Characterization of zoonotic gastric *Helicobacter* species associated with pigs, cats and dogs

Jungang Liang

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Promoters:

Prof. dr. F. Haesebrouck

Dr. Bram Flahou

Faculty of Veterinary Medicine
Department of Pathology, Bacteriology and Avian Diseases

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List of abbreviations

AGS	human gastric adenocarcinoma cell line
ATCC	American type culture collection
BLAST	basic local alignment search tool
bp	base pair
cagA	cytotoxin-associated gene A
CagA	cytotoxin-associated protein A
*cag*PAI	cytotoxin-associated gene pathogenicity island
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EMA	ethidium monoazide
FCS	fetal calf serum
g	gram
g/L	gram per liter
GGT	γ-glutamyl transpeptidase
GSH	reduced glutathione
h	hour
H.	*Helicobacter*
HBSS	Hank's balanced salt solution
HCl	hydrochloric acid
HspA	heat-shock protein A
hsp60	heat-shock protein 60 gene
Ig	immunoglobulin
kb	kilobase
kDa	kilodalton
LEL	lymphoepithelial lesion

L-Gln	L-glutamine
L-Glu	L-glutamate
l	liter
LPS	lipopolysaccharide
M	molar
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MLST	multilocus sequence typing
NapA	neutrophil-activating protein A
NHPH	non-*Helicobacter pylori Helicobacter*
nm	nanometer
°C	degree Celsius
OD	optical density
%	percentage
OMP	outer member protein
OMV	outer membrane vesicles
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	measure of acidity or basicity
PHYLIP	PHYLogeny Interference Package
PI	propidium iodide
rHSGGT	recombinant *H.suis* GGT
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	Real time polymerase chain system

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEM	transmission electron microscopy
Th response	T helper response
Treg	regulatory T cells
U	unit
UBT	urea breath test
µg	microgram
µL	microlitre
µM	micromolar
ureA/B	*urease* subunit A/B
VacA	vacuolating cytotoxin
vol	volume
WT	wild-type

GENERAL INTRODUCTION

The first *Helicobacter* species isolated from the stomach of humans, in 1983, was *Helicobacter* (*H.*) *pylori* (Warren and Marshall, 1983), although these bacteria had been observed in the stomach of humans already many years before (Doenges JL, 1938; Freedburg and Barron, 1940; Palmer ED, 1954; Steer and Colin-Jones, 1975; Fung *et al*, 1979). Infection with this bacterium has subsequently been associated with several human gastric disorders, such as gastritis, peptic ulceration and gastric cancer (Marshall and Warren, 1984). Indeed, the discovery of *H. pylori*, as well as the discovery of its role in gastric pathology, was a major breakthrough in medical science and this led to an exponential increase of research on gastric disease related to *H. pylori* infection. In subsequent years, several other non-*H. pylori* species, most of them associated with domestic animals, were shown to be capable of causing gastric disease in humans.

1. *Helicobacter pylori*

1.1 PREVALENCE, DIAGNOSIS, CLINICAL SIGNIFICANCE AND TREATMENT

Helicobacter pylori is a slightly curve-shaped, microaerophilic, Gram-negative bacterium that typically is localized in human gastric mucus (Testerman *et al*., 2001). This pathogen was previously thought to belong to the "*Campylobacte*r" genus. However, subsequent studies have demonstrated that it belongs to another genus and the name *Helicobacter pylori* was born (Goodwin *et al*., 1989; Karenlampi *et al*., 2004).

Today, infection with *Helicobacter pylori* is highly prevalent in humans world-wide (Bode *et al*.,1998), with a prevalence of 40% to 80%, depending on the geographical location, social habits and general welfare (Pounder and Ng, 1995; Kusters *et al*., 2006). It is presumed that infection is acquired primarily in infancy, as *H.pylori* is strictly transmitted from humans to humans (Drumm *et al*., 1990; Matsui *et al*., 2014). The transmission of *H. pylori* strains appears to occur through person-person contact, fecal-oral, or oral-oral routes and via recently contaminated food and water (Drumm *et al*., 1990; Berg *et al*., 1997). The transmission between family members was elucidated by molecular techniques (Dominici *et al*., 1999; Roma-Giannikou *et al*., 2003; Raymond *et al*., 2004).

In developing countries, more than 80% of the population is infected with *H. pylori*, even at young age. In developed countries, the prevalence of *H. pylori* is in general lower and remains under 40%. It is considerably lower in children and adolescents than in adults and elderly people (Kusters

et al., 2006; Pounder *et al*., 1995). However, low prevalence rates were recently reported in North Sulawesi, Indonesia, reaching only 14.3% for adults and 3.8% for children (Miftahussurur *et al*., 2014). In developed countries, it is thought that the prevalence of *H. pylori* infection is rapidly declining as a result of improvements in personal hygiene and quality of life (Yamaoka Y, 2009).

A variety of noninvasive and invasive methods have been described for the detection of *H. pylori*. The former ones consist of serological tests (Newell and Stacey, 1989) and ^{13}C or ^{14}C urea breath tests (Graham *et al*., 1987; Weil and Bell, 1989). Invasive methods rely on endoscopy and biopsy to provide material for bacterial detection and identification (Goodwin *et al*., 1985), histopathological examination (Gray *et al*., 1986) or rapid urease testing (Marshall *et al*., 1987). However, each of these techniques has some disadvantages. Firstly, they are sometimes too insensitive to confirm complete eradication of the organisms after medical treatment; secondly, they can not detect the coccoid form of the organisms (Steer HW, 1989); and thirdly, they sometimes cannot distinguish *H. pylori* from closely related organisms, for instance from animal sources (Lee *et al*., 1988; Tompkins *et al*., 1988). Several PCR methods were developed for the detection of *H. pylori*, based on the 16S rRNA gene, 26-kDa *ssa* gene, the *ureA* gene, *glmM* gene, etc. (Ho *et al*., 1991; Chisholm *et al*., 2001; Joosten *et al*., 2013). Of these, the *ureC* (*glmM*) gene PCR has been described to be one of the most sensitive and specific for the detection of *H. pylori* in gastric biopsy specimens (Lu *et al*., 1999; Smith *et al*., 2011).

Most infections with *H. pylori* are asymptomatic (Riegg *et al*., 1995; Graham *et al*., 1993) and the majority of carriers develop an asymptomatic chronic gastritis that persists for decades (Farnbacher *et al*., 2010). In up to 20% of the *H. pylori*-infected people, disease develops which mainly includes symptomatic gastritis and peptic ulceration, but also gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma (Devi *et al*., 2006; Farnbacher *et al*., 2010). Only about 1% of *H. pylori*-infected humans develop malignant gastric sequelae, indicating a multi-factorial process that includes host factors (gene polymorphisms) (Rad *et al*., 2004), environmental factors (alcohol and nicotine abuse, tobacco smoking, diet, etc.) (Ogihara *et al*., 2000), and bacterial factors (presence or absence of virulence factors). Gastric cancer is more prevalent in East Asian countries than in Western countries and still a leading cause of cancer death worldwide (Vilaichone *et al*., 2004; Jones *et al*., 2009; Matsunari *et al*., 2012; Matsusaka *et al*., 2014; Bornschein *et al*., 2014).

The genetic disposition of both the bacterial strain (genetic variance of certain bacterial proteins, see below) and the host can increase the potential of gastric cancer formation. Aberrant DNA

methylation in host gene promoter regions is thought to play a crucial role in gastric carcinogenesis. Chronic inflammation of the gastric epithelium due to *H. pylori* infection induces aberrant polyclonal methylation that may lead to an increased risk of gastric cancer (Matsusaka *et al*., 2014; Rickinson, 2014).

As a preventive strategy and measure against *H. pylori* infection, eradication therapy is only recommended for high-incidence regions, especially in Asia and large population studies with an adequate follow-up are required to demonstrate the effectiveness of such an approach applied in Western populations (Bornschein and Malfertheiner, 2014). Diverse drug combinations, for example, clarithromycin with amoxicillin or levofloxacin, or levofloxacin/tetracycline instead of clarithromycin, as well as different therapy durations (i.e. 8 or 14 days), have been proposed, but none has achieved a cure rate close to 100% (Malfertheiner *et al*., 2002; Zullo *et al*., 2013). However, such treatment regimens face increasing challenges, including antimicrobial resistance, as well as recurrence of *H. pylori* infection (Graham *et al*., 1998; Malfertheiner *et al*., 2007; Kepekci *et al*., 1999; Zullo *et al*., 2013). Immunization may be a useful alternative for the control of *H.pylori* infections in humans. The immunoproteome of *H. pylori* in the mouse model appears similar to that in human infection, suggesting that the mouse model is a suitable tool for preclinical screening of vaccine candidates (Bumann *et al*., 2002).

1.2 STRAIN DIFFERENCES AND VIRULENCE FACTORS

H. pylori is a highly diverse gastric pathogen at the genetic level (Devi *et al*., 2006; Covacci *et al*., 1999). It is a bacterium that established itself in the human stomach most likely thousands of years ago and is presumed to have co-evolved with its human host (Devi *et al*., 2006; Moodley *et al*., 2009). Gene recombination and DNA transfer may contribute to genetic variability and enhance its adaptation to new host(s) (Fernandez-Gonzalez *et al*., 2014).

Various studies using molecular techniques have demonstrated that gene content and arrangement of *H. pylori* strains are highly variable (Akopyanz *et al*., 1992; Go *et al*., 1996; Jiang *et al*., 1996). *H. pylori* is one of the most genetically diverse bacterial species displaying from 2.7% to 8.0% of DNA sequence polymorphism, and a high level of genetic diversity has been observed in *H. pylori*, compared to the more conserved *Helicobacter mustelae* (Raymond *et al*., 2004). In addition to genetic recombination, *de novo* mutation plays a significant role in generating the high level of genetic variation in *H. pylori* (Alm *et al*., 1999; Wang *et al*., 1999; Percival and Suleman, 2014; Fernandez-Gonzalez and Backert, 2014). The strains from different geographic areas exhibit clear

phylogeographical differentiation, even to such an extent that the genotypes of *H. pylori* can serve as markers for the migration of human populations (Yamaoka Y, 2009; Moodley *et al*., 2009).

The most widely investigated virulence factor of *H. pylori* is *cagA*, which encodes a highly immunogenic CagA protein and is also a polymorphic gene (Covacci *et al*., 1993; Tummuru *et al*., 1993). The *cagA* gene is located on one side of the *cag* (cytotoxin associated genes) pathogenicity island (PAI) which carries about 30 genes (Akopyanz *et al*., 1992; Backert *et al*., 2008). A functional *cag* PAI in *H. pylori* that can make a syringe-like structure, is capable of penetrating gastric epithelial cells and delivering the CagA effector protein into the host cells. The CagA is shown to be injected into the host cytoplasm through the *cag* PAI type IV secretion system (T4SS) and the injected CagA protein becomes tyrosine-phosphorylated by several host kinases, including *Src* and *Abl* (Hatakeyama M, 2009). This initiates numerous signaling cascades associated with cell proliferation, cell motility, actin cytoskeletal rearrangements, disruption of cell-to-cell junctions, pro-inflammatory responses and suppression of apoptosis (Backert *et al*., 2008). *H. pylori* strains that harbor the *cag* pathogenicity island induce activation of NF-κB and interleukin (IL)-8 secretion in gastric epithelial cells, and are associated with the development of severe inflammation in humans. SHP-2 tyrosine phosphatase acts as an intracellular target of the phosphorylated CagA protein (Yamazaki *et al*., 2003). Interestingly, differences in CagA phosphorylation (and subsequent interaction with SHP-2) have an influence on the biological activity of CagA (Higashi *et al*., 2002). The importance of this finding is confirmed by the observation that *H. pylori* strains that deliver CagA with more phosphorylation motifs are most often associated with gastric cancer (Azuma *et al*., 2002; Argent *et al*., 2004).

