes the expansion of IFN- $\gamma$ -producing cells, while in contrast, it aids in the

decrease of IL-4-secreting cells, which are part of a Th2 immune-response (Amedei et al., 2006). Whether the *H. suis* homolog of HP-NAP inhibits a similar role in the modulation of the host response remains to be elucidated.

Next to a Th1 and Th2 response, **Th17 cells** have also been identified in the response to *H. suis* and *H. pylori* infections respectively in mice and humans (Luzza et al., 2000; Mizuno et al., 2005; Vermoote et al., 2012; 2013). Th17 cells secrete IL-17 (which is known as a pro-inflammatory cytokine) and play a role in the induction of IL-8 secretion and the recruitment of neutrophils to the site of infection. Moreover, a Th17 response, associated with elevated gastric IL-1 $\beta$ , has been described in patients with past *H. pylori* infection, suggesting that persistent inflammation might contribute to the development of gastric cancer (Serelli-Lee et al., 2012). Expression of both IL-17 and IL-6 (promoting Th17 response) mRNA are negatively correlated with *H. suis* colonization in mice (Flahou et al., 2012). Although some authors suggest the opposite, most researchers agree there is a similar, negative correlation between a Th17 response and *H. suis* or *H. pylori* colonization levels (Scott Algood et al., 2009; Kao et al., 2010; Shi et al., 2010; Vermoote et al., 2011; 2012).

Moreover, a significant correlation between the reduction of *H. suis* colonization levels and decreased expression levels of IL-10 was observed during vaccination studies in mice (Vermoote et al., 2011; 2012). IL-10, an anti-inflammatory cytokine is produced by **regulatory T cells** or Tregs (Roncarolo et al., 2006). Tregs are mainly CD4<sup>+</sup> cells, that have a key role in the prevention of unwanted immune-mediated responses, such as allergies and autoimmunity diseases and are currently considered as a major obstacle in *Helicobacter* vaccination studies (Coombes et al., 2005; Hawrylowicz and O'Garra, 2005; Roncarolo et al., 2006). Kao and co-workers showed that, after *in vitro* exposition to *H. pylori* antigens, dendritic cells turned from a Th17/Th1 response toward Treg differentiation, thereby hampering an effective cell-mediated immune response against *H. pylori*, and probably *H. suis* infections too (Kao et al., 2010).

As the **B-cell response** is believed to play an important role in the pathogenesis, Vermoote et al. (2011; 2012) observed a significant correlation between the reduction of gastric *H. suis* colonization and an increase of antigen-specific antibodies in vaccinated mice. These findings correspond with some *H. pylori* vaccination studies, whereas others described a successful immunization of B-cell deficient mice, indicating that antibodies are not essential for protection (Czinn et al., 1993; Ermak et al., 1998; Blanchard et al., 1999; Goto et al., 1999; Sutton et al., 2007; Garhart et al., 2003a; 2003b; D'Elios et al., 2007; Jeremy et al., 2006; Morihara et al., 2007). Indeed, the effect of antibodies on the bacterial colonization remains

controversial. Intra-gastric administration of *H. felis* monoclonal IgA was shown to mediate protection against infection in mice in one report (Czinn et al., 1993). In other reports, specific IgA and IgG was shown to promote bacterial colonization (Akhiani et al., 2004; 2005). Overall, future research should elucidate which role humoral and/or local antibodies play in the host response to NHPH-infections.

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# **Scientific aims**



*Helicobacter (H.) suis* is the most prevalent non-*Helicobacter pylori Helicobacter* (NHPH) species in humans suffering from gastric disorders. *H. suis* gastritis more frequently leads to mucosa-associated lymphoid tissue (MALT) lymphoma than gastritis associated with *H. pylori* infections, which further emphasizes its clinical relevance. The exact source of *H. suis* infections for humans is still not known. However, contact with pigs or pork products represents a plausible mode of transmission. Demonstrating *H. suis* infections in humans with gastric disease is far from being simple. Among other things, it is hampered by the very fastidious nature of this bacterium and its focal distribution in the stomach. Detecting anti – *H. suis* antibodies could offer a valuable alternative.

The **general aims** of the present thesis were to identify potential sources of infection with *H. suis* for humans and to investigate the possibility of improving the diagnostic methods used in human clinical practice. The specific aims are given below.

**The first specific aim** (Chapter 1) was to determine whether *H. suis* bacteria can persist in pork. As *H. suis* is extremely difficult to isolate, the development of a non-culture based method to quantify viable *H. suis* bacteria was needed. Such method will enable to study *H. suis* occurrence in pork and to determine if *H. suis* is still present at retail.

Subsequently, **the aim of the second study** was to examine *H. suis* occurrence on pork carcasses at slaughter, elucidating their potential importance in the food chain (Chapter 2).

In contrast with the well-established diagnostic tests for *H. pylori* infections, the diagnosis of *H. suis* (and by extension all other gastric NHPH) infections in humans, poses a challenge. *Helicobacter suis* specific serology could provide a simple, reliable diagnostic method. The **third specific aim of this thesis** (Chapter 3) was to identify *H. suis*-specific protein antigens which elicit an immune response in infected humans and could therefore be of value as target in future serological test methods.



# **Experimental studies**



# Chapter 1

## Survival of *Helicobacter suis* bacteria in retail pig meat

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**Abstract**

*Helicobacter (H.) suis* colonizes the gastric mucosa of pigs world-wide and is the most prevalent non-*Helicobacter pylori Helicobacter* species in humans. This agent might be transmitted to humans by manipulation or consumption of contaminated pork. *H. suis* is a very fastidious micro-organism and is extremely difficult to isolate. Therefore, we developed a non-culture dependent, quantitative detection method allowing differentiation of viable from dead *H. suis* bacteria in pork. This was established by a combination of ethidium bromide monoazide (EMA) treatment and real-time (RT)-PCR. This EMA RT-PCR was applied to 50 retail pork samples. In two samples, viable *H. suis* bacteria were detected. Sequence analysis of the obtained PCR products confirmed the presence of *H. suis* DNA. Viable *H. suis* bacteria persisted for at least 48 h in experimentally contaminated pork. In conclusion, consumption of contaminated pork may constitute a new route of transmission for *H. suis* infections in humans.

## 1. Introduction

Gastric diseases in humans, such as gastritis, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma, have been commonly associated with *Helicobacter (H.) pylori* infections (Kusters et al., 2006, Marshall and Warren, 1984, Parsonnet et al., 1991a, Parsonnet et al., 1991b, Parsonnet et al., 1994 and Parsonnet, 1994). *H. pylori*, however, is not the only *Helicobacter* species capable of colonizing the human gastric mucosa. Spiral-shaped non-*H. pylori* helicobacters (NHPH) have been demonstrated in 0.1–6.2% of all human gastric biopsies (Baele et al., 2009, Coman et al., 1996, Heilmann and Borchard, 1991, Ierardi et al., 2001, Mention et al., 1999, Stolte et al., 1994, Stolte et al., 1997 and Yali et al., 1998). The pig-associated bacterium *H. suis* comprises 13.9% to 78.5% of human NHPH infections, which makes this species the most prevalent gastric NHPH in humans (De Groote et al., 2005, Haesebrouck et al., 2009, Trebesius et al., 2001 and Van den Bulck et al., 2005). In a recent study, results of multilocus sequence typing revealed that human-and pig-derived *H. suis* strains are closely related, indicating that *H. suis* infections in humans indeed originate from pigs (Liang et al., 2013). It has been suggested that *H. suis* bacteria might be transmitted to humans by consumption of contaminated pork (Van den Bulck et al., 2005). However, nothing is known concerning the presence and persistence of *H. suis* bacteria in pork. Isolation and cultivation of *H. suis* is a very laborious process with a low success rate, rendering detection of live *H. suis* bacteria in food by using culture-based methods not feasible. Using ethidium monoazide (EMA) in combination with real time (RT)-PCR, detection and differentiation of viable vs. dead bacteria in mixed cell populations has been established in several other bacterial taxa (Cawthorn and Witthuhn, 2008, Nocker and Camper, 2006, Nogva et al., 2003 and Rudi et al., 2005). EMA, a fluorescent dye, penetrates compromised cytoplasmic membranes of dead cells and subsequently intercalates into the double helical DNA (Nogva et al., 2003, Rudi et al., 2005, Hixon et al., 1975a and Hixon et al., 1975b). Subsequent photoinduced cross-linking inhibits PCR amplification of DNA from dead cells.

The aim of this study was to determine whether pig meat constitutes a possible source of *H. suis* infections for humans. Therefore, a protocol for pork sample treatment with ethidium monoazide (EMA) in combination with (RT-)PCR was developed for the selective detection of viable *H. suis* bacteria. Using this technique, the persistence of *H. suis* bacteria in pig meat was evaluated and the occurrence in pork at retail was assessed.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

*H. suis* strain HS5, which was isolated from the gastric mucosa of a sow according to the method described by Baele et al. (2008), was used in this study. The bacteria were grown on *Brucella* culture plates (Oxoid, Basingstoke, UK) supplemented with 20% heat inactivated fetal calf serum (HyClone, Logan, UT, USA) and Vitox and Skirrow supplements (Oxoid) topped with *Brucella* Broth (Oxoid). Both agar and broth pH were adjusted to 5 by adding HCl. Growth was established in a microaerobic atmosphere (85% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% O<sub>2</sub>) at 37 °C.

### 2.2. Preparation of viable and dead *H. suis* suspensions

After 72 h of incubation, *H. suis* bacteria were harvested from the liquid phase of the biphasic medium and the concentration of viable bacteria per ml was counted with a Neubauer counting chamber. Tenfold dilution series (10<sup>8</sup> to 1 ml<sup>-1</sup>) of live *H. suis* bacteria were prepared in duplicate (series 1 and 2). All dilutions of series 1 were heated for 10 min at 100 °C. Series 2 included viable *H. suis* bacteria. Viability after heat-treatment was evaluated by examining the motility of the bacteria by light-microscopy and by cell viability assessment using the LIVE/DEAD® BacLight™ Viability Kit (Invitrogen, Carlsbad, CA, USA).

### 2.3. EMA treatment of viable and heat-killed *H. suis* bacteria

Ethidium monoazide (EMA) (Sigma, St. Louis, MO, USA) was dissolved in 4 ml dimethylformamide (DMF) and 1 ml RPMI-1640 Medium (Sigma) to obtain a stock concentration of 1 mg ml<sup>-1</sup>. In different repeats, all dilutions of both series 1 and 2 were treated with final concentrations of either 10 or 20 µg ml<sup>-1</sup> EMA in 24 well cell culture plates. The samples were covered in aluminum foil and placed on a shaker (600 RPM) at room temperature for 10 min. Thereafter, all samples were placed on ice and irradiated for 2, 5 or 10 min with a 500 W halogen light source, which was placed at a distance of 25 cm. This protocol was then repeated, adding the same concentration of EMA to the samples, and applying the same period of incubation and irradiation. Next, all suspensions were transferred to Eppendorf tubes, pelleted by centrifugation at 5000 ×g, and washed twice to remove unbound EMA. Final pellets obtained by 5 min centrifugation at 5000 ×g were resuspended in 250 µl Hanks' Balanced Salt Solution (HBSS–GIBCO®, Invitrogen).

## 2.4. DNA isolation and quantification

DNA extraction of bacterial suspensions was done by using PrepMan Ultra (Invitrogen), according to the manufacturer's protocol. For optimization of the EMA staining technique, conventional PCR (40 cycles; annealing temperature at 60 °C) was performed using the following primers: UreSu 531 FW (5'-CAC CAC CCC GGG GAA GTG ATC TTG-3') and UreSu 783 RV (5'-CTA CAT CAA TCA AAT GCA CGG TTT TTT CTT CG-3'), resulting in a 253-bp PCR product of the *ureA* gene of *H. suis*, as visualized using 1.5% agarose gel electrophoresis. For subsequent experiments the same primer set was used for quantitative RT-PCR. RT-PCR was performed on each sample of both series, to determine the number of *H. suis* bacteria ml<sup>-1</sup>. An external standard, comprising tenfold dilutions of a 1541 bp *ureA-ureB* gene segments, was used starting at 10<sup>10</sup> amplicons for each 10 µl of reaction mixture (primers: forward 5'-GGTGTTGCCCATATGATTCA-3'; reverse 5'-CGAATCCTAGAGTCAGCAA-3'). The amplicons were purified with Invisorb® Fragment Cleanup (according to the manufacturer's instructions; Invitex, Berlin, Germany). The copy number concentration was calculated based on the expected length of the amplicon and its dsDNA amount. The RT-PCR-mix, with a total volume of 10 µl per well, consisted of 5 pmol of UreSu 531 FW and UreSu 783 RV primers, 5 µl of iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and 1 µl extracted DNA. RT-PCR was performed on a CFX96 RT PCR System with a C1000 Thermo Cycler (Bio-Rad). The cycling conditions were an initial cycle at 95 °C for 15 min, followed by 40 cycles at 95 °C for 20 s and 60 °C for 30 s. Both standard and samples were run in duplicate.

## 2.5. Application of EMA RT-PCR to detect viable *H. suis* bacteria in artificially contaminated pork

To determine the usefulness of the EMA RT-PCR technique in detecting live *H. suis* bacteria in pig meat, minced pork was spiked with a known number of either live or dead *H. suis* bacteria. Therefore tenfold dilution series (10<sup>8</sup> to 1 ml<sup>-1</sup>) containing live or dead *H. suis* bacteria were prepared as described before. For 25 g of minced pork, 250 µl of each dilution was added, as well as 5 ml Hank's Balanced Salt Solution (HBSS–GIBCO®, Invitrogen). Minced pork with HBSS was included as a negative control. Next, each spiked pork sample was homogenized in a stomacher for 2 min by using stomacher bags with a built-in filter membrane and with a pore size of 70 µm (BagPage®, Interscience, St Nom la Bretêche, France). All stomacher bags were sealed and incubated at 4 °C. Each pork mixture was

homogenized again as described before, after 0, 24 or 48 h of incubation. Subsequently, 250  $\mu\text{l}$  of the obtained filtered suspensions were treated with EMA twice as described above. In addition, in a second group of spiked pork samples, 250  $\mu\text{l}$  of each suspension was centrifuged for 5 min at 5000  $\times g$ , without prior EMA-treatment. All pellets were washed twice in 1 ml HBSS. After a final centrifugation at 5000  $\times g$  for 5 min, the pellets were treated with PrepMan Ultra (Invitrogen) according to the manufacturer's protocol, whereupon they were subjected to the same RT-PCR as described before.