Not all *H. pylori* strains, however, contain this pathogenicity island. *H. pylori* isolates can be distinguished according to the presence of this island or the CagA effector protein. The severity of *H. pylori*-related disease is reported to correlate with the presence of the *cag* PAI, and infection with *cag* PAI–positive *H.pylori* is associated with gastric mucosal atrophy and gastric cancer. The CagA protein is considered to have oncogenic properties, for instance based on the observation that *cag*A-transgenic mice reval a higher incidence of tumor formation (Miura *et al*., 2009). A large number of studies have shown that *cag*A^+ *H. pylori* strains are more frequently associated with severe forms of disease, such as peptic ulceration (Peek *et al*., 1995; Censini *et al*., 1996; Yamaoka *et al*., 1997). The CagA protein can be divided into two types, i.e. the Western-type and the East-Asian-type, based on the presence of EPIYA motifs. The Western-type CagA has EPIYA-A and EPIYA-B followed by one to five repeat regions of the EPIYA-C sequence, while the strains belonging to the East-Asian –type CagA has EPIYA-A, EPIYA-B, and EPIYA-D (Hatakeyama

M, 2004). With the development of an α-EAS antibody which is only immunoreactive with East-asian-type CagA but not Western-type CagA, comparative research has revealed that East-Asian-type CagA strains are more virulent and more strongly associated with gastric mucosal atrophy and gastric cancer than the Wastern-type CagA or CagA-negative strains (Abe *et al*., 2011). Nevertheless, the frequency of *cag*A carriage among *H. pylori* strains varies considerably depending on the geographic origin of the strain or, more precisely, the ethnic origin of the host. It has been proposed that *H.pylori* strains have co-evolved to colonize specific human host populations via the selection of certain bacterial properties *in vivo* (Covacci and Rappuoli, 1998; Philpott *et al*., 2002).

Besides the *cag* PAI, other virulence genes have also been described in *H. pylori*, including *vacA*, *iceA*, *oipA* and *ggt* (Ben Mansour *et al*., 2010; Matsunari *et al*., 2012). The vacuolating cytotoxin, encoded by the *vacA* gene, is an important virulence factor present in all *H. pylori* strains. This gene plays a role in the development of vacuoles in epithelial cells and in the induction of apoptosis of these cells (Cover *et al*., 2003; Kuster *et al*., 2006). VacA acts as a multifunctional toxin causing alterations in late endosomes and mitochondrial membrane permeability (Cover *et al*., 2005). The *vacA* gene possesses several polymorphic sites, namely the signal (s) region, the midregion (m) and the intermediate region, and allele differences have been described to influence toxicity (Atherton *et al*., 1995). Furthermore, VacA inhibits T-cell proliferation via β2-integrins, supporting the chronicity of *H. pylori* infection (Sewald *et al*., 2008). Another virulence gene is *iceA* (induced by contact with epithelium). This gene has two main allelic variants (*iceA1* and *iceA2*) and the function of these variants is not yet clear. *IceA*1 is upregulated upon contact of *H. pylori* with the gastric epithelium and has been regarded as a marker for peptic ulcer disease. The *oipA* gene encodes one of the outer membrane proteins and is an inflammation-related gene located approximately 100 kb from the *cag* PAI on the *H. pylori* chromosome. OipA induces IL-8 secretion by epithelial cells and active OipA protein production may be "on" or "off" depending on the number of CT dinucleotide repeats in the signal sequence of the *oipA* gene (Ben Mansour *et al*., 2010). All *H. pylori* strains also show γ-glutamyl transpeptidase (GGT) activity. This enzyme causes epithelial cell death, partly dependent on degradation of glutathione or glutamine (Flahou *et al*., 2011; Shibayama *et al*., 2003). In addition, the enzyme inhibits the proliferation of T-cells, which may prevent the generation of an effective host immune response (Schmees *et al*., 2007). Recently, this has been shown to depend on deprivation of the extracellular space from glutamine by the GGT activity (Wüstner *et al*., 2014). *In vitro* experiments in human gastric epithelial cells (AGS cell line) challenged with *H. pylori* wild-type and knockout mutant strains have shown that

induction of interleukin-8 (IL-8) was significantly decreased by knockout of the GGT-encoding gene but not by knockout of the asparaginase-encoding gene. IL-8 induction by infection with the *H. pylori* wild-type strain was significantly decreased by adding glutamine, a substrate of the GGT, during infection (Rimbara *et al*., 2013). These data show the *H. pylori* GGT plays an important role in the *H. pylori*-evoked chronic inflammatory response.

It has been observed in gastric epithelial cells that lipopolysaccaride (LPS) enriched outer membrane vesicles (OMV) are continuously shed from the surface of *H. pylori*. The *H. pylori* VacA has been shown to enhance the association of *H. pylori* OMV with host gastric epithelial cells. Constitutive release of OMV from the surface of *H. pylori* and uptake by epithelial cells might contribute to the pathogenesis of infection through the persistent delivery of some of the above-mentioned virulence factors and antigens to gastric epithelial cells (Parker *et al*., 2010).

1.3 PATHOGENESIS OF *H. PYLORI* INFECTION IN RODENT MODELS

Helicobacter pylori is highly adapted to the human gastric mucosa, yet some isolates are able to colonize the stomach of a variety of large animal hosts including non-human primates, pigs, sheep, dogs and cats (Handt *et al*., 1994; Ferrero and Fox, 2001; Dore *et al*., 2001; Momtaz *et al*., 2014). In addition, certain *H. pylori* strains are capable of colonizing the gastric mucosa of small mammals such as mice (Marchetti *et al*., 1995; Lee *et al*., 1997) and Mongolian gerbils (Watanabe *et al*., 1998).

H. pylori has been shown to persist in its human host for decades. To study the adaptation and persistence process in the stomach, animal models mimicking the human situation are required. Although the frequently used mouse model comes with a large reservoir of genetic tools such as specific transgene and knock-out mouse lines, its major disadvantage should not be neglected, as mice so far cannot persistently be infected with *H.pylori* type I-strains expressing a functional T4SS. The stability of the *cag*-PAI is lost in mice over time of infection (Philpott *et al*., 2002).

The Mongolian gerbil was established as a successful animal model for studying *H. pylori* pathogenicity and for research into agents for the treatment of *H. pylori* infection in humans (Farnbacher *et al*., 2010; Hirayama *et al*., 1996; Watanabe *et al*., 1998). Mongolian gerbils have been successfully infected with classified *H. pylori* type I-strains, expressing a functional T4SS able to translocate the oncoprotein CagA into the host cells, where it can be tyrosine-phosphorylated by host kinases (Hatakeyama M, 2009; Odenbreit *et al*., 2002; Stein *et al*., 2000; Dixon *et al*., 1996). The development of a fundus-dominant gastritis in Mongolian gerbils has been

shown to depend on a functional Cag pathogenicity isoland, whereas less virulent *H. pylori*-strains with a defective T4SS, so called type II-strains, do not result in a corpus-dominant atrophic gastritis, a risk factor for developing gastric adenocarcinoma (Farnbacher *et al*., 2010). In addition, inflammatory changes may progress more rapidly and aggressively than in other laboratory animal models or in man (Hirayama *et al*., 1996; Suzuki *et al*., 2002) and experimental *H. pylori* infection alone has been shown to induce the characteristic pathway (gastritis, atrophy, metaplasia, dysplasia) leading to gastric cancer in this animal species, further underlining this is a suitable model to study long-term *H. pylori* infection (Dixon *et al*, 1994).

Both in naturally and in experimentally infected animals, *H. pylori* infection induces a persistent inflammatory response. Cytokines playing a role in gastric inflammation in *H. pylori*-infected gerbils are the Th17 cytokine IL-17 (Sugimoto *et al*., 2009) and cytokines involved in the generation of a Th1 response, including the signature Th1 cytokine IFN-γ (Wiedemann *et al*., 2009; Yamaoka *et al*., 2005; Shi *et al*., 2010; Sommer *et al*., 1998). In addition, upregulation of IL-1β is often observed in the stomach of *H. pylori*-infected gerbils (Wiedemann *et al*., 2009; Yamaoka *et al*., 2005). This cytokine stimulates gastrin release from antral G cells and inhibits antral D cells to express somatostatin, an inhibitor of gastrin-stimulated acid secretion (Wiedemann *et al*., 2009; Zavros *et al*., 2005). This does, however, not result in increased production of hydrochloric acid due to a modulating effect of IL-1β on the *H. pylori*-mediated H^+/K^+ATPase α-subunit promoter inhibition, contributing to reduced parietal cell H^+/K^+ATPase gene and protein expression and thus to hypochlorhydria (Saha *et al*., 2007).

The peptide hormon gastrin is secreted by G-cells, mainly in the antrum of the stomach, in response to food intake and stimulating the secretion of gastric acid by parietal cells (Dockray *et al*., 1999). Higher gastrin levels can be observed in human patients and animal models and this is considered to be a reaction to the *H. pylori*-induced hypochlorhydria (Tucker *et al*., 2010; Chittajallu *et al*., 1991). In an attempt to repair acid homeostasis, gastrin stimulates histamine release from enterochromaffin-like (ECL) cells, which triggers acid secretion (Furutani *et al*.,2003; Rieder *et al*., 2005; Takashima *et al*., 2001). In *H. pylori*-infected gerbils, IL-1β is up-regulated during *H. pylori* infection and gastrin levels have been described to increase after 16 weeks of infection and mainly in the antrum (Wiedemann *et al*., 2009).

In conclusion, the development of gastric carcinoma is a multi-factorial, long-term process. *H. pylori* infection is a factor which seems to function as a "trigger" during cancer transformation, because the infection can cause genome instability in host cells. Besides strain differences in

pathogenicity, other factors, such as dietary habits (salt, alcohol intake, smoking), ethnic group (gene polymorphisms, e.g. interleukin (IL)-1β) also contribute to the development of gastric cancer (Wiedemann *et al*., 2009; Rimbara *et al*., 2013; Kovalchuk *et al*., 2014).

2. Gastric non-*H. pylori Helicobacter* species

2.1 GASTRIC NHPH INFECTION IN HUMANS

Soon after the discovery of *H. pylori*, other spiral-shaped bacteria, which are morphologically clearly distinct from the typical curve-shaped *H. pylori*, were discovered in a small percentage of humans suffering from gastritis (Debongnie *et al*., 1995), gastric ulceration (Debongnie *et al.*, 1998), and gastric MALT lymphoma (Morgner *et al*., 1995; Heilmann *et al*., 1991; De Bock *et al*., 2006). Initially, the name " *Gastrospirillum hominis*" was proposed in 1989 (McNulty *et al*., 1989). Subsequent studies demonstrated that these organisms were very similar, if not identical, to the spiral-shaped bacteria found in the stomachs of cats, dogs and non-human primates (Heilmann *et al*., 1991). Sequencing of the 16S rRNA gene revealed that these bacteria belonged to the genus *Helicobacter*. In honour of the German pathologist Konrad Heilmann, who first studied the pathology associated with these microorganisms, this bacterium was renamed "*Helicobacter heilmannii*" (Heilmann *et al*., 1991; O'Rourke *et al*., 2004a; Haesebrouck *et al*., 2009). More recent investigations have, however, shown that not one *Helicobacter* species is involved, but instead several distinct *Helicobacter* species which have been detected in the stomachs of different animal species (De Groote *et al*., 2005; van den Bulck *et al*., 2005). These helicobacters include *H. suis* from pigs, which has also been referred to as "*H. heilmannii"* type 1 in previous studies (Baele *et al*., 2008a; Haesebrouck *et al*., 2009), as well as *H. heilmannii* sensu stricto (s.s.) (Smet *et al.*, 2012), *H. felis* (Lee *et al*, 1988), *H. bizzozeronii* (Hanninen *et al*., 1996) and *H. salomonis* (Jalava *et al*., 1997), which are most frequently associated with cats and dogs. This group of highly related cat- and dog-associated *Helicobacter* species is sometimes referred to as the "*H. heilmannii*" type 2 group. It also includes *H. cynogastricus* and *H. baculiformis*, which have, so far, not been detected in humans (Solnick et al., 1993; Van den Bulck *et al.*, 2006; Baele *et al.*, 2008; Haesebrouck *et al*., 2009). In some cases, this entire group of bacteria (including "*H. heilmannii"* type 1 and type 2) is referred to as the *H. heilmannii* sensu lato group (*H. heilmannii* in the wide sense), which is clearly distinct from the single species *H. heilmannii* s.s. mentioned above (Haesebrouck *et al*., 2011). In general, however, this group of related but distinct *Helicobacter* species is better referred to as 'gastric non-*H. pylori* helicobacters' (NHPH), in order to avoid confusion (Haesebrouck *et al.*, 2009). Based on several different studies performed so far,

sequencing of the 16S rRNA gene has been shown not to be able to distinguish the canine and feline gastric *Helicobacter* species. Sequencing of the (partial) *urease A* and *B* subunit genes, on the other hand, allows identification of these bacteria to the species level (Haesebrouck *et al.*, 2011).