## **2.6. Detection of viable *H. suis* in commercial pork samples**

Fifty vacuum wrapped minced pork samples, purchased at 4 different local supermarkets over a period of 14 weeks, were submitted to the double EMA RT-PCR technique as described above. Positive PCR products were subjected to sequence analysis of the *ureA* gene to confirm the identification of *H. suis* bacteria.

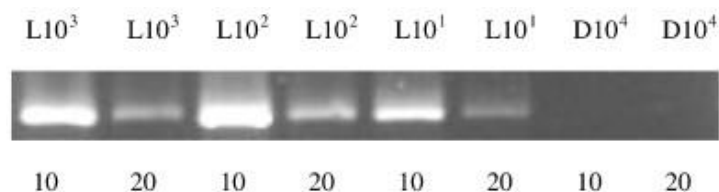
## **3. Results**

### **3.1. Optimization of EMA-PCR for detection of viable *H. suis* bacteria**

Death of 100% of the *H. suis* bacteria by heat-treatment for 10 min at 100 °C could be confirmed, both by microscopic examination of motility of the bacteria and by live/dead staining.

Single EMA-treatment of *H. suis* bacteria was unsuccessful for sound differentiation of viable from dead bacteria (data not shown). When EMA concentrations of 50  $\mu\text{M}$  or higher were used, this resulted in unspecific inhibition of DNA amplification. Using lower concentrations of EMA, however, resulted in an incomplete suppression of PCR products generated from dead cells. By repeating the EMA treatment before PCR-amplification, only samples containing viable *H. suis* bacteria showed a band on an agarose electrophoresis gel. DNA from heat-killed *H. suis* bacteria was not amplified. These results were obtained for both EMA concentrations (10  $\mu\text{g ml}^{-1}$  and 20  $\mu\text{g ml}^{-1}$ ) with an irradiation time of 5 min and are shown in Fig. 1. A light exposure time of 2 min, on the other hand, resulted in an inhibition of amplification of the DNA from the viable bacteria. An irradiation time of 10 min also resulted in insufficient distinction between viable and dead bacteria. Probably, live bacteria were killed by heat during this procedure. Taken together, the double treatment with EMA at a concentration of 10  $\mu\text{g ml}^{-1}$  or 20  $\mu\text{g ml}^{-1}$  gave equivalent results, when an

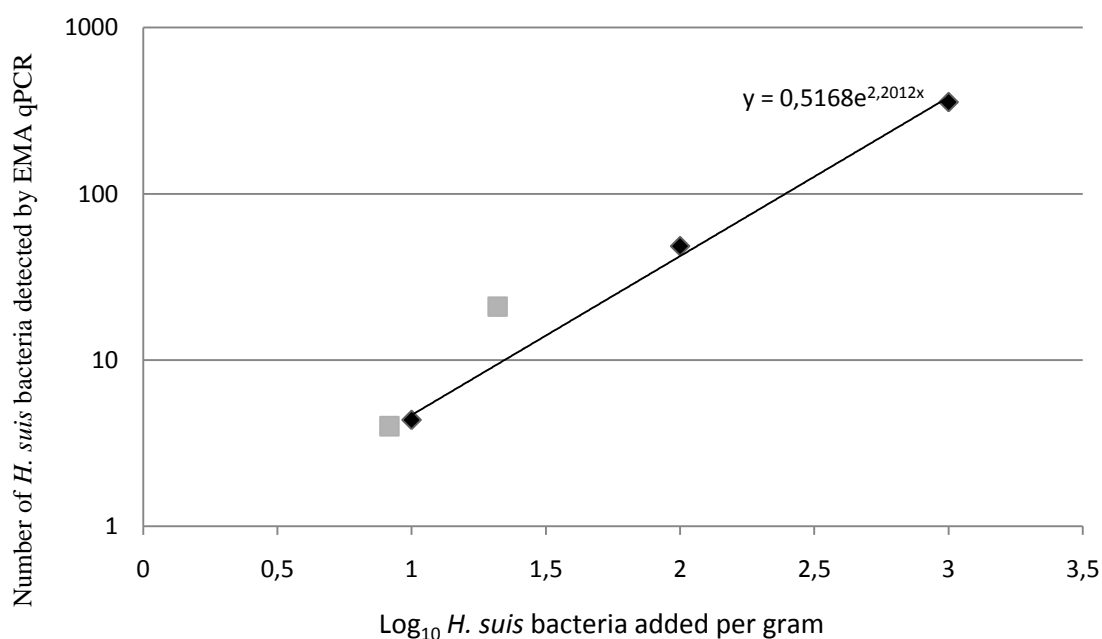
irradiation time of 5 min was used. For further studies, we used the double EMA protocol at  $10 \mu\text{g ml}^{-1}$ .



**Figure 1.** Electrophoresis gel of PCR products after double-EMA treatment of serial dilutions of *H. suis* bacteria. The upper lane shows concentrations of either live (L) or dead (D) *H. suis* bacteria  $\text{ml}^{-1}$ . The lower lane represents the EMA concentration ( $\mu\text{g ml}^{-1}$ ).

### 3.2. Validation of EMA RT-PCR on spiked pork samples and survival of *H. suis* bacteria in pork

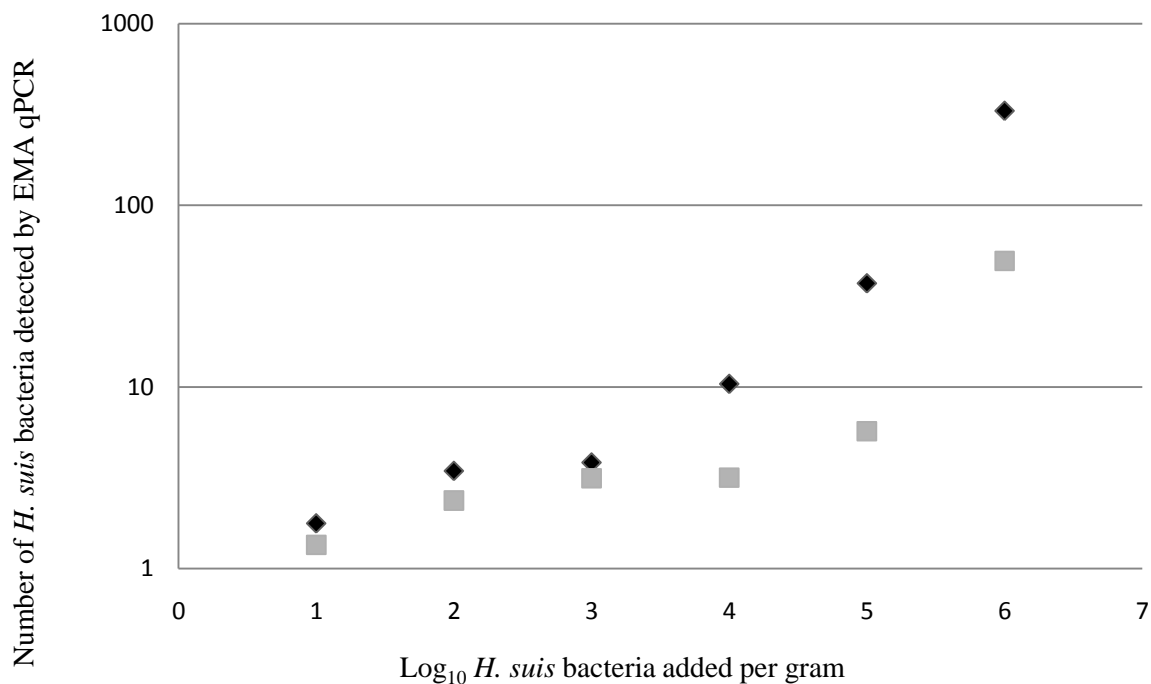
The correlations between EMA RT-PCR results and the number of viable or dead bacteria added per gram pork are shown in Fig. 2, and demonstrate that the developed EMA RT-PCR detects as few as 10 viable bacteria added per gram of minced pork.



**Figure 2.** Scatter plot showing a linear trend between the number of *H. suis* bacteria added per gram of pig meat and the number detected per gram immediately after experimental contamination (shown as black diamonds). The gray cubes show viable *H. suis* bacteria present (4 and 21 per gram) in two retail pork samples.

By treating dead *H. suis* bacteria twice with EMA, no DNA amplification was established.

After 24 and 48 h of incubation at 4 °C, viable *H. suis* bacteria could still be detected in samples with initial concentrations ranging from 10 to 10<sup>6</sup> viable *H. suis* bacteria added per gram (Fig. 3).



**Figure 3.** Survival of *H. suis* bacteria in pork was determined by performing EMA RT-PCR 24 h (shown as black diamonds) and 48 h (shown as gray cubes) after contamination. *H. suis* DNA could not be detected in control groups containing up to 10<sup>6</sup> heat-killed bacteria g<sup>-1</sup> pig meat.

### 3.3. Occurrence of viable *H. suis* bacteria in minced pork at retail

In two out of 50 retail pork samples, DNA from live *H. suis* bacteria was detected with the EMA RT-PCR method, with the presence of 4 and 21 viable bacteria per gram of pork respectively ( Fig. 2). When performing RT-PCR without prior EMA-staining on the two positive pork samples, 4 and 49 bacteria per gram were detected respectively. In none of the other 48 retail pork samples *H. suis* DNA was detected. Sequence analysis of the positive PCR products confirmed the presence of *H. suis* DNA with 100% identity to the *ureA* gene of several known *H. suis* strains (GenBank accession numbers: EF204594.1, EF204593.1, EF204592.1).

#### 4. Discussion

To our knowledge, this study is the first to describe a technique that enables quantification of live *H. suis* bacteria in pig meat. Since RT-PCR, known to be a highly sensitive method for the quantification of foodborne pathogens, cannot distinguish amplified DNA derived from live or dead bacteria, this technique was combined with EMA staining. By treating bacterial samples with EMA before PCR, only DNA from viable cells was amplified, since EMA selectively binds to DNA of dead microorganisms. This detection method was first described by Nogva et al. (2003) and ever since, multiple optimizations for various microorganisms have been described ( Nam et al., 2011, Qin et al., 2012 and Rueckert et al., 2005). However, DNA amplification from viable bacterial cells can be partly suppressed by EMA treatment resulting in an underestimation of the number of viable cells ( Cawthorn and Witthuhn, 2008, Nocker and Camper, 2006, Nocker et al., 2006 and Kobayashi et al., 2009), as was shown in the present study when concentrations higher than  $50 \mu\text{g ml}^{-1}$  were used. On the other hand, by treating bacterial suspensions once with EMA at concentrations below  $50 \mu\text{g ml}^{-1}$ , insufficient differentiation between viable and dead bacteria occurred (data not shown). To overcome these limitations, a double treatment with low concentrations of EMA was developed, as previously performed by Minami et al. (2010).

The EMA RT-PCR was applied to spiked pork samples after 0, 24 or 48 h of cold storage. Survival of *H. suis* bacteria was ascertained up to 48 h post-experimental incubation, which is rather unexpected, since *H. suis* is known to be a very fastidious microorganism. Possible environmental factors contributing to survival of *H. suis* (under the conditions described in the Materials and methods section) are the presence of a moist environment and a low oxygen tension. Indeed, like other gastric *Helicobacter* species, *H. suis* prefers microaerobic conditions ( Baele et al., 2008), which may have been achieved by sealing the sample recipients. In addition, survival of *H. pylori* has been observed in aqueous or moist environments ( Bellack et al., 2006, West et al., 1992 and Böhmeler et al., 1996).

In two out of 50 retail minced pork samples, DNA from viable *H. suis* bacteria was detected with the EMA RT-PCR method, demonstrating the presence of low concentrations of viable bacteria. It has been described that the prevalence of *Salmonella enterica* in minced pork ranges from 0.3 to 4.3%, with a contamination level of  $-2.35 \log(10) \pm 1.09 \text{ CFU g}^{-1}$  ( Delhalle et al., 2009), indicating that the contamination level might be similar for both pathogens. A comparable percentage (2.5%) of minced pork samples are contaminated with

*Campylobacter* bacteria with a contamination level ranging from  $-1.40$  to  $-0.02 \log(10)$  CFU  $g^{-1}$  ( Ghafir et al., 2007).

All results combined, suggest that transmission of *H. suis* bacteria to humans through contaminated pork might occur, indicating that this micro-organism might act as a foodborne pathogen, especially since the dose required to infect humans is unknown.

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# Chapter 2

## **Presence of *Helicobacter suis* on pork carcasses**

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## Abstract

*Helicobacter (H.) suis* is a world-wide spread pathogen which not only colonizes the stomach of pigs, but is also the most prevalent gastric non-*H. pylori Helicobacter* (NHPH) species in humans. *H. suis* infections are associated with gastric lesions both in pigs and in humans. Recently, the presence of viable *H. suis* bacteria has been demonstrated in minced pork, suggesting that manipulation or consumption of contaminated pig meat is a possible route of transmission of this zoonotic agent. The main goal of this study was to determine the extent of pork carcass contamination with *H. suis* at slaughter. In two consecutive studies, the occurrence of *H. suis* DNA was assessed in scalding water, head and mouth swabs, mesenteric lymph nodes, palatine tonsils and on the chest, shoulder and ham region of pork carcasses from three slaughterhouses using qPCR with *ureA* gene based *H. suis*-specific primers. *H. suis* DNA was detected on carcasses in all slaughterhouses, in 8.3% of all 1083 samples. It was found in all sampled matrices, except for the palatine tonsils and scalding water samples. Contamination levels of dressed pork samples did not exceed 184 genomic equivalents per 100 cm<sup>2</sup> (shoulder, ham) or 300 cm<sup>2</sup> (chest). All positive PCR products were subjected to sequence analysis of the *ureA* gene to confirm the identification of *H. suis* bacteria. Using multilocus sequence typing (MLST) on a selection of the positive samples, 5 unique sequence types (STs) could be assigned. Multiple *H. suis* strains were present on samples derived from one specific pig herd. Since *H. suis* DNA was detected in 11% (n: 90) of the mesenteric lymph nodes derived at the slaughterhouse, it was determined whether these organisms can colonize the mesenteric lymph nodes after experimental infection. Despite high-level colonization of the porcine stomachs with the *H. suis* strain, no *H. suis* DNA was detected in the mesenteric lymph nodes at four weeks after experimental infection. This might indicate that its presence in these tissues of slaughtered pigs is due to contamination during the slaughter process, but further studies are necessary to confirm this. In conclusion, we demonstrate a relatively high prevalence of *H. suis* on pork carcasses.