Gastric NHPH infections of the human stomach are consistently accompanied by active chronic gastritis. Gastric erosions are mainly located in the antrum and duodenal ulcers have also been reported in association with gastric NHPH infections. Furthermore, these infections have been associated with low grade MALT lymphoma of the stomach, and the risk of developing MALT lymphoma is higher with gastric NHPH, compared to *H. pylor*i infection (Morgner *et al.*, 1995, 2000; Haesebruck *et al*., 2009). The associated diseases such as gastritis and gastric MALT lymphoma have been described to resolve after eradication of these bacteria which further proves this causal relationship (Morgner *et al*., 2000). Interestingly, a link between *H. suis* infection and idiopathic parkinsonism (IP) (Blaecher *et al*., 2013) has also been reported.

As a strictly human-associated pathogen, *H. pylori* is in general transmitted from human to human. In contrast, infection with gastric NHPH is strongly associated with contact at any age with wild, domestic or companion animals, and especially pigs (Meining *et al*., 1998; Schott *et al*., 2011; Matsui *et al*., 2014). In addition, viable *H. suis* bacteria have recently been demonstrated in commercial pork (De Cooman *et al*., 2013), which might serve as a source of infection for humans. Alhough pigs and pork are considered to be possible sources of human *H. suis* infection, this remained to be confirmed at the onset of the current PhD study.

Compared to the high prevalence of *H. pylori* infection, these gastric non-*Helicobacter pylori Helicobacter* infections are detected in a relatively small percentage of humans, ranging from 0.1-6.0% (Schott *et al.*, 2011; Okiyama *et al*., 2005). In developed countries, some reported prevalence rates of gastric NHPH are 0.1% (in Japan) (Okiyama *et al*., 2005), 0.1% (in Italy) (Lerardi *et al*., 2001), 0.25% (in Germany) (Heilmann *et al*., 1991) and 0.25% (in Belgium) (Debongnie *et al*., 1995), whereas in rural areas or in developing countries, higher prevalences have been reported, for instance 2% in southern China (Chen *et al*., 1998) and 6.2% in Thailand (Yali *et al*., 1998). It most probably represents only the tip of the iceberg because human infection with these gastric NHPH may pass inapparent, or result in mild disease signs which are not further examined (Mazzucchelli *et al*, 1993). Furthermore, even in severe cases, these infections are most probably underdiagnosed, due to the lack of simple diagnostic methods allowing identification of the causal agent to the species level (Haesebrouck *et al.*, 2009). Detection of urease activity, for instance, has

been described not to be a reliable diagnostic method for detection of these infections (Debongnie *et al*., 1995; Boyanova *et al*., 2003; Sykora *et al*., 2003; Matsui *et al*., 2014).

To date and due to the extremely fastidious nature of these microorganisms, only few gastric NHPH strains have been isolated *in vitro* from humans and these few strains have been identified as *H. bizzozeronii* (Andersen *et al*., 1996; Kivisto *et al*., 2010; Jalava *et al*., 2001) and *H. felis* (Wuppenhorst *et al*., 2013).

2.2 *HELICOBACTER SUIS* INFECTION IN PIGS

In addition to the group of zoonotic NHPH colonizing dogs and cats (*H. heilmannii*" type 2 group), the "*H. heilmannii"* sensu lato group also compromises "*H. heilmannii*" type 1 bacteria (Haesebrouck *et al*., 2011) which are identical to *Helicobacter suis*. This microorganism colonizes the stomachs of the majority of pigs. It is also the most common gastric NHPH species in humans suffering from gastric disease (Haesebrouck *et al*., 2009; De Groote *et al*., 1999, 2005; O'Rourke *et al*., 2004a; Hellemans *et al*., 2006; Baele *et al*., 2008a).

Spiral microorganisms were first reported in the stomach of pigs in 1990 and they were referred to as "*Gastropirillum suis*" (Queiroz *et al*., 1990). Subsequent studies based on 16S rRNA gene sequence analysis indicated that this microorganism belongs to the *Helicobacter* genus and therefore it was renamed as "*Candidatus* Helicobacter suis" because at that time it was not possible to culture it *in vitro* (De Groote *et al*., 1999). Following studies further demonstrated that "*H.heilmannii*" type 1, described in humans, was both morphologically and genetically identical to "*Candidatus* Helicobacter suis" (De Groote *et al*., 2005; Hellemans *et al*., 2006). It was not until 2008 that this bacterium was successfully isolated from pigs. Based on sequencing of 16s and 23s rRNA genes, the partial *ureA* and *ureB* genes and partial *hsp60* genes, as well as analysis of the whole-cell protein profiles, this bacterium was characterized as a novel species which was formally named "*Helicobacter suis*" (Fig.1-5) (Baele *et al*, 2008a; Haesebrouck *et al*., 2009)

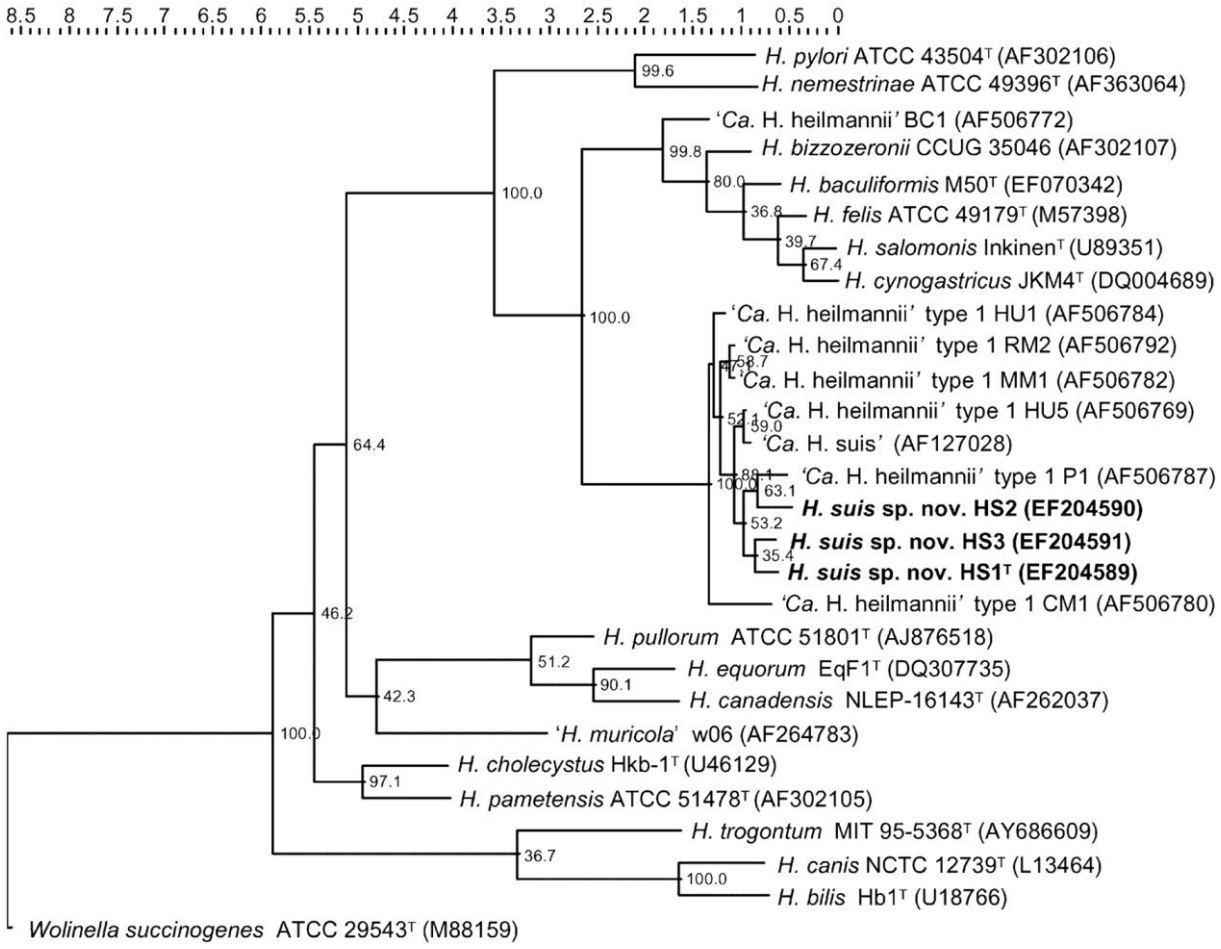

Fig.1. Phylogenetic tree for 28 *Helicobacter* strains based on 16SrRNA gene sequence similarity data. Bar, 8.5% sequence divergence. The numbers immediately to the left of branches indicate the number of times out of 100 the clade was recovered by bootstrap resampling (number of bootstraps: 1000) (Baele et al., 2008).

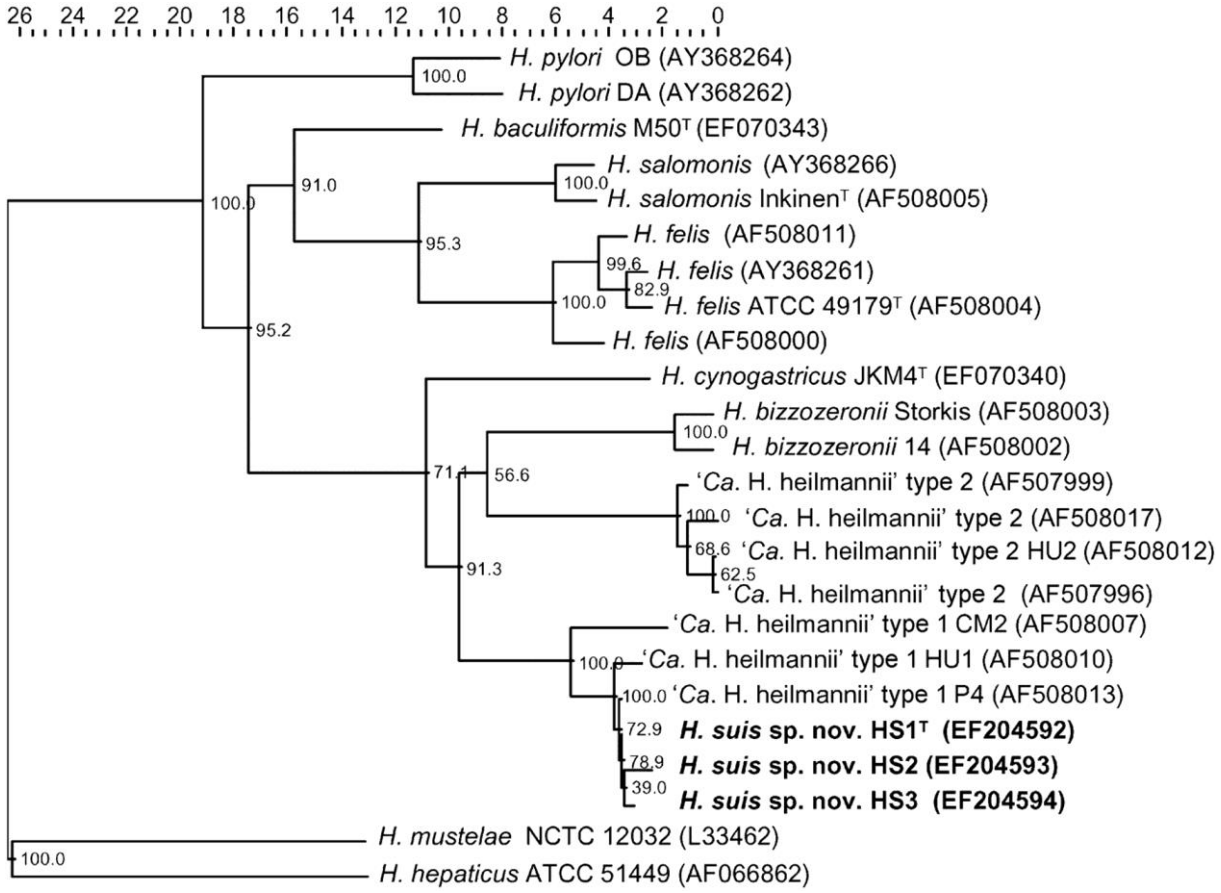

Fig. 2 Phylogenetic tree, reconstructed from genetic distances, based on the partial *ureA* and *ureB* gene sequences for *H. suis* and other urease-positive species of the genus *Helicobacter*. Bootstrap values are indicated. Bar, 26% divergence (Baele et al., 2008).