## 1. Introduction

In 1990, Queiroz et al., studied the role of pigs as a possible reservoir for *Helicobacter* (*H.*) *pylori*-like organisms. Instead of *H. pylori*-like organisms, not previously described Gram negative, large spiral-shaped bacteria were found in the mucus layer of lumen and antral pits of the pig stomachs. Initially, the name “*Gastrospirillum suis*” was proposed. Subsequent characterization, however, showed that this bacterium belonged to the genus *Helicobacter* (De Groote et al., 1999) and after the first successful *in vitro* isolation, the name *H. suis* was accepted (Baele et al., 2008). *H. suis* bacteria have been detected in up to 60% of pig stomachs, with prevalences depending on the age of the animals, as well as on the geographic region (Hellemans et al., 2007 and Kopta et al., 2010). Pigs, however, are not the only possible hosts for *H. suis* bacteria. These organisms have been detected in human gastric biopsies more frequently than any of the other gastric non-*H. pylori Helicobacter* (NHPH) species causing gastric diseases such as gastritis, gastric ulcers and gastric cancer (Debonnie et al., 1995, Debonnie et al., 1998, Haesebrouck et al., 2009, Joo et al., 2007 and Morgner et al., 1995).

The exact routes of transmission from pigs to humans remain unclear. Recently, the presence of viable *H. suis* bacteria has been reported in minced pork, suggesting that manipulation or consumption of contaminated pork is a possible route of transmission of *H. suis* bacteria (De Cooman et al., 2013). To date, there are no other studies reporting the occurrence of *H. suis* bacteria in pork and no information on carcass contamination is available. The contribution of food of animal origin to human infection with *H. suis* therefore remains unclear.

The main aim of this study was, therefore, to investigate *H. suis* occurrence on pork carcasses. Samples were taken at seven stages of slaughter, in three abattoirs, and examined quantitatively for the presence of *H. suis* DNA. Multilocus sequence typing (MLST) was used to obtain insight in the heterogeneity of the *H. suis* population present on pork carcasses.

## 2. Materials and methods

### 2.1. Sample collection and preparation

The slaughter process, which is generally standardized in all Belgian slaughterhouses, is as follows. After stunning, the animals are secured by the hind legs to an overhead conveyer rail. The gas or electric stunned animals are stuck immediately by cutting the main blood vessel in

the chest. After bleeding, the animals are scalded and dehaired mechanically. The pigs are then secured to the overhead conveyer, singed by gas burners, eviscerated, and next cut along the midline by using an automatic splitting saw.

In a first study, 363 samples originating from three pig herds were collected in three different Belgian abattoirs (A, B, C). The samples were taken at five sampling points, but without following the same carcasses through the slaughter process. In each slaughterhouse, 30 samples from the head and 30 samples from the mouth were collected immediately after automatic splitting of the carcasses, using cotton swabs (Cultiplast®, LP Italiana Spa, Milano, Italy) which were moistened in buffered peptone water. Furthermore, 30 mesenteric lymph nodes were removed immediately after evisceration and placed into sterile plastic bags for transport to the laboratory. In the lab, the surface of the lymph nodes was seared and cut aseptically into small pieces. Next, the lymph nodes were homogenized and 40 mg was transferred into 1.5 ml Eppendorf tubes, which were then kept at  $-20^{\circ}\text{C}$  until DNA extraction. Thirty palatine tonsils were excised and treated the same way as the lymph nodes. Finally, 2 l of scalding water was collected from each slaughterhouse, after at least 2 h of slaughter activities.

A second study was carried out in slaughterhouse C that was visited on three occasions. On each occasion, all samples originated from only one pig herd, but again without following the same carcass through the slaughter process. Each time, 30 mesenteric lymph nodes were collected immediately after evisceration, 30 samples from the mouth were taken after mechanical splitting and 30 samples were collected from the ham, shoulder and chest region of carcasses on the slaughterline and once again of carcasses after 6 h of chilling by forced ventilation. Mesenteric lymph nodes were treated as described above. For collection of samples from the mouth and the carcasses, sponge-sticks (3M™ Sponge-Sticks, St. Paul, USA) were used, which were moistened in 10 ml buffered peptone water. A  $100\text{ cm}^2$  area of the ham and shoulder and a  $300\text{ cm}^2$  area of the chest was sampled, according to the EU Decision 2001/471/EC of 8 June 2001. All mouth and carcass samples were stored in plastic, sterile bags at  $-20^{\circ}\text{C}$  until DNA extraction.

## **2.2. DNA extraction**

Head and mouth swabs, collected during the first study, were placed in Eppendorf tubes, containing 250  $\mu\text{l}$  buffered peptone water. After vortexing for 1 min, the swabs were placed in tips, which were then placed in Eppendorf tubes, and then centrifuged at  $9300 \times g$  for 5 min.

The obtained pellet was kept and 100  $\mu$ l Prepman Ultra (Invitrogen, Carlsbad, CA, USA) was added. Further conditions used for DNA extraction, were according to the manufacturer's protocol.

One liter of each scalding water sample, collected during the first study, was filtered using the PowerWater® DNA Isolation kit (Mobio Laboratories, Inc., Carlsbad, USA), applying small adaptations to the protocol, using a 0.22  $\mu$ m filter membrane and adding an additional centrifugation step at 800  $\times$ g for 5 min.

For DNA extraction from the tonsils and mesenteric lymph nodes, collected during the first and second study, the Isolate Genomic DNA Mini Kit (Bioline, London, UK) was used, following the manufacturer's instructions for isolation of DNA from animal tissue.

The mouth and carcass samples that had been collected during the second study and stored in plastic bags, were defrosted overnight at 4 °C. Then, 10 ml of buffered peptone water was added and the samples were homogenized in a stomacher for 90 s. Thereafter, most of the fluid was recovered from the bags and placed into 15 ml Falcon tubes for centrifugation (30 min at 3724  $\times$ g). The obtained supernatant was removed, and 50  $\mu$ l of PrepMan Ultra (Invitrogen) was added to the pellet. DNA extraction was performed according to the manufacturer's instructions.

### **2.3. Quantitative polymerase chain reaction**

After DNA extraction, quantitative polymerase chain reaction (qPCR) was performed on all samples, using the same primers (UreSu 531 FW and UreSu 783 RV) and conditions as described by De Cooman et al. (2013). Briefly, qPCR involved an initial cycle at 95 °C for 15 min, followed by 40 cycles of denaturation (at 95 °C for 20 s), primer annealing (at 60 °C for 30 s) and chain extension (at 73 °C for 30 s). An external standard, comprising tenfold dilutions of a 1541 bp *ureA-ureB* gene segment, was used starting at 10<sup>5</sup> amplicons for each 10  $\mu$ l of reaction mixture. The copy number concentration was calculated based on the expected length of the amplicon and its dsDNA amount. Sequences of the primers used, are given in Table 1. Both standard and samples were run in duplicate. Sequence analyses of the *ureA* gene were performed on 21 positive PCR-products to confirm the identification of *H. suis* bacteria.

Primer	Sequence (5' to 3')	Amplicon size (bp)
UreSu 531 FW	CACCACCCCGGGGAAGTGATCTTG	253
UreSu 783 RV	CTACATCAATCAAATGCACGGTTTTTCTTCG	
UreSu 428 FW	GGTGTTGCCCATATGATTCA	1541
UreSu 1968 RV	CGAATCCTAGAGTCAGCAA	

**Table 1.** Oligonucleotide primers used for amplification and quantitation (UreSu 531 FW and UreSu 783 RV) and establishing an external standard (UreSu 428 FW and UreSu 1968 RV) (De Cooman et al., 2013).

## 2.4. Multilocus sequence typing

The five mouth swab samples with the highest quantity of *H. suis* equivalents on qPCR (originating from two pig herds of the second study) were selected for further typing using multilocus sequence typing (MLST). The variable regions of seven housekeeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureAB* and *yphC*) were amplified and sequenced according to the method described by Liang et al. (2013). Amplification was established with Accuzyme DNA polymerase, using the same conditions as previously described. Purification of PCR products and sequencing were performed by GATC Biotech (Cologne, Germany). Electropherograms were exported and converted to Kodon software (Applied Maths, Sint-Martens-Latem, Belgium).

## 2.5. Experimental *H. suis* infection in pigs

Twenty-four pigs were inoculated with *H. suis* strain HS1 (Baele et al., 2008) at the age of four weeks. All animals received  $10^9$  to  $2 \times 10^9$  *H. suis* bacteria *per os* twice, with an interval of 48 h. At the age of eight weeks, the animals were euthanized and stomach samples were collected from the mucosa of the antrum, corpus and fundus, from each animal, as well as 3 mesenteric lymph nodes (one close to the stomach, one close to the jejunum and one close to the rectum). DNA was extracted from these samples, using the protocol described above for the tonsils and mesenteric lymph nodes. DNA extracts were frozen at  $-20^\circ\text{C}$  until further analysis. For the detection of *H. suis* DNA, qPCR was performed using the same primers and conditions as described before.

All laboratory animal experiments were approved by the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

### 3. Results

#### 3.1. Detection of *H. suis* DNA on pork carcasses at slaughter

In the first study, *H. suis* DNA was detected in samples from all three slaughterhouses, in 10% of the head swabs (n: 90), 14.4% of the mouth swabs (n: 90) and 13.3% of the mesenteric lymph nodes (n: 90) (Table 2). No *H. suis* DNA was detected in the palatine tonsils and scalding water samples.

**Table 2.** Presence of *H. suis* DNA on pork carcasses using *H. suis*-specific primers (first study).

		Mouth swabs	Head swabs	Mesenteric lymph nodes	Palatine tonsils	Scald tank water
Slaughterhouse A	Number of positive samples	6 (n:30)	4 (n: 30)	3 (n: 30)	0 (n: 30)	0 (n: 1)
	Mean number of bacteria detected	$1.4 \times 10^2$ /ml	$3.1 \times 10^1$ /ml	9.0/mg	0/mg	0/ml
Slaughterhouse B	Number of positive samples	4 (n: 30)	4 (n: 30)	6 (n: 30)	0 (n: 30)	0 (n: 1)
	Mean number of bacteria detected	$2.5 \times 10^2$ /ml	8.6/ml	$2.8 \times 10^1$ /mg	0/mg	0/ml
Slaughterhouse C	Number of positive samples	3 (n: 30)	1 (n: 30)	3 (n: 30)	0 (n: 30)	0 (n: 1)
	Mean number of bacteria detected	$1.8 \times 10^1$ /ml	2.1/ml	0.34/mg	0/mg	0/ml

In the second study, *H. suis* DNA was detected in all five dressed pork carcass areas, both before and after chilling. Overall, 7.8% of the 720 samples contained *H. suis* DNA. The mouth and chest were identified as the two most frequently contaminated areas pre-chilling and shoulder and chest areas post-chilling (Table 3).

**Table 3.** Comparison of PCR-positive pork carcass samples by sampling area on 3 different occasions (I, II and III; second study).

	Number of positive samples at:								Total
	Mouth	Ham bc <sup>a</sup>	Chest bc	Shoulder bc	Shoulder ac <sup>b</sup>	Chest ac	Ham ac	Lnn.	
I	8 (n: 30)	2 (n: 30)	0 (n: 30)	0 (n: 30)	1 (n: 30)	0 (n: 30)	0 (n: 30)	3 (n: 30)	14 (n: 240)
II	9 (n: 30)	1 (n: 30)	5 (n: 30)	2 (n: 30)	1 (n: 30)	2 (n: 30)	1 (n: 30)	3 (n: 30)	24 (n: 240)
III	6 (n: 30)	2 (n: 30)	2 (n: 30)	3 (n: 30)	1 (n: 30)	0 (n: 30)	0 (n: 30)	4 (n: 30)	18 (n: 240)
Total	23 (n: 90)	5 (n: 90)	7 (n: 90)	5 (n: 90)	3 (n: 90)	2 (n: 90)	1 (n: 90)	10 (n: 90)	56 (n: 720)

<sup>a</sup> bc: before forced ventilation chilling.

<sup>b</sup> ac: after 6 h of forced ventilation chilling.

Quantitative examination of the samples revealed 4.1 to 103.0 genomic equivalents of *H. suis* per 100 cm<sup>2</sup> (shoulder and ham) or 300 cm<sup>2</sup> (chest) for the samples taken before chilling and approximately 2.9 to 184.0 genomic equivalents of *H. suis* per 100 or 300 cm<sup>2</sup> for the swabs taken after forced air chilling.

Sequence analyses confirmed that all positive PCR-products contained *H. suis* DNA.

### 3.2. Multilocus sequence typing

*H. suis* from five mouth swabs from two pig herds were analyzed for genetic relatedness using MLST. Hereby, five different sequence types (STs) were identified, of which none had been previously included in the PubMLST database. No ST was found in more than one pig herd. Within the same pig herd, two to three different STs were found (Table 4).

**Table 4.** Determination of allele numbers and sequence types for 5 *H. suis* strains present on mouth swabs derived from two pig herds (p1 and p2)

	<i>atpA</i>	<i>efp</i>	<i>mutY</i>	<i>ppa</i>	<i>trpC</i>	<i>ureA</i>	<i>yphC</i>
Lie-1 (p1 mouth 18)	1	2	11	2	1	5	7
Lie-2 (p1 mouth 27)	1	4	11	1	1	1	3
Lie-8 (p2 mouth 4)	4	2	11	1	1	1	1
Lie-9 (p2 mouth 5)	1	2	11	1	2	1	3
Lie-10 (p2 mouth 17)	1	2	11	1	2	1	1

### 3.3. Absence of *H. suis* DNA in mesenteric lymph nodes after experimental infection

Twenty three out of the twenty four orally inoculated pigs tested positive for the presence of *H. suis* DNA in the gastric mucosa. The two most frequently colonized regions were the antrum and the fundic gland region, with mean densities of  $1.8 \times 10^7$  and  $1.3 \times 10^7$  bacteria/mg tissue respectively. In contrast, in none of the mesenteric lymph nodes *H. suis* DNA was detected.

## 4. Discussion

*H. suis* is a very fastidious micro-organism requiring a highly enriched biphasic medium at pH 5 and a microaerobic atmosphere (Baele et al., 2008). Up till now, isolation of this agent has been only successful from the gastric mucosa of pigs strongly colonized with this micro-organism and even from this tissue, it is a long and laborious process (Vermoote et al., 2011).