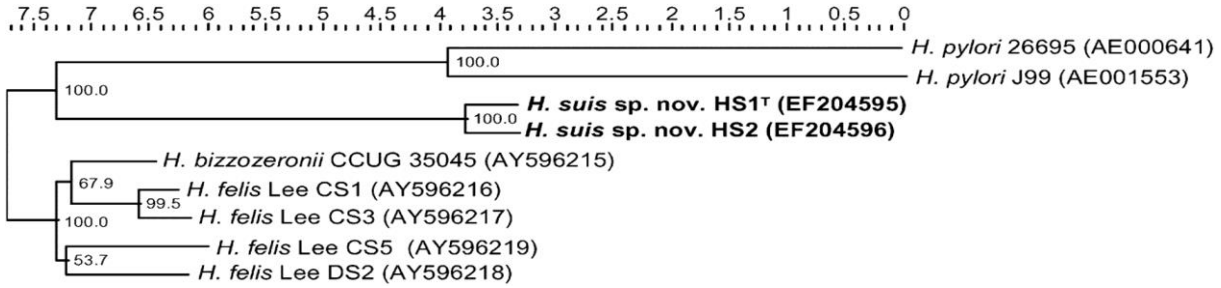

Fig. 3 Phylogenetic tree for nine *Helicobacter* strains based on 23S rRNA gene sequence similarity data. Bootstrap values are indicated. Bar, 7.5% sequence divergence (Baele et al., 2008).

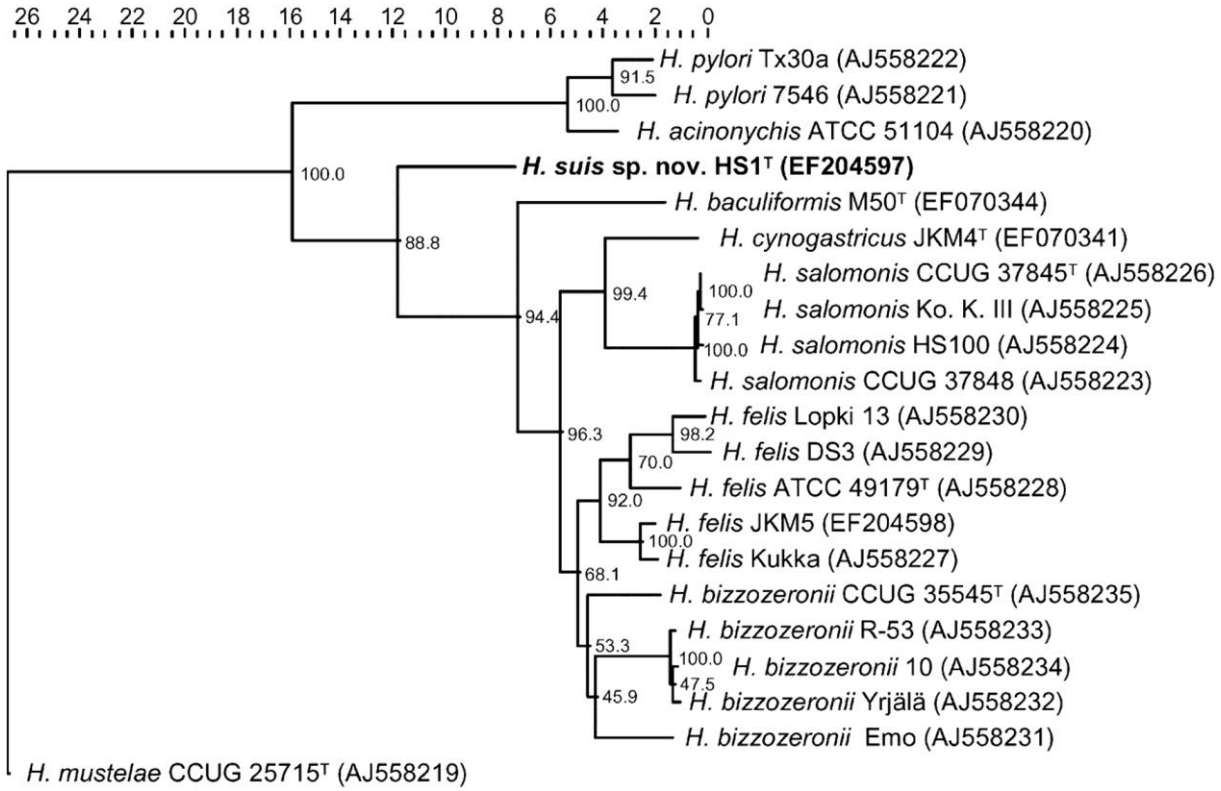

Fig. 4 Phylogenetic tree, reconstructed from genetic distances, based on the partial *hsp60* gene sequences for *H. suis* and other species of the genus *Helicobacter*. Bootstrap values are indicated. Bar, 26% divergence (Baele et al., 2008).

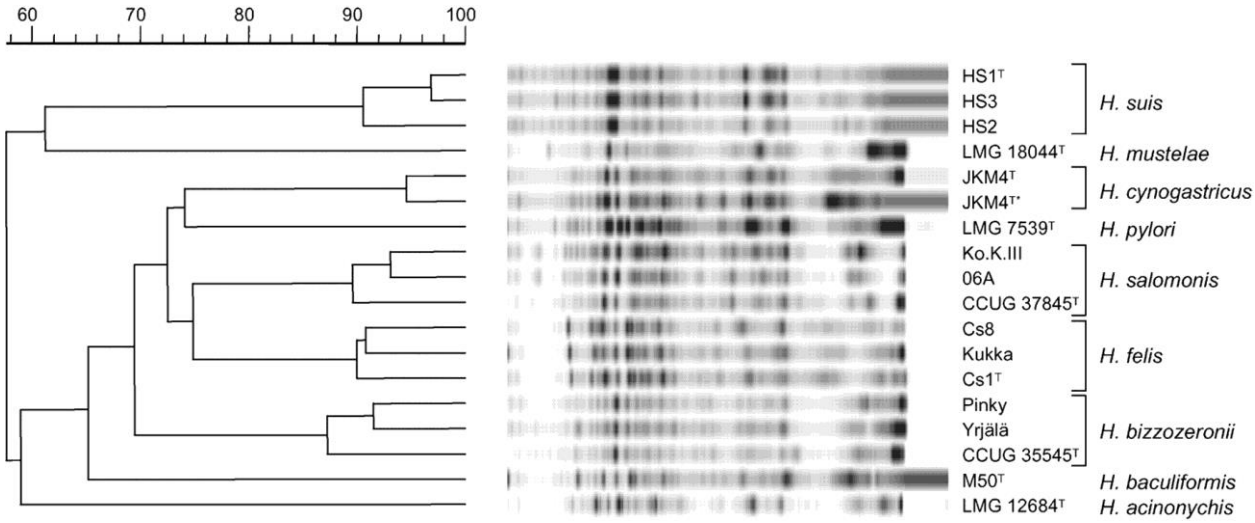

Fig. 5 Dendrogram derived from the numerical analysis of the whole-cell protein profiles of *H. suis* strains HS1^{T}, HS2 and HS3, as well as other *Helicobacter* reference strains (Baele *et al*., 2008).

2.2.1 Prevalence of *Helicobacter suis* in pigs

In pigs, the prevalence of a *H. suis* infection is very low before weaning. After weaning, the infection rate gradually increases (Hellemans *et al*., 2007). In contrast, *H. suis* infection is very common in pigs at slaughter age world-wide, but the prevalence observed varies from one country to another. In countries all over the world, the prevalence of *Helicobacter suis* in the stomachs of pigs at the age of slaughter ranges from 8% to 95%, and most studies report prevalence rates of 60% or more (Barbosa *et al*., 1995; Cantet *et al*., 1999; Choi *et al*., 2001; De Groote *et al*., 2000; Grasso *et al*., 1996; Magras *et al*., 1999; Melnichouk *et al*., 1999; Mendes *et al*., 1991; Park *et al*., 2000, 2004; Queiroz *et al*., 1990, 1996; Roosendaal *et al*., 2000; Utriainen and Hanninen, 1998; Guise *et al*., 1997). At the onset of the current PhD study, little or no information was available on the variability and spread of *H. suis* strains within a certain pig population.

2.2.2 Detection of *H. suis* infection

The methods used for diagnosis of *H.suis* infection include the urea breath test (UBT) (not used in pigs), the rapid urease test performed on gastric biopsies, immunohistochemistry, histological staining (including silver staining), mouse inoculation assays, electron microscopy, protein profiling, DNA hybridization, and PCR amplification. Until now, species-specific (RT)-PCR assays have been proven to be the most sensitive diagnostic tool for the detection of *H. suis* infection. Several species-sensitive primer pairs recognizing several genes, including 16S rRNA and *urease* genes have been developed (Table 1) (Joosten *et al*., 2013a; Blaecher *et al*., 2013; Matsui *et al*., 2014; De Groote *et al*., 2000; O'Rourke *et a*l., 2004b). Investigations based on non-PCR diagnostic methods probably underestimate the true infection rate with this bacterium (Hellemans *et al*., 2007).

In addition to species-specific PCR assays, amplification and sequencing of certain genes can be used for identification of the *Helicobacter* species involved. 16S rRNA gene sequencing can be used to discriminate *H. suis* from other members of the *Helicobacter heilmannii* sensu lato group, but this gene cannot distinguish among the dog-and cat-associated *H. felis, H.bizzozeronii, H. salomonis, H. cynogastricus, H. baculiformis and H. heilmannii* s.s. (O'Rourke *et al*., 2004; Haesebrouck *et al*., 2011). For a more reliable differentiation between *H. suis* and other gastric NHPH, sequencing of the *hsp60* or *gyrB* gene is useful. Sequencing of the genes encoding the urease A and B subunits seems currently to be the most suitable method because sequences of

these genes are available for all *Helicobacter heilmannii* sensu lato species (Haesebrouck *et al*., 2011; Kivisto *et al*., 2010; O'Rourke *et al*., 2004). In addition, whole cell protein profiling may also be useful, but it is only applicable if pure *in vitro* cultures are available, which is a serious drawback when working with these fastidious microorganisms (Haesebrouck *et al*., 2009).