As it is not feasible to isolate these bacteria from other matrices, such as carcass samples, qPCR was used during this study for the detection of *H. suis* DNA.

To our knowledge, this is the first report describing the presence of *H. suis* DNA on pork carcasses at slaughter. An overall prevalence of 4.3% on dressed pork carcasses was detected, which is comparable to that of well-known zoonotic bacteria such as *Salmonella* (1.9%–6%), *Yersinia* (0–0.3%) and *Campylobacter* (8.8%) (Bohaychuk et al., 2011, Bonardi et al., 2003, Davies et al., 2000, Gürtler et al., 2005, Käsbohrer et al., 2000 and Nauta et al., 2013). Besides, the *H. suis* genomic equivalents on the contaminated carcasses were in the range of those for *Campylobacter* (Ghafir et al., 2007). This further indicates that contaminated pig meat may constitute a source of *H. suis* infection for humans.

Considering that the pork carcasses were scalded at 58 °C, followed by singeing by gas burners prior to evisceration, one could hypothesize that carcass contamination is due to spilling of the stomach content during evisceration. This might explain why the chest and shoulder regions were more frequently contaminated than the ham. Another possibility is that the carcass is contaminated during splitting, when the splitting saw reaches the mouth of the pigs. The oral cavity may already be contaminated by regurgitation after securing the pigs upside down to an overhead conveyer rail. Contamination of carcasses by spilling intestinal content during evisceration, as for example has been suggested for *Salmonella enterica* (Bonardi et al., 2013) and *Arcobacter* spp. (Ohlendorf and Murano, 2002 and Van Driessche et al., 2004), is rather unlikely, since *H. suis* DNA has never been identified in feces after experimental infection of pigs (unpublished results).

Although *H. suis* DNA was detected in 13.3% of mesenteric lymph nodes of slaughter pigs, its presence in mesenteric lymph nodes of pigs at four weeks after experimental infection with *H. suis* was not demonstrated. These data suggest that the presence of *H. suis* DNA in mesenteric lymph nodes is not a result of tissue colonization, but rather occurs during slaughter. On the other hand, long term colonization studies of mice with the closely related *H. heilmannii*, have shown migration of these bacteria in macrophages to lymphoid tissue along the gastro-intestinal tract (unpublished results). Further studies are necessary to determine if this also occurs in pigs colonized with *H. suis* for longer periods of time.

By performing MLST on five selected samples, a high diversity among the strains derived from the same pig herd and all slaughtered in the same abattoir was demonstrated. This indicates that the contamination of carcasses does not originate from the slaughterhouse environment.

Cooling combined with drying reduced the number of detectable *H. suis* genomic equivalents on the pork carcasses. This reduction might be caused by firm adhesion of the bacteria on the skin. Alternatively, the bacteria may be able to shelter in the hair follicles and can therefore not be reached by swabbing, as has been demonstrated for many microorganisms by Cason et al. (2004). In other related microaerophilic bacteria such as *Arcobacter* spp. (Van Driessche and Houf, 2007) and *Campylobacter* spp., as well as in *Salmonella enterica* (Epling et al., 1993 and Oosterom et al., 1985) forced cooling is not fully capable of eliminating carcass or environmental contamination. Further research is necessary in order to assess the actual risk imposed to consumers of pork and to identify the exact route(s) of contamination during the slaughter process.

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# Chapter 3

## ***Helicobacter suis* antigens recognized by sera of *Helicobacter* infected human patients**

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### **Abstract**

*Helicobacter (H.) suis* is the most prevalent non-*H. pylori* *Helicobacter* (NHPH) species colonizing the stomach of humans. Development of a *H. suis*-specific serological test would seriously simplify the diagnosis in humans. In order to investigate the feasibility of such a test, *H. suis*-specific antigens were identified by performing two-dimensional gel electrophoresis of *H. suis* proteins present in whole-cell lysate and one-dimensional gel electrophoresis of *H. suis* proteins recombinantly expressed in *Escherichia coli*, followed by immunoblotting with sera from human patients infected with either *H. suis*, *H. heilmannii* or *H. pylori*. Several *H. suis* whole-cell lysate derived protein spots between ~20 kDa and ~30 kDa and at ~60 kDa were only recognized by the serum of an *H. suis* infected patient. The small subunit (20 kDa) of *H. suis*  $\gamma$ -glutamyl transpeptidase was recognized by sera of *H. suis* or *H. heilmannii* infected humans, but not by sera from *H. pylori* infected patients. These results indicate that development of a serological test allowing to differentiate NHPH or *H. suis* infected patients from patients infected with *H. pylori* is feasible.

### **Keywords**

Non-*Helicobacter pylori* helicobacters, *Helicobacter suis*, zoonotic agent, immunoproteomics, serodiagnosis

## 1. Introduction

*Helicobacter (H.) pylori* has been recognized as an important factor in the development of gastric disease in humans (Atherton, 1997; De Luca and Iaquinto, 2004; Cover and Blaser, 2009). Bacterial gastric disorders in humans have, however, also been associated with non-*Helicobacter pylori* helicobacters (NHPH), with a prevalence ranging between 0.2 and 6% (Haesebrouck et al., 2009). Of all NHPH, *H. suis* is the most frequently diagnosed species in human gastric mucosal specimens. *H. suis*, a large spiral-shaped bacterium, primarily colonizes the stomach of pigs worldwide, with a prevalence often exceeding 60% in pigs at slaughter age (Hellemans et al., 2007; Baele et al., 2008; Haesebrouck et al., 2009). Direct or indirect contact with pigs is considered to be the main infection route for humans (Haesebrouck et al., 2009). Recently, viable *H. suis* bacteria were detected in pork, indicating that this agent might also be transferred to humans through the food chain (De Cooman et al., 2013).

*H. suis* infected humans may not only develop gastritis and peptic ulcer disease, but they have been shown to carry a higher risk of developing gastric mucosa-associated lymphoid tissue (MALT) lymphoma, compared to *H. pylori* infected humans (Stolte et al., 1997; Debongnie et al., 1998; Morgner et al., 2000; Stolte et al., 2002). At present, various tests for the diagnosis of *H. pylori* infections are readily available. The diagnosis of NHPH infections, however, is often hampered by the focal distribution of the bacteria in the stomach and the very fastidious nature of these species. For example, the accuracy of Urea Breath tests may be lower than is the case for *H. pylori* infected patients (Haesebrouck et al., 2009; Matsumoto et al., 2014). Furthermore, microscopic visualization of NHPH microorganisms is difficult and the morphological differences between NHPH species are in general not obvious (Debongnie et al., 1994; Happonen et al., 1998). At present, no test for the identification of antibodies against NHPH in blood samples of humans or animals is available. Obviously, there is a need for a simple and reliable tool to diagnose NHPH infections, preferably with identification up to the species level. In order to obtain the knowledge needed to develop an antigen-based *H. suis*-specific diagnostic test, *H. suis*-specific antigens that are recognized by antibodies of infected human patients need to be identified.

The present study aimed at identifying *H. suis*-specific antigenic proteins eliciting an immune response in infected humans, thus holding promise as potential target proteins for species-specific diagnosis. Two approaches were used. In a first series of experiments, immunogenic protein patterns of whole bacterial cell lysates were compared after probing

with sera from human patients infected with *H. suis*, *H. heilmannii* or *H. pylori*. In a second approach, the reaction of these sera was studied against three specific *H. suis* proteins which were recombinantly expressed in *Escherichia coli*: urease subunit B (UreB), neutrophil-activating protein A (Nap A) and gamma-glutamyl transpeptidase (GGT). These proteins were chosen because they are considered to play a role in the interaction of *H. suis* with its hosts (Flahou et al., 2011; Vermoote et al., 2011; Zhang et al. 2015).

## **2. Materials and methods**

### **2.1. Bacterial strains**

In the following experiments, *H. suis* strain HS5bLP (HS5, GenBank: ADHO00000000), *H. heilmannii* ASB1.4 (Genbank: HE984298), *H. bizzozeronii* R1051 and the Sydney strain of *H. pylori* (strain SS1) were used. *H. suis* strain HS5bLP (Liang et al., 2015) was isolated from the stomach of a sow, according to the method described by Baele et al. (2008). *H. heilmannii* ASB1.4 was isolated from the gastric mucosa of a kitten with severe gastritis (Smet et al., 2013) and *H. bizzozeronii* strain R1051 was isolated from canine gastric biopsies (Hänninen et al., 1996). The Sydney strain of *H. pylori* (strain SS1) shows a high colonizing ability in mice (Lee et al., 1997).

### **2.2. Patients**

A total of 118 patients undergoing upper gastroscopy for gastrointestinal complaints, such as dyspepsia, stomach ache, halitosis or vomiting, were recruited for this study. Patients who had received any antimicrobial therapy during the 4 weeks prior to gastroscopy, or who received active immunosuppressive therapy, were excluded from this study. All experiments were approved by the Ethics Committee of Ghent University (2011/393) and the local Ethics Committee of the Maria Middelaes hospital. After consent to participation in this study, 10 mL venous blood was collected from each patient and, after centrifugation, sera were gathered and stored at -20°C until further examination. During gastroscopy, biopsy specimens were obtained from the antrum, corpus and duodenal region, and stored at -20°C in an Eppendorf tube, containing 600 µL ethanol.

### **2.3. DNA isolation and quantification**

DNA extraction from the biopsy specimens was performed by using the DNeasy Tissue kit according to manufacturer's instructions (Qiagen, Valencia, CA). After DNA extraction, a

nested polymerase chain reaction (PCR) was performed, using the primers and conditions as shown in Table 1. Sequence analysis was performed on any positive PCR product, to further identify the gastric helicobacters up to the species level.

**Table 1.** All human stomach biopsy specimens were subjected to a nested PCR, in order to detect a fragment of the urease gene. During the first run, primers developed by O'Rourke et al. (2004)\* were applied. The conditions used during the second run, were described by Flahou et al. (2014)\*\*.

	Primer FW	Primer RV	PCR-conditions
<b>O'Rourke*</b>	SOL U430	SOL 1735	5' 94°C
	gCK gAW TTg ATg CAA gAA gg	CTT CgT gRA TTT TAA RRC CAA T	35 x (1' 94°C - 1' 52°C - 1' 72°C) 5' 72°C
<b>UreNHPH_short**</b>	UreNHPH_shortF	UreNHPH_shortR	5' 94°C
	CDg TRM gNT TTg ARC CNg g	gTD gTD ggD CCr Tac ATW gA	40 x (1' 94°C - 1' 57°C - 1' 72°C) 5' 72°C

#### 2.4. Serum samples

In a previous study, Joosten et al. (2013) diagnosed a *H. suis* infection in a pig veterinarian, suffering from reflux oesophagitis and dyspepsia. A blood sample of this patient, as well as samples of any patient for whom a gastric *Helicobacter* infection was identified during the current study, were included. After centrifugation of the blood samples (1000xg, 4°C, 10 min), sera were collected and frozen at -70°C until further use. Serum originating from a patient without previous history of gastric upset and for whom no *Helicobacter* DNA was found during examination of gastric biopsy specimens, was included as a negative control. As a positive control for *H. suis* infection, pooled sera from mice, intranasally immunized with *H. suis* whole-cell lysate, were used (Vermoote et al., 2012).

#### 2.5. Two-dimensional gel electrophoresis (2D-PAGE)

*H. suis* HS5bLP, *H. heilmannii* ASB1.4, and *H. bizzozeronii* R1051 strains were grown on their respective biphasic medium, as described previously (Flahou et al., 2010; Smet et al., 2012). Once a volume of 50 mL was obtained for each of the three strains, with a concentration of  $10^6$ - $10^8$  bacteria per mL, the bacteria were harvested by centrifugation (5000 xg, 4°C, during 10 min), and 4 washing steps with Hank's Balanced Salt Solution (HBSS) were performed. *H. pylori* SS1 was grown on Brain Heart Infusion (BHI) agar supplemented with 10% horse blood and Vitox supplement (Oxoid, Basingstoke, UK). Cultures were

harvested by washing all culture plates with HBSS, obtaining a volume of 50 mL, with a concentration of  $10^6$ - $10^8$  bacteria per mL. The ReadyPrep™ Sequential Extraction Kit (Bio-Rad, Hercules, CA, USA) was used to extract both soluble and insoluble proteins from each of the four bacteria (Vermoote et al., 2012). Next, the protein concentration was determined using the Bradford protein quantification method (Bradford Protein Assay (Bio-Rad, Hercules, CA, USA)). All proteins were stored at  $-70^{\circ}\text{C}$  until further use.

In parallel, a total of 100  $\mu\text{g}$  of HS5bLP, ASB1.4, R1051 and SS1 proteins were transferred into LoBind Eppendorf tubes (Eppendorf AG, Hamburg, Germany). Next, 160  $\mu\text{l}$  rehydration buffer (7M ureum, 2M thiouream, 2% CHAPS, 0.2% carrier ampholyte pH 3-4, 100 mM dithiothreitol (DTT) and bromophenol blue) was added to each Eppendorf tube. Each solution was passively absorbed onto a ReadyStrip™ (IPG Strips, 11 cm, pH 3-10, Bio-Rad) during 6 h, after which iso-electric focusing was performed in a Protean IEF Chamber (Bio-Rad) as described by Van Steendam et al. (2010). Each strip was then set to equilibrate, during 15 min, in 1.5% DTT in equilibration buffer (50 mM TrisHCl, pH 8.8 6 M urea, 20% glycerol, 2% SDS), and next, in 4% iodoacetamide in equilibration buffer during another 15 min. Then, gel electrophoresis was performed on a 10% TrisHCl SDS-PAGE, at 150 V during 20 min, followed by 60 min at 200V. For each gel which was run to be immunoblotted afterwards, another gel was performed in parallel. The latter was first fixed in 10% MeOH, 7% acetic acid, and next stained with Sypro® Ruby Protein Gel staining (Bio-Rad). After staining, HS5bLP, ASB1.4, and R1051 proteins were visualized on the respective gels, using the VersaDoc Imaging System (Bio-Rad).

## **2.6. 2D Western blotting**

Proteins were transferred from the gels to a nitrocellulose membrane (Bio-Rad), as described previously (Van Steendam et al., 2010). Membranes were treated with 5% nonfat dry milk Blotting-Grade blocking buffer (Bio-Rad), and incubated overnight (ON) with a 1/200 serum sample diluted in blocking buffer. After washing the membranes with 0.3% Tween-20 (Bio-Rad) diluted in PBS, they were incubated for 1 h with goat anti-human immunoglobulin G (IgG), or in case of the positive control murine serum, with goat anti-mouse IgG conjugated with horseradish-peroxidase (HRP) (1/1000 diluted in blocking buffer, Pierce, Rockford, IL, USA). After two washing steps with 0.3% Tween-20 of 5 min each, membranes were treated with Supersignal West Dura Extended Duration Substrate (Pierce). Next, patterns were visualized, using the VersaDoc Imaging system.

## 2.7. In-Gel Digestion and LC-MS/MS Analysis

In-gel digestion of proteins was performed as described by Cheung et al. (2009). Identification of the peptides was performed, as previously described by Van Steendam and co-workers (2012), using the TripleTOF 5600 (ABSciex) appliance. Data analysis was performed by automated database searching (MASCOT Daemon 2.3., Matrix Science, London, UK) against the *H. suis* protein database from NCBI. Peptide mass tolerance and fragment mass tolerance was set at 25 ppm and 0.2 Da, respectively. Maximum two miscleavages were allowed. Proteins were only considered to be correctly annotated when the significance was below 0.01 ( $p < 0.01$ ) and at least three peptides passed the required bold red criteria from Mascot Daemon, indicating that at least three peptides had rank 1 and a significance below 0.01.

## 2.8. Preparation of recombinant *H. suis* UreB, NapA and GGT

The gene encoding the *H. suis* urease subunit B (UreB) (GenBank locus tag HSUHS5\_0285) and the gene encoding the *H. suis* neutrophil-activating protein A (NapA) sequence (GenBank locus tag HSUHS5\_0015) were amplified by PCR and cloned into the pET-24d vector and the pENTR™/TEV/D-TOPO® vector, respectively (with a transfer from the latter into the pDEST17™ destination vector). The obtained rUreB was expressed in *E. coli* strain BL21 (DE3) and the rNapA in *E. coli* strain BL21-AI™, using the *E. coli* Expression System with Gateway® Technology (Invitrogen, Carlsbad, CA, USA). All conditions used to perform expression of UreB and NapA proteins, were previously described by Vermoote and co-workers (2012). The  $\gamma$ -glutamyl transpeptidase (GGT) of the HS5bLP strain (GenBank GU972556) was expressed in the *E. coli* Expression System with Gateway® Technology, as described elsewhere (Flahou et al., 2011). rUreB, rNapA and rGGT protein concentrations were determined with the RC DC Protein Assay (Bio-Rad).

## 2.9. One-dimensional gel electrophoresis (1D-PAGE) and Western blotting of *H. suis* rUreB, rNapA and rGGT

1D-PAGE was performed using 10% polyacrylamide TGX™ precast gels (Bio-Rad). Briefly, 10  $\mu$ g rUreB, rNapA or rGGT was diluted (1:1) in 2x Laemmli sample buffer (Bio-Rad), and loaded into each well of the precast gel. Gels were run at 300 V for 25 min, using 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 as a running buffer. Staining of the gels and blotting procedures were done as described above.

### 3. Results

#### 3.1. Diagnosis of gastric *Helicobacter* infections

During the current study, *H. heilmannii* infection was identified by performing nested PCR and subsequent sequence analysis in a male patient, suffering from dyspepsia, halitosis and nausea. Furthermore, in 18 of 118 (15%) patients, *H. pylori* infection was identified by PCR and DNA sequence analysis. Blood samples from the *H. heilmannii* and 5 *H. pylori* infected patients, as well as from the previously identified *H. suis* infected patient (Joosten et al.,2013) were collected before an anti-*Helicobacter* therapy was initiated.

#### 3.2. *H. suis*, *H. heilmannii*, *H. bizzozeronii* and *H. pylori* immunoproteomics

Whole-cell proteins from *H. suis* strain HS5bLP were initially separated on 2D-PAGE. Fig 1A shows the complex 2D profile of HS5bLP extracted proteins after Sypro® Ruby Protein Gel staining (Bio-Rad). The protein spots were separated over a molecular weight (Mr) range of 20-120 kDa and a pI range of 3-10. The representative 2D-immunoblot probed with serum of the *H. suis* infected patient, is shown in Fig 1B. Numerous protein spots were recognized by the *H. suis* infected patient's serum sample. In comparison, no immunoreactive pattern was visualized after loading serum samples from the *H. heilmannii* infected patient (Fig 1C). Fig 1D shows the pattern as was visualized after loading serum obtained from one of the patients with *H. pylori* infection, with a distinct immunoreaction at ~50 kDa. Similar patterns were obtained for the serum samples of the other *H. pylori* infected patients. On the immunoblot probed with negative control serum, no immuno-reactive spots were observed (results not shown). Nine differentially expressed protein spots which showed a strong reactivity with serum from the *H. suis*-infected patient, but not with serum from patients infected with *H. pylori* or *H. heilmannii*., were excised from the accompanying gel (Fig 1A), digested and identified by LC-MS/MS analysis (Table 2).

During a second experiment, whole bacterial cell protein of *H. heilmannii* strain ASB1.4, *H. bizzozeronii* strain R1051, and *H. pylori* strain SS1 were separated on 2D-PAGE as well. After performing 2D Western blotting, the serum sample of the patient infected with *H. suis* was loaded. The resulting immunoreactive pattern was restricted to the ~50 kDa region, for the blots containing *H. heilmannii* strain ASB1.4 and *H. bizzozeronii* strain R1051 proteins (Fig 2A-B). No immunoreactive pattern was visualized after loading the serum sample on the blot containing *H. pylori* strain SS1 proteins (Fig 2C).

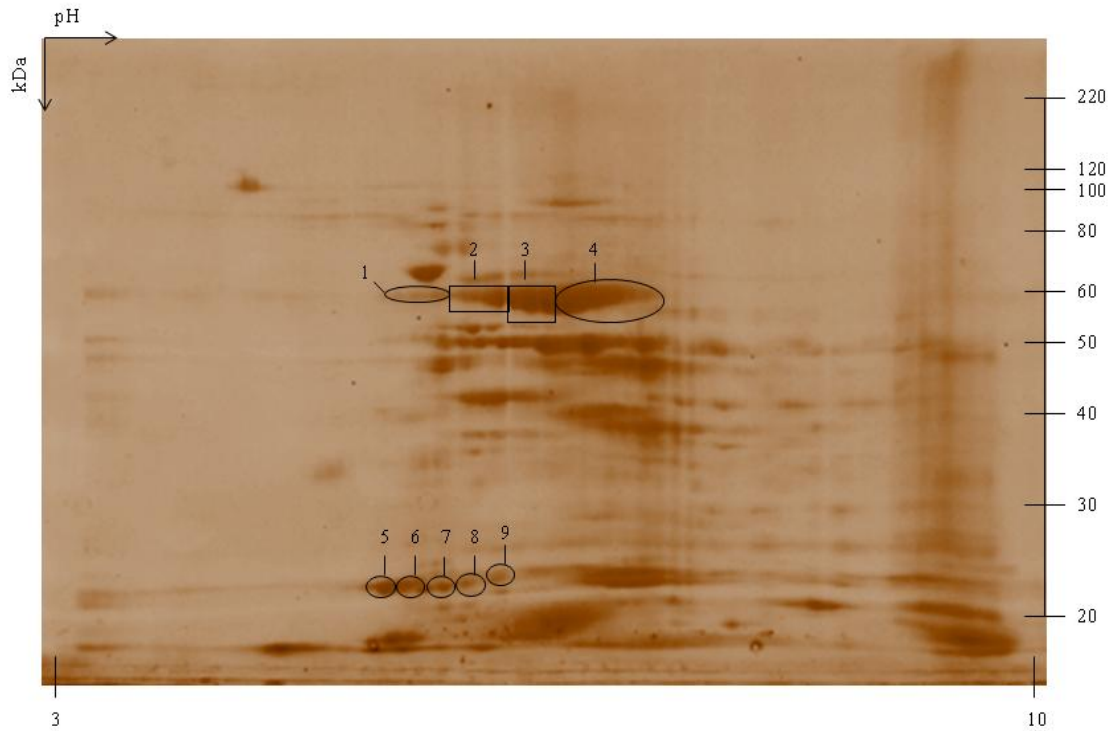


Fig 1A The *H. suis* protein profile obtained after 2D-PAGE. Nine protein spots (1-9) were excised (circled in the figure), based on the results of the 2D-immunoblot loaded with serum from a *H. suis* infected pig veterinarian. No immunoreaction was detected at the same position on the immunoblot after loading serum samples of *H. pylori* or *H. heilmannii* infected patients. In table 2, the corresponding protein identifications are listed.

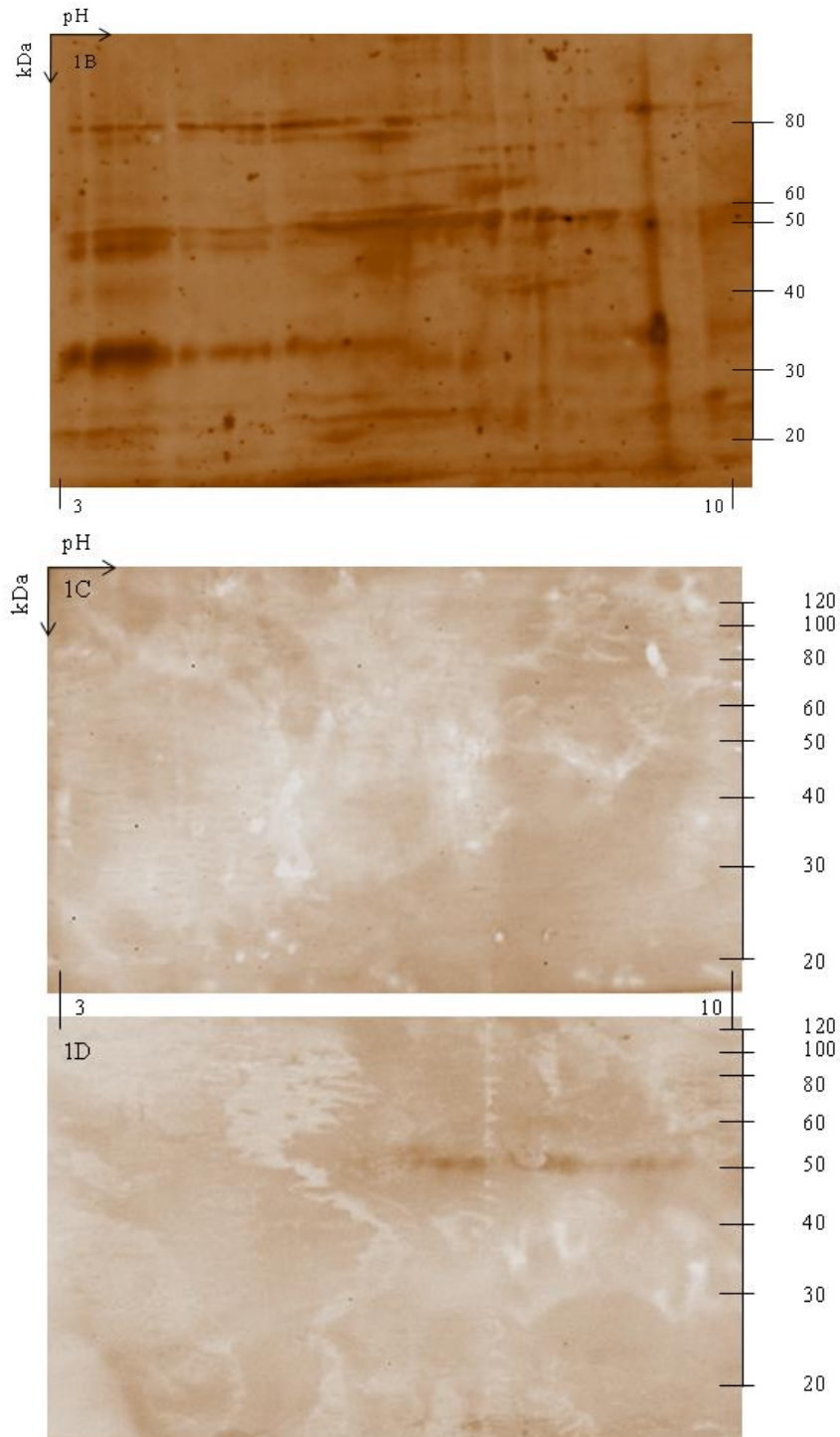


Fig 1B-1C-1D: 2D-immunoblots obtained by loading HS5bLP-protein extract, and subsequent probing with the serum sample from the *H. suis* infected patient (Fig 1B), the serum sample of the *H. heilmannii* infected patient (Fig 1C), and the serum sample of a *H. pylori* infected patient (Fig 1D).

Table 2 Putative identification of immuno-reactive protein-spots. The identified spots correspond to positions 1-9, as depicted in SDS-PAGE from figure 1A and were only found after loading the serum sample of the *H. suis* infected patient. Identification was done by LC-MS/MS and this resulted in the identification of more than one possible protein per spot. In this table, the most abundant protein is mentioned first for each spot.