Table 1. Development of *H.suis* species-specific PCR primers

primers	sequence (5'--3')	polarity	amplicon size in bp	annealing temp	Gene	Reference
V832f	TTG GGA GGC TTT GTC TTT CCA	forward	433	**60°C**	*16S rRNA*	De Groote et al., 2000
V1000f	AGG AAT TCC CTA GAA ATA GGG	forward			*16S rRNA*	De Groote et al., 2000
V1261r	GAT TAG CTC TGC CTC GCG GCT	reverse			*16S rRNA*	De Groote et al., 2000
BF_HsuisF1	AAA ACA MAG GCG ATC GCC CTG TA	forward	150	**62°C**	*ureAB*	Blaecher et al., 2013
BF_HsuisR1	TTT CTT CGC CAG GTT CAA AGC G	reverse			*ureAB*	Blaecher et al., 2013
UreNHPHF	CDG TRM GNT TTG ARC CNG G	forward	379	**57°C**	*ureAB*	Flahou et al., 2014
UreNHPHR	GTD GTD GGD CCR TAC ATW GA	reverse			*ureAB*	Flahou et al., 2014
UreSu 531 FW	CACCACCCCGGGGAAGTGATCTTG	forward	253	**60°C**	*ureA*	De Cooman et al., 2013
UreSu 783 RV	CTACATCAATCAAATGCACGGTTTTTTCTTCG	reverse			*ureA*	De Cooman et al., 2013
carR2F	TAGCTCTAGGGTGCAGGGAATA	forward	220		*carR*	Matsui et al., 2014
carR2R	CTCACCCACACCTGCTAGTATTTT	reverse			*carR*	Matsui et al., 2014

2.2.3 Pathogenesis of *H. suis* infection

Colonization and associated disease

In young animals, *H. suis* colonization is mainly observed in the antrum of the stomach (Hellemans *et al*., 2007; De Bruyne *et al*., 2012). When animals grow older, higher colonization rates are gradually observed in the fundic gland region (Hellemans *et al*., 2007; unpublished observations). In pigs, *H. suis* infection is associated with lymphoplasmacytic gastritis which is most pronounced in, but not limited to the antrum, as well as ulceration of the pars nonglandularis, which is a region of keratinized epithelium surrounding the oesophageal opening, where *H. suis* probably does not

colonize (Choi *et al*., 2001; Haesebrouck *et al.*, 2009; Mendes *et al*., 1991; Queiroz *et al*., 1996; Roosendaal *et al.*, 2000; De Bruyne *et al*., 2012; Hellemans *et al*., 2007). Ulceration of the pars oesophagea is a worldwide cause of economic loss in the pig industry (Friendship RM, 2003). Lesions vary from mild hyperkeratosis to deep erosive lesions (Roels *et al*., 1997) and mortality rates exceeding 1% have been described as a result of bleeding from these erosive lesions (Ayles *et al*., 1996). The presence of severe ulcers has also been associated with loss in daily weight gain of pigs (Ayles *et al*., 1996). However, Grasso *et al*. (1996), Guise *et al*. (1997), Melnichouk *et al*. (1999), Park *et al*. (2004), and Szeredi *et al*. (2005) did not find this association. The exact role of *H. suis* in the pathogenesis of gastric ulceration remains partly to be elucidated, but most likely, *H. suis*-induced changes of gastric acid secretion are involved. Interestingly, our research group showed that *H. suis* infection not only causes gastritis but also a reduced daily weight gain in experimentally infected pigs, which is however not necessarily associated with gastric ulceration (Bruyne *et al*., 2012).

In previous studies, mice and Mongolian gerbils have been described to be good models to investigate *H. suis*-induced gastric pathology (Flahou *et al*., 2010a; Nakamura *et al*., 2008). The suitability of the Mongolian gerbil model to study the pathogeneis of *H. suis* infections (in humans) is illustrated by the observation of its colonization and the inflammation evoked (Flahou *et al*., 2010b). In gerbils, *H. suis* colonization and the associated inflammation were observed predominantly in the antrum and a narrow zone in the fundus near the forestomach/stomach transition zone, whereas in mice, bacteria colonized the entire glandular stomach and elicited a chronic inflammation mainly in the fundus. In humans infected with *H. suis* and other gastric NHPH, the colonization and inflammation are mainly observed in the antrum of the stomach and this is less pronounced or absent in the fundic gland region (corpus) of the majority of these patients (Haesebrouck *et al*., 2009; Debongnie *et al*., 1998; Morgner *et al*., 1995, 2000). Perhaps these differences, compared to *H. pylori* infection, may be explained by the absence of the *cag* pathogenicity island in *H. suis* and other NHPH, since the presence of this virulence factor has been shown to be important for colonization of the fundic gland region (Wiedemann *et al*., 2009; Vermoote *et al.*, 2011).

Genome analysis of *H. suis* has shown that this bacterium contains the majority of genes known to be essential for colonization in the stomach, including the urease system, alpha carbonic anhydrase, sheathed flagella and the pH taxis *tlpB* gene (Vermoote *et al*., 2011). However, the *H. suis* genome lacks genes coding for BabA and SabA adhesins, which play a major role in *H. pylori* adhesion to the human gastric mucosa (Yamaoka and Alm, 2008). *H. suis*, however, has been

shown to adhere to thus far unidentified host mucin structures (unpublished results), which indicates that adherence to host mucins most likely plays a role in the initial colonization and long-term persistence of *H. suis* in the stomach, and that it is mediated by adhesins other than BabA and SabA. Possible candidate adhesins of *H. suis* are outer membrane proteins, including the HpaA, HorB and Hof proteins. Indeed, the genes encoding these OMPs have been shown to be present in the genomes of *H. suis* and other NHPH (Arnold *et al*., 2011; Schott *et al*., 2011; Smet *et al*., 2013).

A factor essential for colonization and survival of *H. suis* in its hostile niche, the stomach, is the urease enzyme consisting of two subunits, UreA and UreB (Andrutis *et al*., 1995; Eaton and Krakowka, 1994). The urease-mediated production of ammonia has been shown to reduce the viability of gastric epithelial cells (Smoot *et al*., 1990). Other studies have suggested that ammonia accelerates cytokine-induced gastric epithelial cell apoptosis (Igarashi *et al*., 2001). However, some *H. suis* strains detected in humans in Japan (for example, *H. suis* strain SNTW101) display no detectable urease activity, although the genes are present (Okiyama *et al*., 2005; Matsui *et al*., 2014).

Immune response and host cell death

Once experimental animals are successfully colonized with *H. suis*, an inflammatory response is evoked. To date, only some of the virulence mechanisms of *H. suis* have been elucidated, and it is currently not known whether differences in terms of virulence are present among *H. suis*, as has been described for *H. pylori*. In addition, no clear typing method has been developed for *H. suis*, which makes it difficult to distinguish between different *H. suis* strains.

In mice exprimentally infected with *H. suis*, a stronger inflammatory response is described in BALB/c mice, compared to C57BL/6 mice (Flahou *et al*., 2010b). Although most *H. suis* strains used in this study induced a similar immune response, some differences could nevertheless be observed. *H. suis* strain HS6, for instance, was shown not to induce upregulation of IL-4, IL-6 and IL-10, in contrast to most other strains (Flahou *et al*., 2012). Inflammation observed in mice was mostly limited to the fundus, and after long-term infection, a higher number of B cells was detected suggesting *H. suis* infection is mainly associated with a Th2 response. In contrast to the predominant Th17/Th1 response evoked by a *H. pylori* infection (Robinson and Atherton, 2010), *H. suis* infections have indeed been shown to induce a Th17/Th2 response in mice (Flahou *et al.*, 2012). *H. pylori* harbors some virulence factors that can modulate the host immune response towards a Th1 response, including the *cag* pathogenicity island, the outer inflammatory protein A,

OipA, and the neutrophil-activating protein, HP-NAP (Amedei *et al*., 2006; D'Elios *et al*., 2007; Yamauchi *et al*., 2008). Except for HP-NAP, *H. suis* lacks homologs of these factors, which could possibly explain the absence of a Th1 response associated with a *H. suis* infection in mice and the higher risk of developing gastric MALT lymphoma during gastric NHPH infection, compared to *H. pylori* infection. Indeed, the development of low-grade B cell MALT lymphoma needs CD40-mediated signaling, as well as Th2-type cytokines, rather than a Th1-predominant response (Greiner *et al*., 1997; Knorr *et al*., 1999). Also in Mongolian gerbils experimentally infected with *H. suis*, a clear inflammatory response develops. In general, a more rapid development of inflammatory changes is observed in this animal species compared to mice (Court *et al*., 2002). Most infected gerbils show an obvious lymphocytic infiltration in the antrum and a narrow zone at the forestomach/stomach transition zone. The diffuse infiltration with lymphocytes and the presence of large lymphoid aggregates/follicles aggravates during the course of infection and is accompanied by destruction of the gastric glands and disruption of the lamina muscularis mucosae. From 9 weeks post infection onwards, lymphocytic aggregates were shown to contain a majority of CD20-positive B cells, mostly organized in germinal center-presenting lymphoid follicles. In gerbils infected for 8 months, these germinal centers were often large, hyperproliferative and irregular. Additionally, severe destruction of the normal antral architecture at the inflamed sites and development of MALT lymphoma-like lesions, such as lymphoepithelial lesions and infiltration of the tunica muscularis, were observed in some gerbils. The location and the induced pathology, including development of gastric MALT lymphoma (like lesions), resemble those observed in humans infected with NHPH.

As described above, differences in the host response evoked by different *H. pylori* strains have been associated with the presence, absence or functionality of several virulence-associated genes, such as *cag*PAI, *vac*A and *ggt* (Yamauchi *et al*., 2008; Gebert *et al*., 2003; Beigier-Bompadre *et al*., 2011). The first two factors, as well as other virulence-related genes are missing in *H. suis*. *H. suis* does, however, contain GGT activity, similar to *H. pylori* and some other *Helicobacter* spp. This enzyme has been described to impair proliferation of Jurkat T cells, as well as primary stimulated T cells and this effect could be modulated by adding 2 important substrates of the enzyme, reduced glutathione or glutamine (Zhang *et al*., 2013). However, using different concentrations of whole-cell lysate from a *H. suis ggt* deletion mutant strain did not completely abolish the inhibitory effect on Jurkat T cell proliferation. This suggests that putative virulence factors, other than GGT, are involved and contribute to the inhibition of lymphocyte proliferation (Zhang *et al*., 2013). At the same time, outer membrane vesicles (OMV) of *H. suis* were shown to

be a potential transport tool carrying GGT across the gastric epithelium to lymphocytes residing in the deeper mucosal layers (Parker *et al*., 2010; Zhang *et al*., 2013). Besides the GGT, *H. suis* also contains a homolog of the neutrophil activating protein of *H. pylori* (HP-NAP) and the *H. pylori* flavodoxin A (FldA)- encoding gene (Vermoote *et al*., 2011a). The *H. pylori* FldA is associated with the development of MALT lymphomas in humans (Chang *et al*., 1999). The exact role of HP-NAP and FldA as putative *H. suis* virulence factors in gastric diseases remains to be elucidated.