Spot nr	<i>H. suis</i> protein	Mascot score
1	Chaperonin GroEL	2706
	Chaperone protein DnaK	1158
	Flagellin B	344
	Urease subunit beta	256
	Heat shock protein 90 (HtpG)	241
	F0F1 ATP synthase subunit beta	241
	Trigger factor	202
	Elongation factor G	176
	GTP-binding TypA/BipA translation elongation factor	101
	Flagellin B	63
2	Chaperonin GroEL	5400
	Heat shock protein 90 (HtpG)	449
	Flagellin A	433
	ATP-binding ABC transporter	197
	Hypothetical protein	194
	Elongation factor G	157
	Urease subunit beta	96
	GTP-binding TypA/BipA translation elongation factor	82
3	chaperonin GroEL	9367
	flagellin B	895
	Urease subunit beta	789
	heat shock protein 90 (HtpG)	171
	ATP-binding ABC transporter	81
4	Chaperonin GroEL	9932
	Urease subunit beta	1978
	Flagellin B	793
	Transketolase A	200
	Leucyl aminopeptidase	169
	1-deoxy-D-xylulose-5-phosphate synthase	86

	Aspartyl-tRNA synthetase	68
	Glutamine synthetase	63
	SPFH domain-containing protein	
5	Tellurium resistance protein TerD	2118
	Inorganic pyrophosphatase	417
	Flagellin B	265
	Co-chaperone and heat shock protein 24	161
	Hypothetical protein HSUHS1_0896	72
	Chemotaxis protein	68
6	Tellurium resistance protein TerD	2503
	Inorganic pyrophosphatase	903
	Flagellin B	585
	ATP-dependent Clp protease proteolytic subunit	335
	Co-chaperone and heat shock protein 24	218
	Leucyl aminopeptidase	203
7	Inorganic pyrophosphatase	1449
	Tellurium resistance protein TerD	1339
	ATP-dependent Clp protease proteolytic subunit	307
	Co-chaperone and heat shock protein 24	179
	Flagellin A	170
	Leucyl aminopeptidase	169
	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	131
8	Inorganic pyrophosphatase	1368
	Flagellin B	514
	Tellurium resistance protein TerD	479
	Co-chaperone and heat shock protein 24	466
	Leucyl aminopeptidase	319
	Adenylate kinase	297
	ATP-dependent Clp protease proteolytic subunit	252
	Peptidoglycan-associated lipoprotein precursor	210
	Hypothetical protein HSUHS5_0940	207
	5'-methylthioadenosine/S-adenosylhomocysteine	177

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	nucleosidase	
	Molybdenum cofactor biosynthesis protein	145
9	Adenylate kinase	1268
	Inorganic pyrophosphatase	760
	Hypothetical protein HSUHS5_0940	723
	ATP-dependent Clp protease proteolytic subunit	645
	Transcription elongation factor GreA	605
	Flagellin A	568
	Molybdenum cofactor biosynthesis protein	456
	Chaperonin GroEL	388
	Tellurium resistance protein TerD	273
	Biotin carboxyl carrier protein	258
	Phosphoserine phosphatase (serB)	246
	Peptidoglycan-associated lipoprotein precursor	219
	Purine-binding chemotaxis protein	189
	Co-chaperone and heat shock protein 24	178
	Peroxiredoxin	160
	Leucyl aminopeptidase	125
	2-oxoglutarate-acceptor oxidoreductase subunit OorC	54

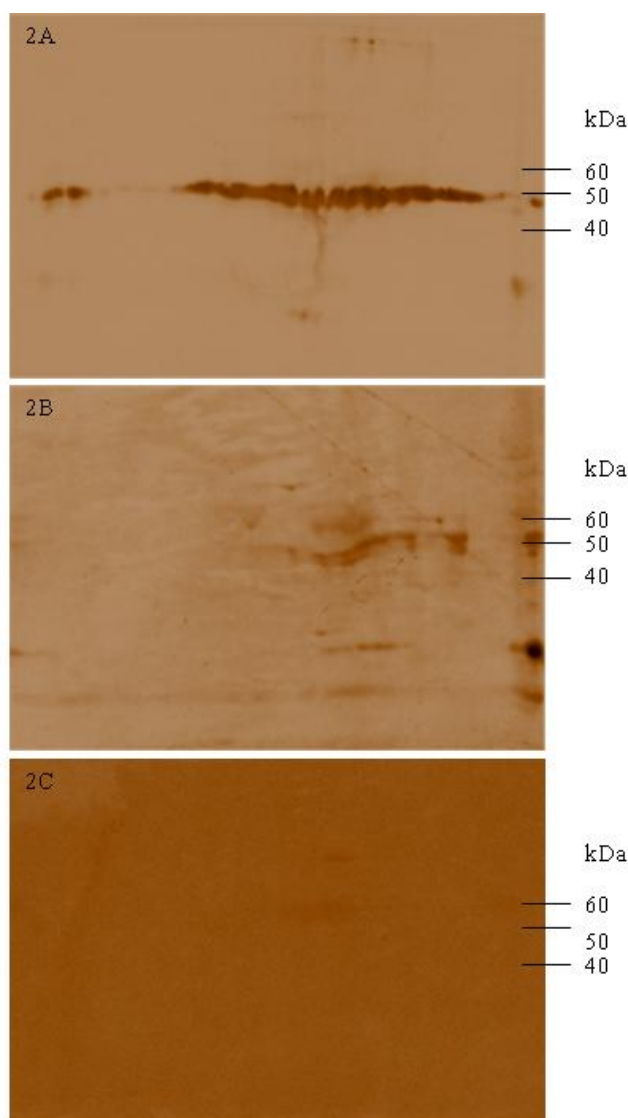


Fig 2A-C: 2D-immunoblots obtained after loading of *H. heilmannii* strain ASB1.4-protein extract (2A), *H. bizzozeronii* strain R1051 (2B), and *H. pylori* SS1 and loading with the serum sample of the patient infected with *H. suis*.

### 3.3. 1D- PAGE analysis of *H. suis* rUreB, rNapA and rGGT

For rUreB, reactivity was detected in the serum of the *H. suis* infected patient, one of the 5 *H. pylori* infected patients and the murine positive control serum, with a distinct band visible at ~63 kDa. For rNapA, reactivity was detected for the very same serum samples as for rUreB, with a band visible at ~60 kDa. As previously described by Flahou and co-workers (2011), recombinant *H. suis* GGT is secreted as a pro-form (~60 kDa), which is subsequently autoprocessed into a large and a small subunit (~40 kDa and ~20 kDa, respectively). Immunoreactivity was shown for the sera of all *H. pylori* infected patients only at ~60 kDa, and for the serum of the *H. heilmannii* infected patient only at ~20 kDa. In comparison, a strong

immunogenic reaction was visualized both at ~20 kDa and ~60 kDa for the serum obtained from the *H. suis* infected patient and for the murine positive control serum (Fig 3).

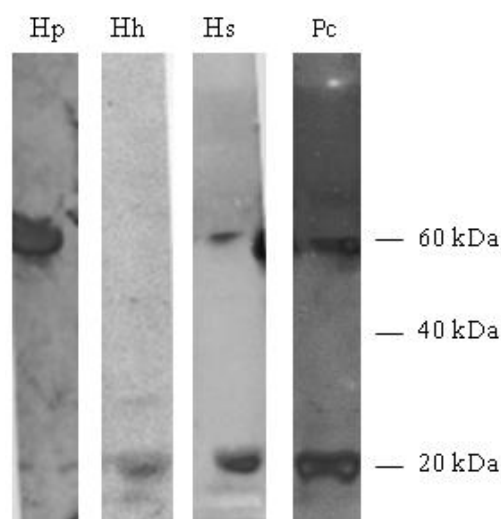


Fig 3: 1D-PAGE immunoblotting of *H. suis* rGGT, probed with serum of a patient infected with *H. pylori* (Hp), *H. heilmannii* (Hh), *H. suis* (Hs) or *H. suis* immunized mice (positive control Pc). No immunoreactivity against rGGT is seen against the ~40 kDa large subunit. Only in the lanes probed with serum from patients with a NHPH-infection and with the positive control serum, immunoreactivity is seen at ~20 kDa. Immunoreactivity at ~60 kDa was shown for the lanes probed with positive control serum and with sera from *H. pylori* and *H. suis* infected patients.

#### 4. Discussion

The main aim of the current study was to identify serologic markers which could be of value in future diagnosis of *H. suis* infections in humans. Serological tests were of the first methods used for the diagnosis of *H. pylori* infections and are still valued ever since, especially in epidemiological assays (Burucoa et al., 2013). Serology is indeed the only method applicable for large-scale, population-based screening (Formichella et al., 2013). For *H. suis*, serological diagnosis may even be more important than for *H. pylori* as demonstration of *H. suis* in gastric samples is hampered by its patchy distribution in the stomach and the very fastidious nature of this species. The focal colonization pattern of *H. suis* most probably also accounts for the lower accuracy of Urea Breath Tests than is the case for *H. pylori* infected patients (Haesebrouck et al., 2009).

In a first approach, we studied the reaction pattern of sera obtained from human patients infected with *H. suis*, *H. heilmanni* or *H. pylori* against proteins present in whole-cell

lysate of several NHPH and *H. pylori*. This allowed to select 9 immuno-reactive protein spots between ~20kDa and ~30kDa and at ~60kDa which were recognized by serum from the *H. suis* infected patient, but not by serum from patients infected with other *Helicobacter* species. For each of these spots, however, the identification by LC-MS/MS led to the correspondence of more than one protein on the same position in the gel and immuno-reactivity visualized on a blotting membrane does not necessarily result from the most abundant protein identified on this position. Further studies are necessary to determine the usefulness of these proteins as antigens in *H. suis*-specific serological tests.

In our second approach, three proteins were selected for recombinant expression, purification and use in 1D-PAGE blots. For UreB, which is involved in acid-resistance of the bacteria, a marked reactivity was detected in the serum sample of the *H. suis* infected patient, the serum sample of a *H. pylori* infected patient, and the positive control serum originating from lysate-immunized mice. NapA is a putative virulence factor of *H. suis* that has, so far, been detected in all *H. suis* strains investigated. For this protein, reactivity was detected in the same serum samples as when using rUreB. These findings contrast with the results obtained after experimental infection of mice with *H. suis*, since antibodies to UreB were not detected in sera of these animals collected at four weeks after infection (Vermoote et al., 2012). This may indicate that the immune response after infection with *H. suis* may be host-specific or may depend on the infection stage.

Immunoreactivity to *H. suis* GGT was also observed in the present study. Previous studies have identified the *H. suis* GGT as an important factor involved in death of gastric epithelial cells and modulation of the host immune response (Flahou et al., 2011; Zhang et al., 2013). This enzyme was shown to be synthesized as a precursor enzyme of ~60 kDa, with subsequent processing into a large (~40 kDa) and a small subunit (~20 kDa), which has also been described for other bacterial GGT's such as the *H. pylori* and *E. coli* GGT (Suzuki and Kumagai, 2002; Boanca et al., 2006; Flahou et al., 2011). In the present study, *H. suis* GGT immuno-reactivity was observed when using the serum originating from the *H. suis* infected patient at ~20 kDa and ~60 kDa. The same pattern was observed in blots incubated with the positive control serum pool, whereas only at ~20 kDa reactivity was observed for the serum sample originating from the *H. heilmannii* infected patient and only at ~60 kDa for the serum samples of all included *H. pylori* infected patients. These observations might suggest that the 40 kDa GGT subunit does not contain an antigenic determinant or epitope. Seen the fact that only the 60 kDa subunit was recognized by the sera of *H. pylori* infected humans, one might conclude that only the proenzyme presents the antigenic determinant recognized by serum

from *H. pylori* infected patients, in contrast to serum from *H. suis* infected patients, recognizing possibly the same epitope in the pro-enzyme as well as the small subunit after autoprocessing of the proenzyme. Formichella et al. (2013) noticed that *H. pylori* GGT was recognized in half of the patients, but described a stability problem when applying test strips, which was probably due to the autoprocessing of the 60-kDa precursor, which in turn influences the detectability of the antigen.

Our finding that none of the sera of patients infected with *H. pylori* recognized the 20 kDa subunit of the *H. suis* rGGT, which was recognized by human patients infected with *H. suis* or *H. heilmannii* as well as by sera of *H. suis* immunized mice, may indicate that this antigen might be useful to differentiate infections with NHPH from *H. pylori* infections in human patients. Combining it in a multiplex protein array with *H. suis* rUreB and rNapA, which in the present study were not recognized by the serum of the *H. heilmannii* infected patient, as well as with proteins detected in the whole-cell lysate derived spots that were differentially recognized by the serum of the *H. suis* infected patient (our first approach), might allow development of a *H. suis*-specific serological test. Such test will have to be validated by comparing the antibody binding patterns of sera from several *H. suis* infected patients, as well as sera from healthy subjects and patients infected with other gastric *Helicobacter* species, including *H. pylori*. One of the major challenges to be countered consists of collecting sera of human patients infected with NHPH, since such sera are not readily available.

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# **General discussion**



Even though *H. pylori* is considered as the primary causative agent of infectious gastric diseases in humans, awareness of the clinical relevance of non-*Helicobacter pylori Helicobacter* (NHPH) infections has increased over the past 25 years. These infections most likely originate from animals, but the exact routes of transmission from animal to man remain unclear. In addition, the diagnosis is more complex than is the case for *H. pylori* infections, which often results in the lack of an exact species identification. In this thesis, we focused on the transmission, the detection and diagnosis of the most prevalent NHPH species in humans, *H. suis*. In the next paragraphs, new insights and future perspectives resulting from the present research will be discussed.

### **1. The importance of pork in the transmission of *H. suis* to humans**

Only a few years ago, a new isolation method was successfully applied for the *in vitro* cultivation of *H. suis* from the stomach mucosa of pigs (Baele et al., 2008). Since the description of this method, only 20 *H. suis* strains have been isolated from porcine stomachs, which demonstrates the long and laborious process to obtain pure cultures of this fastidious and slow-growing microorganism. Given this low success rate from an organ which is the primary habitat of this micro-organism where it is usually present in high numbers, culture-based methods are not considered as appropriate detection methods of viable *H. suis* bacteria in complex matrices, such as meat. In contrast, culture methods are considered the gold standard in prevalence studies of other important foodborne pathogens, such as *Campylobacter* and *Salmonella*, on pork carcasses and meat (Van Loock et al., 2000; Butzler, 2004; Jasson et al., 2010). However, while bacteria used to be considered viable when they could be cultured, today's idea of viability assessment tends to be different, certainly since the existence of a viable but non culturable (VBNC) state was hypothesized (Shahamat et al. 1989). Bacteria that have entered this state are no longer culturable from the environment, although they remain viable and may be capable of establishing infection in a host (Oliver, 2005).

In order to determine whether pork meat constitutes a possible source of *H. suis* infections for humans, a new, non-culture dependent method, which differentiates and quantifies viable from dead *H. suis* bacteria, was needed. One of the most successful recent approaches to detect viable bacteria using non-culture dependent methods, is based on bacterial membrane integrity (Nocker et al., 2007). In **Chapter 1**, the elaboration of such a technique was described, by combining qPCR with a prior ethidium monoazide (EMA) treatment. The main advantage of using an EMA qPCR can be found in the speed and

sensitivity of the molecular detection, while at the same time providing information on viability. Using the developed EMA qPCR, we demonstrated that *H. suis* survives in experimentally contaminated pork samples for up to 48 h. This method may prove useful for further studies concerning *H. suis* survival in different environmental conditions and matrices, which is vital to identify potential sources of infection. The survival of *H. suis* in a certain matrix may depend on the availability of a suitable niche with specific requirements of moisture and oxygen levels. Hence, it was surprising that DNA derived from viable *H. suis* bacteria was detected in minced pork at retail level. In the current study, only low concentrations of viable *H. suis* bacteria were demonstrated, which further decreased over time. Therefore, although survival of *H. suis* was demonstrated, multiplication in pork (as is possible for certain foodborne pathogens, such as *Salmonella*) is highly unlikely. Since the dose required to infect humans is unknown for all *Helicobacter* spp. and at this stage, there is no information on possible factors hampering or facilitating survival, no clear conclusion can be drawn concerning the true hazard of contaminated meat as a vehicle for *H. suis* infections in humans.

Consumption of pork is particularly prominent in Europe, and the prevalence of *H. suis* infections in pigs at slaughter age is 60% or more (Haesebrouck et al., 2009) and pork carcasses were shown to be frequently contaminated (**Chapter 2**). Therefore, one might assume that the prevalence of *H. suis* infections in humans is also expected to be high. However, in **Chapter 3**, the prevalence of NHPH-infections among 118 patients suffering from severe gastric upset was reported to be lower than 1%, which correlates with previous studies conducted in Western countries (Hilzenrat et al., 1995; Mention et al., 1999; Ierardi et al., 2001; Boyanova et al., 2007; Iwanczak et al., 2012). Only a single patient was diagnosed with *H. suis* infection in recent studies performed by our team (Joosten et al., 2013). This apparently low prevalence at present in Europe may be in contrast with other parts of the world. In China and Thailand, the presence of NHPH in gastric biopsies was shown 2% and 6%, respectively, in patients suffering from severe gastric upset (Yali et al., 1998; Yang et al., 1998). Recently, Liu et al. (2014) showed that NHPH infections are common among Chinese patients co-infected with *H. pylori*, with *H. suis* being the most prevalent NHPH species, infecting 6.94% of all *H. pylori*-positive patients. Although coinfections with *H. pylori* and NHPH species have previously been described by other researchers, some authors suggested a negative correlation between *H. suis* and *H. pylori* colonization (De Groote et al., 2005; Ojano et al., 2012; Yakoob et al., 2012; Martin et al., 2013). Whether or not the higher occurrence reported in Asian countries originate from a higher colonization capacity among Asian strains,

could be due to an underestimation in European countries, or could be inherent to the food culture and cultural food processing activities is unclear and could be the aim of future studies.

## **2. The role of slaughter in the spread of *H. suis* through the meat supply chain**

Using qPCR, *H. suis* DNA was detected on dressed pork carcasses (**Chapter 2**), which adds strength to the hypothesis of *H. suis* transmission through pork consumption. No differentiation between viable or dead bacteria was made during this study, since the time span between collection of the carcass samples and processing of these samples was a limiting factor, which could render false negative results. The shoulder and chest area were the two most frequently contaminated regions on the carcasses. We therefore hypothesize that carcass contamination is either caused by spilling stomach content after cutting the esophagus at evisceration or, more likely, during splitting, when the splitting saw reaches the mouth of the pigs, that may already be contaminated by regurgitation as pigs are secured upside down. Although in theory, the carcass splitter should be disinfected with water at 82°C between each carcass, this procedure is not always feasible in practice. The water used is, however, frequently at a lower and ineffective temperature (Botteldoorn et al., 2004; De Sadeleer et al., 2008). Moreover, in many slaughterhouses a mechanical carcass splitter is used, which is not disinfected in between each carcass.

In order to control carcass contamination, it is crucial to first identify the sources and routes of contamination throughout the slaughter process. The current study however, did not aim to identify the exact route(s) of (cross-)contamination. Future studies are needed to assess the role of the different steps at slaughter and their impact on carcass contamination. This can be achieved by following individual pigs from the lairage area up until the respective carcasses hanging in the chilling room. Taking samples of the same pig, does not only allow to identify risk points along the slaughter line, but also to study the genotypic diversity of *H. suis* in the slaughterhouse. Again, the low success rate of *H. suis* isolation has hampered research, by narrowing the options in the development of a genotyping method. Only recently, a multilocus sequence typing (MLST) method, allowing typing of *H. suis* directly in stomach samples, was developed (Liang et al., 2013). Employing this technique, the authors revealed that *H. suis* is a genetically heterogenic bacterial species. In addition, strain typing was applied to four *H. suis* strains colonizing human patients, which showed a very close relationship to porcine *H. suis* strains. (Liang et al., 2013; Flahou et al., 2015, personal communications). The same technique was successfully applied in the present study to characterize *H. suis* from five selected mouth swab samples at the slaughterhouse (**chapter 2**).

Five different sequence types (STs) were identified and within samples derived from the same pig herd, two or three different STs were found. Although only a low number of *H. suis* was examined, the high diversity on carcass level might indicate contamination of carcasses does not originate from the slaughter environment and agrees with the previous findings of Liang et al. (2013) concerning the high diversity at pig herd level. On the contrary, MLST analysis was not successfully applied to a selection of the dressed carcass swabs. Most probably, this was due to the low concentration of *H. suis* DNA obtained from the sponge samples. Therefore, no comparison between genotypes of *H. suis* bacteria present in the mouth could be made with those present on the carcasses. At this stage, for example, it is unclear whether positive mouth and/or lymph node samples originating from one pig can be related to contaminated contiguous pig carcasses along the slaughterline. Therefore, whether or not there is a causal linkage between the contamination of carcasses and the mouth or lymph nodes of successive pigs remains unclear.

In a similar study design, investigating the prevalence of *Salmonella* contamination at slaughter, a contaminated lairage area was shown to be related to positive carcasses after polishing, before opening the belly of the pig (De Busser et al., 2011). Several authors have emphasized the role of the lairage pens in (cross)contamination of *Salmonella* on pork carcasses due to re-shedding by carrier animals under stressful conditions (Hurd et al., 2001; De Sadeleer et al., 2008; De Busser et al., 2011). Measures taken during slaughter procedures in avoidance of fecal contamination not only reduce the spread of *Salmonella* on pig carcasses, but have shown to drastically limit *Campylobacter* contamination on pork meat as well (Borch et al., 1996). Both pathogens, however, are associated with the porcine intestine and are primarily shed in the faeces. Since at this moment, there are no indications for fecal transmission of *H. suis* in pigs, we believe this pathway is of less importance in carcass contamination.

### **3. Feasibility of developing a diagnostic test based on serology**

Many serological tests are available for the diagnosis of *H. pylori* infections (Vaira et al., 1999; Burucoa et al., 2013; Formichella et al., 2013). In contrast, no such test identifying antibodies against NHPH species in blood of humans or animals is available yet.

Several techniques can be applied for antibody detection. Most tests are based on enzyme-linked immunosorbent assay (ELISA) formats, using whole cell lysates or purified antigens (Vaira et al., 1999). Latex agglutination tests have been demonstrated to be more suitable as near patient tests, because they are technically simple to perform and provide a

result within minutes. Only recently, a highly sensitive and specific line immunoassay based on recombinant *H. pylori* proteins, which are known to be involved in virulence, colonization, and immune evasion, was developed (Formichella et al., 2013). The main advantage of this test principle over ELISA, is the possibility of a separate application of different single antigens, which allows screening for diverse virulence factors from different *H. pylori* strains. Overall, the performance of serology is highly dependent on the choice of antigens. The antigens used should not cross-react with proteins of other bacterial species in order to increase the specificity. Moreover, they should be highly conserved in different strains of the same species to be applicable in different patient populations. In **Chapter 3**, a small *H. suis* GGT subunit was identified to elicit an immune response only in NHPH-infected humans, holding promise as a potential target protein for the differentiation of gastric NHPH infections from *H. pylori* infections. Furthermore, in the present study *H. suis* rUreB and rNapA were recognized by the serum of one *H. pylori* infected patient and a *H. suis* infected patient, but not by the serum of a *H. heilmannii* infected patient. The proteins identified during this study seem to be appropriate targets to be included in a serological test kit, since they do not react with sera from non-infected patients. Moreover, according to their function and to previously performed genome sequence analysis (Vermoote et al., 2011), these proteins are probably highly conserved among different *H. suis* strains. Therefore, by combining the small GGT subunit in a multiplex protein array with *H. suis* rUreB and rNapA, the development of a *H. suis*-specific serological test might be feasible. Further research on variations in these antigens amongst *H. suis* strains is however necessary. Furthermore, the clonal makeup and composition of an individual's immune system depends both on genetic and environmental factors, which in turn can lead to complexities in standardizing a serological test. Therefore, at this moment the major challenge in the development and validation of such a test consists of collecting a sufficient number of sera from human patients infected with *H. suis* and other gastric *Helicobacter* species.

#### **4. Future perspectives**

From the results of the present and other recent research, some perspectives to future work can be formulated. First of all, the prevalence of *H. suis* during different stages at the slaughterhouse and the environmental factors contributing to *H. suis* contamination of pig carcasses should be further examined. Possible environmental factors contributing to the survival of *H. suis* bacteria include the presence of a moist environment, a low oxygen tension and the ability to form biofilms.

A sequence library representing the genetic diversity among *H. suis* strains colonizing the stomach of humans, might enable to comprehend whether certain *H. suis* strains are more able to jump from pigs to humans than others. A comparative genome analysis of these strains with the those present on pork carcasses, might further elucidate the true importance of pork consumption in the transmission of *H. suis* infections. Strain typing without the need for prior cultivation has been made possible by the development of an MLST technique (Liang et al., 2013). At the moment, the biggest obstacle remains the acquirement of a sufficient number of *H. suis* isolates from humans.

The applicability and suitability of *H. suis* rGGT, rUreB and rNapA in diagnostic assays, determining the presence of antibodies in human sera, should be further evaluated. Since a negative correlation between *H. suis* and *H. pylori* colonization has been proposed and a steady decline in *H. pylori* prevalence in Western society has been observed (Martin et al., 2013; Dore et al., 2015), *H. suis* infections might emerge in the future, which in turn again emphasizes the need for a species-specific serologic test.

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# Summary



Since the discovery of *Helicobacter (H.) pylori* infections and their association with gastric diseases in humans, many new gastric *Helicobacter* species have been described. These so-called gastric non-*Helicobacter pylori* (NHPH) species are believed to primarily colonize the stomach of domesticated and wild animals. Meanwhile, 5 NHPH species have been found to colonize the human stomach as well, causing gastritis, gastric ulcers and gastric cancer. *H. suis* is regarded to be the most prevalent NHPH species in the gastric mucosa of humans suffering from gastric disease. *H. suis* is a Gram-negative, spiral-shaped bacterium that colonizes the stomach of the majority of slaughter pigs worldwide. In pigs, *H. suis* infections have been associated with ulcers of the gastric non-glandular mucosa, chronic gastritis and a decrease in daily weight gain. Pigs are considered the main source of *H. suis* infection for humans. The exact routes of *H. suis* transmission to humans, however, remain unclear. Since close contact with animals seems to be the most obvious manner for humans to contract NHPH infections, it is remarkable that pig-related species are more prevalent in humans than those species associated with cats or dogs. This might indicate that other routes, such as the consumption of contaminated pork, may contribute to the transmission of *H. suis* infections to humans as well. Therefore, the first aim of this thesis was to determine the potential of pork as a source of *H. suis* infection for humans.

First, the presence of viable *H. suis* bacteria in retail pork was determined. Since isolation and cultivation of *H. suis* is a very laborious process with a low success rate, detection of viable *H. suis* bacteria in pork by using culture-based methods is not feasible. In order to determine whether pork constitutes a possible source of *H. suis* infections for humans, a non-culture dependent, quantitative detection method allowing differentiation of viable from dead *H. suis* bacteria in pork had to be developed. In chapter one, the elaboration of a protocol for pork sample treatment with ethidium monoazide (EMA) in combination with qPCR was described. After validation on experimentally contaminated pork, this EMA qPCR was applied to 50 retail pork samples. In two of these pork samples, DNA derived from viable *H. suis* bacteria was detected, demonstrating the presence of low concentrations of viable bacteria. Sequence analysis of the positive PCR products confirmed the presence of *H. suis* DNA. Using the same technique, the persistence of *H. suis* bacteria in experimentally contaminated pork was established for at least 48 h. In conclusion, consumption or manipulation of contaminated pork may constitute a new route of transmission for *H. suis* infections in humans.

The main goal addressed in chapter two, was to determine the occurrence of *H. suis* on pork carcasses during slaughter practices. In two consecutive studies, samples were taken at different stages of slaughter (scalding water, head and mouth swabs, mesenteric lymph nodes, palatine tonsils and on the chest, shoulder and ham region of pork carcasses) in three slaughterhouses, and examined for the presence of *H. suis*, using qPCR. *H. suis* DNA was detected in all slaughterhouses, in 8.3% of all 1083 samples. In the first study, *H. suis* DNA was detected in 10% of the head swabs (n: 90), 14.4% of the mouth swabs (n: 90) and 13.3% of the mesenteric lymph nodes (n: 90). In order to elucidate whether true colonization occurs in the lymph nodes, the presence of *H. suis* DNA was assessed at the mesenteric lymph nodes of 24 pigs after 4 weeks of experimental infection. Despite high-level colonization of the porcine stomachs with the *H. suis* strain, no *H. suis* DNA was detected in the mesenteric lymph nodes. This might indicate that its presence in the mesenteric lymph nodes of slaughtered pigs is due to contamination during the slaughter process, or that longer infection periods are necessary for the colonization of lymph nodes. No *H. suis* DNA was detected in the palatine tonsils and scalding water samples taken at three different slaughterhouses.

In the second study, *H. suis* DNA was detected in all five dressed pork carcass areas, with the mouth and chest being the two most frequently contaminated areas pre-chilling and the shoulder and chest the two most frequently contaminated areas post-chilling. Contamination levels of dressed pork samples did not exceed 184 genomic equivalents per 100 cm<sup>2</sup> (shoulder, ham) or 300 cm<sup>2</sup> (chest). All positive PCR products were subjected to sequence analysis of the *ureA* gene, confirming the identification of *H. suis* DNA. Using multilocus sequence typing (MLST) on five selected samples, a high diversity among the strains derived from the same pig herd (and all slaughtered in the same abattoir) was demonstrated. In conclusion, we demonstrate a relatively high presence of *H. suis* on pork carcasses. To which extent the consumption of contaminated pork contributes to the transmission of *H. suis* infections from pigs to humans remains unclear. Furthermore, the current study does not identify the exact route(s) of carcass contamination during the slaughter process. These considerations might encompass the focus of future studies.