Besides clearly triggering the host immune response, *H. suis* infection also causes death of gastric epithelial cells. Cell death plays an important role in the development of several gastric pathologies, including gastric ulceration, gastric atrophy and gastric cancer (Shirin and Moss, 1998; Dixon MF, 2001). Interestingly, *H. suis* interacts with host cells in a different way than the well-known *H. pylori*. It is most often observed in the vicinity of or inside the canaliculi of acid-producing parietal cells in infected humans (Joo *et al*., 2007). In *H. suis*-infected pigs as well as in rodent models, these bacteria also show a clear tropism for these acid-secreting parietal cells (Flahou *et al*., 2010b; Hellemans *et al*., 2007; Nakamura *et al*., 2003). Often, these parietal cells show signs of degeneration or oncosis. This increased cell death has been shown to be accompanied by an increased proliferation rate of mucosal epithelial cells (Flahou *et al*., 2010b). For *H. pylori*, several virulence factors have been described to cause gastric epithelial cell death, including the VacA (Cover *et al*., 2003). Apart from VacA, *H. pylori* GGT has been shown to be involved in gastric epithelial cell death (Shibayama *et al*., 2003; Gong *et al*., 2010). For *H. suis*, the GGT is the only factor with a confirmed role in epithelial cell death, which was demonstrated in recent work using human-derived AGS cells. As an important virulence factor, *H. suis* GGT was identified to be involved in the induction of gastric epithelial cell death and the mechanism of its function was elucidated (Flahou *et al*., 2011). Depending on the amount of GGT added to a cell culture, apoptosis or necrosis was induced. In addition, supplementation of recombinant *H. suis* GGT-treated cells with glutathione, a strong antioxidant under physiological conditions, strongly enhanced the observed induction of cell death, revealing that *H. suis* GGT-mediated degradation of glutathione and the resulting formation of glutathione degradation products plays a direct and active role in the induction of gastric epithelial cell damage and/or apoptosis or necrosis (Flahou *et al*., 2011).

2.2.4 Control of *H. suis* infection

As mentioned above, *H. suis* is the most prevalent gastric NHPH species. This zoonootic bacterium is most likely transmitted through contact with pigs (Meining *et al*., 1998). The bacterium should potentially also be considered a food-borne pathogen, since live *H. suis* bacteria have been detected in commercial pork (De Cooman *et al*., 2013). Most likely, the presence of *H.suis* in the tissues of slaughtered pigs is due to contamination during the slaughter process (De Cooman *et al*., 2014). The exact routes of *H. suis* transmission to humans remains however to be confirmed. The association between pigs/pork and *H. suis* infection however suggests that some groups are at higher risk, including veterinarians, pig farmers and abattoir workers.

The fact that *H. suis* can cause severe gastric disorders in humans and pigs justifies the development of a preventive strategy. In order to determine the best strategy, however, it's important to gain insight into the epidemiology of *H. suis,* not only for pig-human transmission, but also with regards to transmission within the pig population. Possible control strategies may include the development of a *Helicobacter suis* vaccine. Some progress has been made in this field, using both lysate- and subunit-based vaccines, but a long way lies ahead (Flahou *et al*., 2009; Vermoote *et al*., 2013). In addition, most current antibacterial vaccines in swines induce only partial protection (Haesebrouck *et al*., 2004). But even if prophylactic vaccination would prove not to prevent a *H. suis* infection, the decreased colonization due to immunization may be economically attractive. Vaccination in production animals to protect humans from zoonotic disease has been done successfully for *Salmonella* Enteritidis in laying hens, and is the perfect illustration of the"one health concept" (Cogan and Humphrey, 2003).

Human patients suffering from severe gastric pathologies associated with *H. suis* can often be treated with the same therapeutic regimens as used for *H. pylori,* in general comprising 2 antimicrobial agents (for instance clarithromycin, amoxicillin, and/or metronidazole) and a proton-pump inhibitor (Morgner *et al*., 2000). In pigs, however, antimicrobial therapy seems to be impractical due to the high prevalence rate of *H. suis* infection (Haesebrouck *et al*., 2009). In addition, antimicrobial-based therapy in pigs is not recommended, partly due to increased human health risks associated with antimicrobial use in food producing animals, including the spread of antimicrobial resistance in pathogens and in bacteria belonging to the normal microbiota (Jensen *et al*., 2008; Vermoote *et al*., 2011b).

2.3 DOG- AND CAT-ASSOCIATED GASTRIC NHPH

Thus far identified gastric NHPH species present in dogs and cats include *H. felis, H. bizzozeronii, H. salomonis, H. heilmannii* sensu stricto, *H. cynogastricus* and *H. baculiformis.* This group of *Helicobacter* species is sometimes referred to as the *"H. heilmannii"* type 2 group. All these species are phylogenetically highly related to each other (Solnick *et al*., 1993) and they differ from each other in less than 3% of the 16S rRNA gene sequences (Solnick *et al*., 1993). The first gastric NHPH species isolated from cats was *H. felis* in 1988 (Lee *et al*., 1988), the first gastric NHPH species isolated from dogs was *H.bizzozeronii* in 1996 (Hanninen *et al*., 1996). After them, the first NHPH species isolated from humans was *H. bizzozeronii* in 2001 (Jalava *et al*., 2001). More recently, *H. cynogastricus* (Van den Bulck *et al*., 2006) and *H. baculiformis* (Baele *et al*., 2008b) were isolated from the stomach of a dog and a cat, respectively. Until now, *H. cynogastricus* and *H. baculiformis* have not yet been detected in the human gastric mucosa. '*Candidatus* H. heilmannii' has been detected on several occasions in humans, wild felines, canines and felines, and this species was isolated successfully from feline gastric mucosa in 2012 and subsequently renamed *H. heilmannii* (sensu stricto) (Haesebrouck *et al.*, 2011; Smet *et al*., 2012).

Regarding infection with members of the *Helicobacter heilmannii* sensu lato group in carnivores, prevalence rates of 41~ 100% have been reported in dogs, and 57 ~100% in cats (Ricci *et al*., 2007; Simpson *et al.*, 1999; Hwang et al., 2002; Chung *et al*., 2014). The results of urease mapping in dogs and cats indicated that *Helicobacter* colonization in the anatomical fundus region is more dense compared with the density in the body (corpus) and antrum. Due to the confusion in nomenclature, more research is needed on the exact prevalence of infection with these different *Helicobacter* species in dogs and cats. In any case, several reports have shown that co-infection with more than one of these species occurs (Van den Buck *et al*., 2005; De Bock *et al*., 2007). In cats and dogs, infection with members of the *Helicobacter heilmannii* sensu lato group has been associated with the development of mild to moderate gastritis (Eaton *et al*., 1996; Neiger *et al*., 1998).

2.3.1 *Helicobacter heilmannii* sensu stricto

Prevalence and clinical significance

The prevalence of *H. heilmannii* s.s. ranges from 20 to 100% in cats and dogs (Haesebrouck *et al*., 2009; O'Rourke et al., 2004a; Hwang *et al*., 2002; Neiger *et al*., 1998; Van den Bulck *et al*., 2005). After numerous fruitless efforts performed in various research groups, several *H. heilmannii* s.s.

strains (ASB1 ^{T}, ASB2, ASB3), were isolated successfully from feline stomachs in 2012 (Smet *et al*., 2012). Isolates ASB1, ASB2 and ASB3 showed 98% similarity with each other, and 91% similarity with *ureAB* gene sequences from "*Candidatus* H.heilmannii" strains, detected in human and wild feline gastric mucosa (O'Rourke *et al*., 2004b). Moreover, these three isolates clustered with these "*Candidatus* H.heilmannii" strains (Figure 6).

Besides in wild felines and in humans, this bacterium is the most commonly found in the gastric mucosa of cats (and dogs), with a prevalence ranging from 20 to 100% (Joosten *et al*., 2013). Like other gastric NHPH, infection with this bacterium is associated with chronic active gastritis in cats (Hwang *et al*., 2002; Joosten *et al*., 2013). In experimental animal models, *H. heilmannii* s.s. mainly colonizes the antrum of the stomach and infection triggers the development of a chronic inflammation in this region (Joosten *et al*., 2013). In humans, *H. heilmannii* s.s. has been detected in 8-19% of gastric biopsies with histological evidence of a NHPH infection (Haesebrouck *et al*., 2009; Trebesius *et al*., 2001; Van den Bulck *et al*., 2005). Infection with this bacterium is associated with the development of gastritis, peptic ulcer disease and MALT lymphoma (Haesebrouck *et al*., 2009; Trebesius *et al*., 2001; Van den Bulck *et al*., 2005; Baele *et al*., 2009; O'Rourke *et al*., 2004a). A recent case report on infection with this bacterium in humans showed the presence of multiple gastric ulcers near the pyloric ring and development of chronic gastritis with prominent lymphoid follicles, without atrophy (Matsumoto *et al*., 2014). In this patient, as well as in other gastric NHPH-infected humans, urea breath test as well as the presence of serum anti-*H. pylori* IgG were negative (Matsumoto *et al*., 2014).

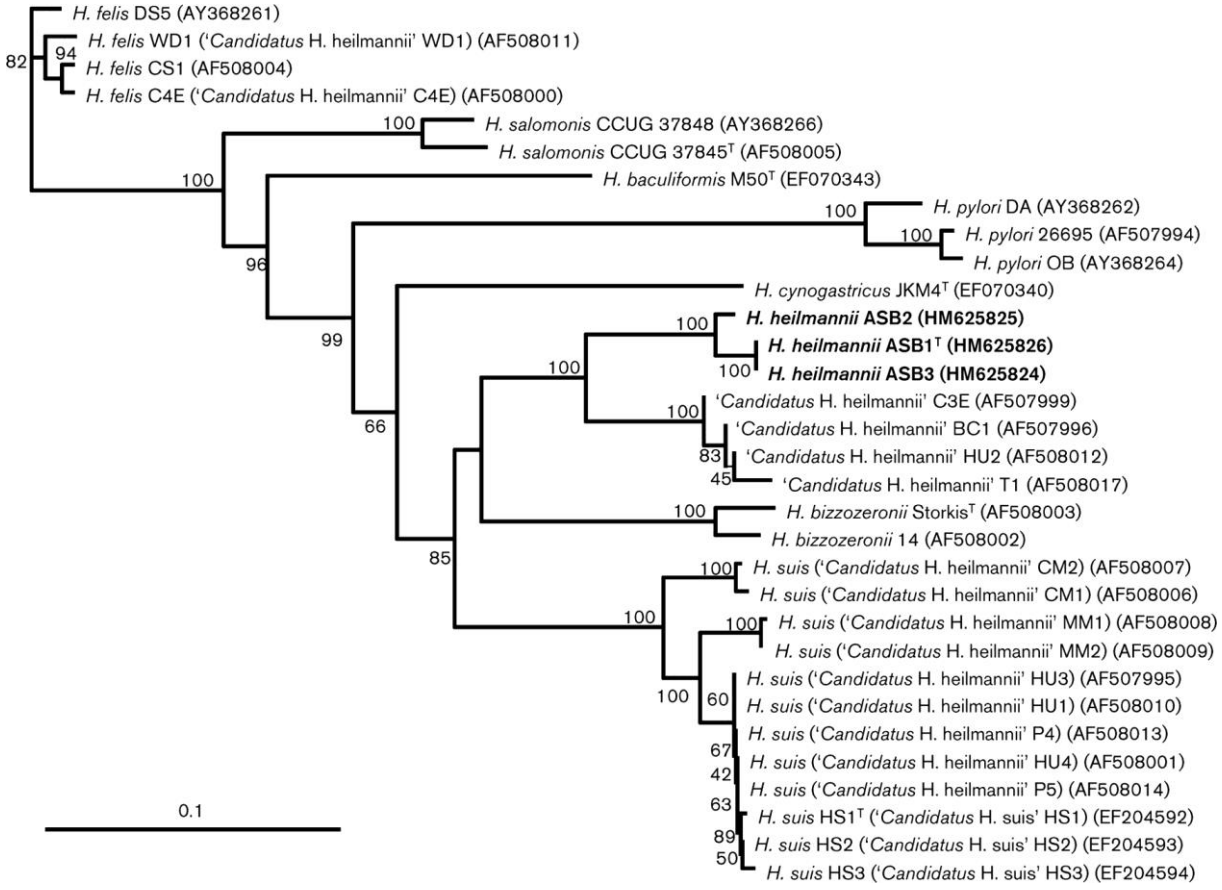

Fig.6 A phylogenetic tree, reconstructed from genetic distances, based on partial *ureA* and *ureB* gene sequences of *H. heilmannii* s.s. and other urease-positive gastric *Helicobacter* species (Smet *et al*., 2012).