Various tests for the diagnosis of *H. pylori* infections are readily available. In contrast, the focal distribution of NHPH bacteria in the human stomach and the very fastidious nature of these species often hamper the diagnosis of these infections in humans. At present, no test for the detection of antibodies against *H. suis*, or NHPH-species in general, in blood samples of humans or animals is available. In order to obtain the knowledge needed to develop an *H.*

*suis*-specific serological diagnostic test, *H. suis*-specific antigens that are recognized by antibodies of infected human patients need to be identified. The second aim of this thesis, was therefore to identify *H. suis* antigens which could be of value in future serological diagnosis of *H. suis* infections in humans (chapter three). For this purpose, two approaches were used. In a first series of experiments, *H. suis*-specific antigens which are recognized by sera of infected patients, were identified by performing two-dimensional gel electrophoresis of *H. suis* proteins, which were probed with sera from human patients infected with *H. suis*, *H. heilmannii* or *H. pylori*. In a second approach, the reaction of these sera was studied, using one-dimensional gel electrophoresis (followed by immunoblotting) of *H. suis* proteins urease subunit B (UreB), neutrophil-activating protein A (Nap A) and gamma-glutamyl transpeptidase (GGT).

During the first approach, nine immuno-reactive protein spots between ~20kDa and ~30kDa and at ~60kDa (which were recognized by serum from a *H. suis* infected patient, but not by serum from patients infected with other *Helicobacter* species) were selected for further identification. For each of these spots, however, the identification by LC-MS/MS led to the correspondence of more than one protein on the same position in the gel.

In our second approach, immunoreactivity to all three selected *H. suis* proteins was observed. In previous studies *H. suis* GGT was shown to be synthesized as a precursor enzyme of ~60 kDa, with subsequent processing into a large (~40 kDa) and a small subunit (~20 kDa). During the current study, the small subunit (20 kDa) of *H. suis* rGGT was recognized by sera of *H. suis* or *H. heilmannii* infected humans, but not by sera from *H. pylori* infected patients. In contrast, reactivity was only observed at ~60 kDa for the serum samples of all included *H. pylori* infected patients. Reactivity for the very same protein was observed after applying the serum of the *H. suis* infected patient as well, while for the *H. heilmannii* infected patient, no immunoreaction was observed. For *H. suis* rUreB, a marked reactivity was detected in the serum sample of the *H. suis* infected patient and the serum sample of one *H. pylori* infected patient. For *H. suis* rNapA, reactivity was detected in the same serum samples as for rUreB.

Combining *H. suis* rGGT in a multiplex protein array with *H. suis* rUreB and rNapA, might therefore allow the development of a *H. suis*-specific serological test. Such test will have to be validated by comparing the antibody binding patterns of sera from several *H. suis* infected, NHPH infected and *H. pylori* infected patients and of sera from healthy humans.



# **Samenvatting**



Sinds de ontdekking van *Helicobacter (H.) pylori* infecties en hun associatie met maagaandoeningen bij de mens, werden verschillende nieuwe gastrale *Helicobacter* species beschreven. Deze zogenaamde gastrale non-*Helicobacter pylori Helicobacter* (NHPH) species koloniseren voornamelijk de maag van gedomesticeerde en wilde dieren. Ondertussen werd reeds voor 5 NHPH species aangetoond dat ze ook de maag van de mens kunnen koloniseren en gastritis, maagulcera en maagkanker kunnen veroorzaken. *H. suis* wordt beschouwd als de meest frequent voorkomende NHPH species bij mensen met maagaandoeningen. *H. suis* is een Gram negatieve, spiraalvormige bacterie die wereldwijd de maag van de meerderheid van de slachtvarkens koloniseert. Bij varkens worden *H. suis* infecties geassocieerd met maagulcera ter hoogte van het non-glandulaire deel van het maagslijmvlies, chronische gastritis en een daling van de dagelijkse gewichtstoename. Varkens worden beschouwd als de belangrijkste bron van *H. suis* infecties voor de mens. Het is onduidelijk hoe de mens besmet wordt met *H. suis*. Aangezien nauw contact met dieren de meest voor de hand liggende manier is voor het oplopen van NHPH infecties, is het opmerkelijk dat de varken-gerelateerde species, *H. suis*, vaker voorkomt bij de mens dan *Helicobacter* species die vooral met katten of honden geassocieerd worden. Dit kan erop duiden dat ook andere routes een rol zouden kunnen spelen in de overdracht van *H. suis* infecties naar de mens. Hierbij kan onder andere gedacht worden aan overdracht via besmet varkensvlees. Het eerste doel van deze thesis was dan ook nagaan of varkensvlees een potentiële bron van *H. suis* infecties voor de mens kan zijn.

In een eerste studie werd de aanwezigheid van levende *H. suis* bacteriën bepaald in varkensvlees. Aangezien isolatie en cultivatie van *H. suis* een zeer moeizaam proces is met een lage kans op slagen, is de detectie van levende *H. suis* bacteriën in varkensvlees met behulp van cultuur-afhankelijke methodes niet haalbaar. Om te bepalen of varkensvlees een mogelijke bron vormt voor *H. suis* infecties bij de mens, was er dus nood aan een cultuur-onafhankelijke, kwantitatieve detectiemethode, die toelaat een onderscheid te maken tussen levende en dode *H. suis* bacteriën in varkensvlees. In hoofdstuk één wordt de uitwerking van een dergelijke methode beschreven. Hierbij werd gebruik gemaakt van ethidium monoazide (EMA) in combinatie met qPCR. Na validatie op experimenteel besmet varkensvlees, werd deze EMA qPCR methode toegepast op 50 stalen varkensgehakt dat aangekocht werd in 3 verschillende winkels. In twee van deze 50 stalen werd DNA afkomstig van levende *H. suis* bacteriën gedetecteerd, waarbij lage concentraties aan levende bacteriën werden aangetoond. De aanwezigheid van *H. suis* DNA werd bevestigd door middel van sequentieanalyse van de

positieve PCR producten. Met behulp van EMA qPCR werd bovendien aangetoond dat *H. suis* bacteriën gedurende ten minste 48 uur overleven in experimenteel besmet varkensvlees. Als conclusie kan gesteld worden dat consumptie of manipulatie van besmet varkensvlees een mogelijke bron van *H. suis* infecties kan zijn voor de mens.

In hoofdstuk twee werd het voorkomen van *H. suis* op varkenskarkassen tijdens het slachtproces nader onderzocht. In twee opeenvolgende studies werden in drie slachthuizen stalen genomen tijdens verschillende stadia van het slachtproces (broeibakwater, kop en muil swabs, mesenteriale lymfeknopen, tonsillen en ter hoogte van de borst, schouder en ham regio van varkenskarkassen). Deze werden onderzocht op de aanwezigheid van *H. suis* DNA, met behulp van qPCR. *H. suis* DNA werd teruggevonden in alle slachthuizen en dit in 8,3% van de 1083 onderzochte stalen. In de eerste studie werd *H. suis* DNA aangetoond in 10% van de kop swabs (n: 90), 14,4% van de muil swabs (n: 90) en 13,3% van de mesenteriale lymfeknopen (n: 90). Om na te gaan of echt kolonisatie optreedt in de lymfeknopen, werd de aanwezigheid van *H. suis* DNA onderzocht in de mesenteriale lymfeknopen van 24 varkens, op 4 weken na een experimentele *H. suis* infectie. Niettegenstaande een hoge graad van *H. suis* kolonisatie werd vastgesteld ter hoogte van de maag van de varkens, werd geen *H. suis* DNA gedetecteerd in de mesenteriale lymfeknopen. Dit kan erop wijzen dat de aanwezigheid van *H. suis* DNA in de mesenteriale lymfeknopen van geslachte varkens het gevolg is van contaminatie tijdens het slachtproces. Het is evenwel ook mogelijk dat de mesenteriale lymfeknopen pas gekoloniseerd worden op een later tijdstip na infectie. Er werd geen *H. suis* DNA teruggevonden in de tonsillen en het broeibakwater.

Gedurende de tweede studie, werd *H. suis* DNA gedetecteerd ter hoogte van alle vijf de bemonsterde zones op het varkenskarkas. De muil en borst waren de frequentst gecontamineerde zones voor het koelen. Na koeling, werd *H. suis* DNA het frequentst aangetoond ter hoogte van de schouder en borst. Het aantal genomische equivalenten dat werd gedetecteerd in de karkasstalen bedroeg niet meer dan 184 per 100 cm<sup>2</sup> (schouder, ham) of 300 cm<sup>2</sup> (borst). Alle positieve PCR producten werden onderworpen aan een sequentieanalyse van het *ureA* -gen, waarbij de identificatie van *H. suis* DNA werd bevestigd. Met behulp van “multilocus sequence typing” (MLST) op vijf geselecteerde stalen, werd aangetoond dat in hetzelfde slachthuis, diverse *H. suis* stammen kunnen aanwezig zijn op karkassen afkomstig van dezelfde varkensstapel. Als conclusie kan gesteld worden dat *H. suis* DNA frequent kan aangetoond worden op varkenskarkassen. De mate waarin consumptie van besmet varkensvlees bijdraagt tot de overdracht van *H. suis* infecties naar de mens blijft onduidelijk.

Bovendien kan op basis van de resultaten bekomen tijdens de huidige studie, niet nagegaan worden hoe karkassen exact besmet worden tijdens het slachtproces. Dit zou het onderwerp van toekomstige studies kunnen uitmaken.

Voor de diagnose van *H. pylori* infecties zijn diverse laboratoriumtesten beschikbaar. Het stellen van de diagnose van een infectie met NHPH species bij de mens is daarentegen veel moeilijker. Dit is onder andere te wijten aan de zeer focale kolonisatie van NHPH bacteriën in de maag, waardoor deze kiemen niet altijd terug te vinden zijn in een biopt dat genomen wordt tijdens gastroscopie. Ook testen die gebaseerd zijn op de urease activiteit van deze kiemen kunnen om dezelfde reden een vals negatief resultaat geven. Bovendien zijn gastrale NHPH bacteriën bijzonder moeilijk te kweken in het laboratorium en voor sommige species zoals *H. suis*, is isolatie vanuit stalen van mensen tot nu toe nog niet gelukt. Op dit moment is er ook geen test beschikbaar die toelaat om antistoffen tegenover *H. suis* of andere NHPH species aan te tonen in het bloed van mensen of dieren. Een eerste stap in de ontwikkeling van een dergelijke test, is de identificatie van *H. suis*-specifieke antigenen, die worden herkend door antilichamen van geïnfecteerde patiënten. Dit vormde de derde doelstelling van deze thesis (hoofdstuk 3). Voor dit doel werden twee benaderingen gevolgd. In een eerste reeks van experimenten werden *H. suis*-specifieke antigenen, die herkend worden door antistoffen in het serum van geïnfecteerde patiënten, geïdentificeerd via tweedimensionale gelelektroforese van *H. suis* eiwitten, gevolgd door immunoblotting. Voor deze toepassing werd gebruik gemaakt van sera afkomstig van patiënten besmet met *H. suis*, *H. heilmannii* of *H. pylori*. In een tweede benadering werd de reactie van deze sera bestudeerd tegenover drie recombinant aangemaakte *H. suis* eiwitten, namelijk urease subunit B (UreB), neutrofiel-activerend eiwit A (NAP-A) en gamma-glutamyl transferase (GGT). Hierbij werd gebruik gemaakt van één-dimensionale gelelektroforese, gevolgd door immunoblotting.

Tijdens de eerste benadering, werden negen immuno-reactieve proteïne spots geselecteerd tussen ~ 20kDa en ~ 30kDa en ter hoogte van ~ 60kDa. Het betrof hier spots die herkend werden door het serum van een met *H. suis* geïnfecteerde patiënt, maar niet door serum van patiënten geïnfecteerd met andere *Helicobacter* species. Door toepassing van massaspectrometrie werd evenwel aangetoond dat elk van deze spots meerdere eiwitten bevatte.

In de tweede reeks experimenten, werd immunoreactiviteit tegen elk van de drie geselecteerde recombinante *H. suis* eiwitten waargenomen. Uit eerdere studies blijkt dat het *H. suis* GGT gesynthetiseerd wordt als een precursor enzyme van ~ 60 kDa dat nadien gesplitst

wordt in een grote (~ 40 kDa) en een kleine subeenheid (~ 20 kDa). Tijdens de huidige studie werd de kleine subeenheid (~ 20 kDa) van het *H. suis* rGGT herkend door sera van patiënten die besmet waren met *H. suis* of *H. heilmannii*, maar niet door sera van met *H. pylori* geïnfecteerde patiënten. Voor de serumstalen van de met *H. pylori* geïnfecteerde patiënten werd enkel reactiviteit ter hoogte van ~ 60 kDa waargenomen. Hetzelfde eiwit werd eveneens herkend door het serum van de met *H. suis* besmette patiënt, maar niet door het serum van de met *H. heilmannii* besmette patiënt. Het *H. suis* rUreB werd herkend door antistoffen in het serumstaal van de met *H. suis* besmette patiënt en het serumstaal van één patiënt die besmet was met *H. pylori*. Voor *H. suis* rNapA werd reactiviteit waargenomen bij gebruik van dezelfde serumstalen als voor rUreB.

Deze resultaten kunnen erop duiden dat door het combineren van *H. suis* rGGT met *H. suis* rUreB en rNapA in een multiplex protein array, de ontwikkeling van een *H. suis*-specifieke serologische test mogelijk is. Deze test moet dan gevalideerd worden met sera afkomstig van niet besmette mensen en met sera van patiënten die besmet zijn met met *H. suis*, andere NHPH en *H. pylori*.

# **Curriculum Vitae**



Lien De Cooman werd geboren op 2 augustus 1984 te Gent. Na het beëindigen van het secundair onderwijs aan het De La Sallecollege te Gent, startte ze de studies diergeneeskunde aan de Universiteit Gent. In 2010 behaalde ze het diploma van Master in de Diergeneeskundige Wetenschappen, evenals het diploma van Proefdierkunde aan de Faculteit Diergeneeskunde van de Universiteit Gent.

Geboeid door het wetenschappelijk onderzoek, startte ze in datzelfde jaar een doctoraatsstudie bij de Vakgroep Pathologie, Bacteriologie en Pluimveeziekten aan de Faculteit Diergeneeskunde van de Universiteit Gent. Gedurende 4 jaar voerde ze onderzoek uit naar de overdracht en diagnostiek van *Helicobacter suis*-infecties bij de mens, hetgeen leidde tot dit proefschrift. Deze studie werd gefinancierd door de Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu (FOD VVVL) en het bijzonder onderzoeksfonds van de Universiteit Gent.

Lien De Cooman is auteur en medeauteur van verschillende publicaties in internationale wetenschappelijke tijdschriften. Daarnaast nam ze actief deel aan internationale en nationale congressen.



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