Pathogenesis and strain differences

Whole genome sequencing of *H. heilmannii* s.s. strain ASB1 has revealed that this bacterium prossesses a homologue for several known virulence factors, such as GGT, the immunomodulator NapA, the flavodoxin FldA, the plasminogenbinding proteins PgbA and PgbB, the collagenase PrtC, the carcinogenic factor Tipα, the proline oxidase PutA, and the secreted serine protease HtrA. This genome encodes several outer membrane proteins but lacks the Bab and SaB adhesins. It also possesses a complete *comB* system conferring natural competence but it lacks a Cag pathogenicity island as well as a homologue of genes encoding VacA (Smet *et al*., 2013).

In an experimental infection study in Mongolian gerbils, high levels of antral colonization were observed for several *H. heilmannii* s.s. strains, whereas others showed much lower colonization

rates or were even shown not to be able to colonize the Mongolian gerbil stomach (Joosten *et al*., 2013). Later, it was found that the latter, however, most likely belongs to another *Helicobacter* species, which was provisionally named *"H. ailurogastricus"* (Joosten *et al*., 2014). In addition, an antrum-dominant chronic (active) gastritis as well as the presence of lymphocytic aggregates were seen and no inflammation was detected in the fundic gland region of the stomach (Joosten *et al*., 2013b). No significant differences were observed in the stomach of infected animals versus controls for IL-5, IL-6, IL-10, IL-12p40, IL-17 and TNF-α. Possibly, the absence of a Th17 response might explain the low number of infiltrating neutrophils in the antral mucosa of the gerbils with gastritis. The Th1 cytokine IFN-γ, a signature marker of the Th1-polarized response (Crabtree *et al*., 2004; Bamford *et al*., 1998), exhibited even a decreased expression in the antrum of the stomachs of experimentally infected gerbils.

In contrast, expression levels of IL-1β were shown to be up-regulated in the antrum of the stomach after challenge. This pro-inflammatory cytokine plays a role in the acute phase of inflammation (Yamaoka *et al*., 2005) and it is a potent inhibitor of gastric acid secretion (El-Omar *et al*., 2001). Indeed, RT-PCR showed a mild decrease in the expression of gastric H^+/K^+ ATPase, responsible for gastric acid secretion, in the antrum of the gerbils after infection. In addition, virulent *H. heilmannii* s.s strains stimulate the secretion of gastrin, which normally enhances the secretion of gastric acid by parietal cells. In the study by Joosten *et al*. (2013), however, this did not result in increased production of hydrochloric acid, most likely due to a modulating effect of IL-1β on the expression of the H+/K+ ATPase α-subunit promoter, contributing to reduced parietal cell H+/K+ ATPase gene and protein expression and thus to hypochlorhydria.

To date, few studies have identified gastric NHPH to the species level by genetic analysis (Wuppenhorst *et al*., 2013; O'Rourke *et al*., 2004b; Dieterich *et al*., 1998; Matsumoto *et al*., 2009; Kivisto *et al*., 2010), let alone to the strain level. In addition, few or no information is available on possible differences in pathogenicity and sensitivity to eradication therapy between different strains of the same species. Like for most gastric NHPH, little is known on the transmission and epidemiology of *H. heilmannii* s.s. As this bacterium has a natural nonhuman reservoir, transmission from animals to humans is likely to occur. Considering the high prevalence in cats and dogs and much lower infection rate in humans, the transmission of this bacterium from animals to humans does not seem to to be very effective (Hermanns *et al*., 1995; Hilzenrat *et al*., 1995). Although the owners of household pets probably acquire these strains directly from their pet(s), other possibilities such as human-human and human-animal transmission, cannot be excluded (Dieterich *et al*., 1998; Eaton *et al*., 1996; Grasso *et al*., 1996; Queiroz *et al*., 1996).

3. Strain typing of gastric *Helicobacter* species

During the last decades, numerous techniques and methods have been developed for bacterial strain typing, based both on phenotypic and genotypic approaches. The majority of these methods are based on differences in the genetic content of the pathogen.

Amplified-fragment length polymorphism (AFLP) analysis is based on the ligation of adapter oligonucleotides to restriction enzyme fragments, which are then used as target sites for amplification by PCR (Vaneechoutte M, 1996), whereas PCR-restriction fragment length polymorphism (PCR-RFLP) analysis is based on the amplification by PCR of a number of known genes, which are subsequently digested with restriction enzymes (Liveris *et al*., 1995). Another technique using restriction enzymes is pulsed-field gel electrophoresis (PFGE). First, genomic DNA is digested with a rare cutting restriction enzyme, generating large DNA fragments which can subsequently be separated by electrophoresis using an electrical field that changes over time (Maule J, 1998). Fingerprinting by random amplified polymorphic DNA (RAPD) (Mazurier SI and Wernars K, 1992) and enterobacterial repetitive intergenic consensus (ERIC)-PCR (Versalovic *et al*., 1991) is done by amplifying random DNA fragments using short primers or genomic regions lying between certain repetitive sequences, respectively. The techniques described above have all been optimized for use with *H. pylori* (Gibson et al., 1998; Röesler et al., 2009; Finger et al., 2006), although they have not been applied extensively.

Whole-genome microarrays have also been used occasionally to investigate *H. pylori* strain differences. DNA segments representative of the whole genome of a reference strain are first spotted on an array (Tulpan, 2010). Marked DNA segments from a different strain are allowed to hybridize with these segments and visualized, which allows identification of core and strain-specific genes (Salama *et al*., 2000)

Another approach is to analyze well-defined loci of a certain bacterial species. Multiple-Locus VNTR (Variable Number of Tandem Repeats) Analysis (MLVA) (Keim *et al.*, 2000) utilizes differences in the number of repeats in certain genomic regions containing a repeat sequence . This type of sequence is prone to slipped strand mispairing and recombination events. By measuring the number of repeats, strains can be differentiated. For this purpose, the VNTR regions are amplified with specific (marked) primers and the length of the resulting fragments is determined by gel or capillary electrophoresis. Multilocus enzyme electrophoresis (MLEE) is based on protein electrophoresis and comparison of the electrophoretic mobilities of multiple core metabolic

enzymes (Selander *et al*., 1986). This technique has some disadvantages, including the fact that 'silent mutations' may not induce changes in encoded amino acids, and the fact that environmental conditions can induce alterations in the phenotype of the enzymes, which may affect the reproducibility.

Multilocus sequence typing (MLST) was first proposed by Maiden in 1998 as an improvement of multilocus enzyme electrophoresis (MLEE) (Maiden *et al*., 1998). MLST is a relatively simple technique, by which alleles of several housekeeping genes, essential for the metabolism of the bacterium, are directly assessed. First, gene fragments from approximately 500 bp are selected for PCR amplification. These PCR products are then subjected to nucleotide sequencing and based on the precise nucleotide sequence, allele numbers are assigned. Each unique allele combination determines the sequence type (ST) of a strain (Figure 7).

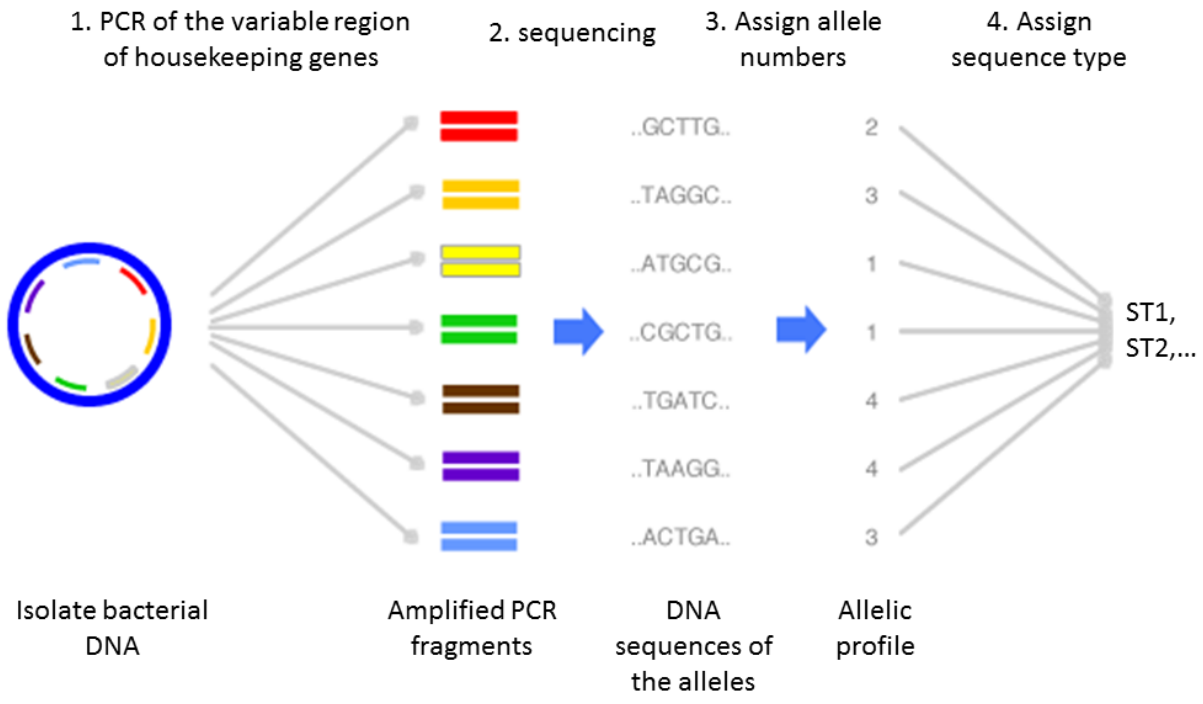

Fig.7 Graphic representation of the steps involved in bacterial Multilocus Sequence Typing (adapted from: http://beta.mlst.net/Instructions/default.html)

The housekeeping genes chosen are 'universal', preferably spread throughout the genome and highly conserved in the bacterial species. Their nucleotide sequences should reveal an appropriate (limited) level of variation between different strains within the same species (Cooper *et al*., 2004;

Pullinger *et al*., 2006). In general, 7 housekeeping genes are selected and more can be included to increase the resolution. If this does not provide satisfactory discrimination, loci with a higher variability such as antigen-encoding genes may also be included (Feavers *et al*., 1999), although some authors postulate this technique should no longer be called MLST. Two of the main advantages of MLST are the portability of nucleotide sequence data, which allows results from different laboratories to be compared without exchanging strains (Griffiths *et al*., 2010; Kilian *et al*., 2012; Laukkanen-Ninios *et al*., 2011) and the fact that it is a culture-independent method, which can be applied onto various biological materials, which is an important advantage when working with fastidious micro-organisms like *Helicobacter*.

Multilocus sequence typing was originally used in the study of *Neisseria meningitidis* and it has subsequently been applied to investigate the molecular epidemiology of numerous pathogenic bacteria (Devi *et al*., 2006; Coffey *et al*., 2006; Platonov *et al*., 2000; Kotetishvili *et al*., 2002; Diancourt *et al*., 2005; Momynaliev *et al*., 2005; Margos *et al*., 2008; Griffiths *et al*., 2010; Kilian *et al*., 2012; Laukkanen-Ninios *et al*., 2011), with the purpose of tracing the infectious source (Kotetishvili *et al*., 2002) and routes of infection (Raymond *et al*., 2004; Osaki *et al*., 2013; De Cooman *et al*., 2014), but also as a means of resolving population biology and/or phylogeographical differentiation of bacterial species (Pullinger *et al*., 2006). To date, MLST is the method that has been the most often used to investigate *H. pylori* strain differences. This has even been shown to help in tracing of the migration of human populations thousands of years ago (Yamaoka Y, 2009; Moodley *et al*., 2009).

In more recent years, whole-genome sequencing has been used to distinguish *Helicobcter* strains (Satou *et al*. 2014; Krebes *et al*., 2014). Analysis of these genomes can be performed on the basis of the nucleotide sequence or using a gene-by-gene approach, which is named whole-genome MLST (wgMLST) (Maiden et al., 2013).

An overview of strain typing methods used for *H. pylori* is shown in Table 1, including the assessment of possible drawbacks and strengths of each technique.

Table 2. Characteristics of various typing methods used for *H. pylori*. Characteristics are rated as follows: ++: very strong; +: strong; ±: variable; -: weak; --: very weak (Adapted from Vranckx et al., 2012)

Typing method	Pure culture required	Discriminatory power	Ease of performance	Ease of interpretation	Interrun-repeatability	Interlaboratory repeatability	Cost-effectiveness
AFLP	Yes	+	±	-	±	-	±
PCR-RFLP	No	-	+	+	±	-	+
PFGE	Yes	+	±	+	+	-	±
RAPD	Yes	-	++	±	--	--	++
ERIC-PCR	Yes	+	++	±	+	±	+
Microarray	Yes	++	--	--	±	±	--
MLVA	No	++	+	++	++	+	+
MLST	No	++	+	++	++	++	-
WGS*	Yes	++	±	±	++	++	±

*WGS: whole-genome sequencing

When we started our PhD studies, techniques for typing of *H. suis* strains or other NHPH were not yet available, with the exception of AFLP performed on *H. suis* strains HS1, HS2 and HS3 (Baele *et al*., 2008).

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SCIENTIFIC AIMS

Besides the well-known *Helicobacter pylori*, other non-*H. pylori Helicobacter* (NHPH) species also cause gastric disease in humans. This group of NHPH comprises several *Helicobacter* species naturally colonizing the stomach of various animal species. *Helicobacter suis* colonizes the stomachs of pigs with infection rates of up to 95% in certain herds. The infection in pigs may result in chronic gastritis, ulceration of the *pars oesphagea* of the stomach and decreased daily weight gain. So far, little information is available on the epidemiology of *H. suis* infection in pigs and humans. In addition, little is known on *H. suis* strain diversity, both on the genotypic and phenotypic level, in these populations. The **primary aim** of this thesis was to develop an unambiguous and discriminatory typing scheme for *H. suis*, which should allow us to increase our understanding of the epidemiology and population structure of this bacterium.

For primary isolation and subsequent cultivation of *H. suis*, strict biphasic conditions are required. These consist of a solid medium with on top of it a liquid medium, making it impossible to obtain single colonies. Hence, biphasic culture may contain more than one *H. suis* strain. This is undesirable for studies aiming for instance at investigating *H. suis* strain differences. Also, to construct and isolate deletion mutants for genes of interest, culture on dry agar plates as single colonies is preferred. Therefore, the **second aim** of this thesis was to establish a protocol allowing to obtain pure *in vitro* cultures of *H. suis* by single colony isolation and purification.

Besides *H. suis*, the group of gastric NHPH contains various *Helicobacter* species naturally colonizing dogs and cats. Most of these species, including *H. felis, H. bizzozeronii* and *H. salomonis*, have already been cultured and used in various experimental set-ups. Recently, *H. heilmannii* sensu stricto and the putative new species "*H. ailurogastricus"*, which mainly colonize the stomachs of cats, have been isolated in this laboratory. Similar to *H. suis*, little information is available on the strain diversity of these *Helicobacter* species, both on the genotypic and phenotypic level. Therefore, the **third aim** of this thesis was to develop an unambiguous and discriminatory typing scheme for *H. heilmannii* sensu stricto and "*H. ailurogastricus"*.

EXPERIMENTAL STUDIES

CHAPTER 1

Multilocus Sequence Typing of the Porcine and Human Gastric Pathogen *Helicobacter suis*

Jungang Liang, Richard Ducatelle, Frank Pasmans, Annemieke Smet, Freddy Haesebrouck, Bram Flahou

Department of Pathology, Bacteriology and Avian Disease, Faculty of Veterinary Medicine. Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

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Abstract

Helicobacter (*H.) suis* is a Gram-negative bacterium colonizing the majority of pigs, in which it causes gastritis and decreased daily weight gain. *H. suis* is also the most prevalent gastric non-*H. pylori Helicobacter* sp. in humans, capable of causing gastric disorders. To gain insight into the genetic diversity of porcine and human *H. suis* strains, a Multilocus Sequence Typing (MLST) method was developed. In a preliminary study, 7 housekeeping genes (*atpA, efp, mutY, ppa, trpC, ureI, yphC*) of 10 *H. suis* isolates cultured *in vitro* were investigated as MLST candidates. All genes displayed several variable nucleotide sites, except the *ureI* gene, which was replaced by part of the *ureAB* gene cluster of *H. suis*. Subsequently, internal gene fragments, ranging from 379-732 bp and comprising several variable nucleotide sites, were selected. For validation of the developed MLST technique, gastric tissue from 17 *H. suis*-positive pigs from 4 different herds and from 1 *H. suis*-infected human patient was used for direct, culture-independent strain typing of *H. suis*. In addition to the 10 unique sequence types (STs) among the 10 isolates grown *in vitro*, 15 additional STs could be assigned. Individual animals were colonized by only 1 *H. suis* strain, whereas multiple *H. suis* strains were present in all herds tested, revealing *H. suis* is a genetically diverse bacterial species. The human *H. suis* strain showed a very close relationship to porcine strains. In conclusion, the developed MLST scheme may prove useful for direct, culture-independent typing of porcine and human *H. suis* strains.

Introduction

Helicobacter suis (*H. suis*) is a Gram-negative, motile, tightly-coiled, spiral-shaped and microaerophilic bacterium that colonizes the gastric mucosa of the majority of pigs worldwide (Grasso *et al.*, 1996; Park *et al*., 2004; Hellemans *et al.*, 2007). Its reported prevalence depends on the study. Mostly, however, this bacterium is detected in more than 60% of pigs at slaughter age (Grasso *et al.*, 1996; Park *et al*., 2004; Hellemans *et al.*, 2007; Haesebrouck *et al.*, 2009). *H. suis* infection in pigs is associated with chronic gastritis and decreased daily weight gain (De Bruyne *et al.*, 2012). In addition, associations have been made between a natural *H. suis* infection and the presence of ulcers in the *pars oesophagea* of the stomach (Barbosa *et al.*, 1995; De Groote *et al.*, 2000; Roosendaal *et al.*, 2000). *H. suis* is also of zoonotic importance, as it is the most prevalent gastric non-*H. pylori Helicobacter* (NHPH) species in humans (Haesebrouck *et al.*, 2009; Van den Bulck *et al.*, 2005). Pigs are considered to be an important source of infection for humans. Besides direct contact with animals, the consumption of raw or undercooked pig meat may also be a source of human infection (Meining *et al.*, 1998; De Cooman *et al.*, 2011).

The first isolation of *H. suis* was described only in 2008 (Baele *et al.*, 2008). In subsequent years, we gathered a total of 10 isolates, cultured *in vitro*, of this extremely fastidious micro-organism. All isolates originate from pig stomachs and until now, no isolates have been obtained from infected humans. To our knowledge, the above mentioned *in vitro* isolates are the only ones available world-wide and still now, *H. suis* isolation remains difficult and time-consuming. Often, mucus scrapings from half of a porcine stomach are needed to successfully isolate a new *H. suis* strain.

In order to gain insight into the strain diversity of both human and porcine *H. suis* strains, several typing methods can be used. However, given the extremely fastidious nature of this microorganism, a culture-independent method should be used, allowing typing of *H. suis* directly in stomach samples. Multilocus Sequence Typing (MLST), introduced in 1998, has been widely used in molecular epidemiology and population biology of bacterial species (Maiden *et al.*, 1998; Woo *et al.*, 2011; Chen *et al.*, 2006; Diancourt *et al.*, 2005) and has proven its usefulness for typing strains of other *Helicobacter* species (Devi *et al.*, 2007; Rimbara *et al.*, 2012; Yamaoka *et al.*, 2009). In addition, this technique uses the unambiguousness and portability of nucleotide sequence data, which allows results from different laboratories to be compared without exchanging strains (Griffiths *et al.*, 2010; Kilian *et al.*, 2012; Laukkanen-Ninios *et al.*, 2011). Although it also has

some drawbacks, including a relatively high cost (Kilian *et al.*, 2012), MLST is considered to be a gold standard for strain typing of bacterial species.

Our aim was thus to develop a robust *H. suis* MLST technique, which can be applied to biological tissue, without the need for cultivation.

Materials and Methods

H. suis isolates cultured *in vitro* and DNA extraction

H. suis strains HS1-HS10 were isolated from the gastric mucosa of sows as described previously (Baele *et al.*, 2008). Bacteria were grown microaerobically (85% N_2, 10% CO_2, 5% O_2; 37°C; 48-96h) on biphasic Brucella (Becton Dickinson, Franklin Lakes, NJ, USA) culture plates (with a pH adjusted to 5) supplemented with 20% fetal calf serum (HyClone, Logan, UT, USA) and Vitox supplement (Oxoid), as described previously (Flahou *et al.*, 2010). Bacterial genomic DNA of all *H. suis* strains was extracted as described by Wilson (Wilson, 1994).

Development of a *Helicobacter suis* Multilocus Sequence Typing method

PCR assays were performed on a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). Different DNA polymerases (Taq, Pwo and Accuzyme) were used in this study, depending on the application. PCR products were first purified using a MSB Spin PCRapace kit (STRATEC Molecular GmbH, Berlin, Germany). Purified PCR products were sequenced using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, Ca) and sequences were determined on an automatic DNA sequencer (ABI Prism™ 3100 Genetic Analyzer, Applied Biosystems). The electropherograms were exported and converted to Kodon software (Applied Maths, Sint-Martens-Latem, Belgium).

Based on the whole genomic DNA sequence of *H. suis* strains 1 and 5 (Vermoote *et al.*, 2011), seven housekeeping genes used for *H. pylori* multilocus sequence typing (*atpA, efp, mutY, ppa, trpC, ureI* and *yphC*) were initially selected for the design of a multilocus sequence typing method for *Helicobacter suis*. The complete coding regions of these genes were amplified from 10 *H. suis* isolates cultured *in vitro* using Pwo polymerase with 3'-5' proofreading exonuclease activity. Final reaction mixtures containing a total of 25 µl were prepared in 2 parts. Part 1 contained 6.25 µl dNTP's (1 mM each), 0.3 µl of each primer at a concentration of 0.6 pmol/µl, 5.15 µl Aq HPLC and 0.5 µl DNA sample. Part 2 included 0.25 µl Pwo DNA polymerase (5U/µl stock solution), 2.5µl 10X buffer containing $MgSO_4$ and 9.75 ul Aq HPLC. Briefly, following an initial 3 min

denaturation at 94°C, amplification was done by 10 cycles of 94°C for 30 s, 53°C to 58°C for 30 s and 72°C for 90 s, followed by 25 or 30 cycles of 94°C for 30 s, 53°C to 58°C for 30 s and 72°C for 90 s with an additional 5 s elongation time for each cycle. A final extension step was performed at 72°C for 10 min. Alternatively, amplification was done using Accuzyme DNA polymerase. Final reaction mixtures (10 µl) included 5 µl Accuzyme mix, 0.1 µl of each primer at a concentration of 0.5 pmol/µl, 3.8 µl Aq HPLC and 1.0 µl DNA sample. Briefly, following an initial denaturation for 3 min at 94°C, amplification was done by 10 cycles of 94°C for 30 s, 53°C to 58°C for 30 s and 72°C for 90 s, followed by 25 or 30 cycles of 94°C for 30 s, 53°C to 58°C for 30 s and 72°C for 90 s with an additional 5 s elongation time for each cycle. A final extension step was performed at 72°C for 20 min. Purification of PCR products and sequencing were performed as described above. Primers used for amplification and sequencing, as well as annealing temperatures, are shown in Table 1.

For all genes, except the *ureI* gene, variable sites were detected. Based on the position of these sites, internal gene fragments (450-732 bp) were selected (Table 2). Amplification was done using Pwo or Accuzyme DNA polymerases with 3'-5' proofreading activity. Primers used for amplification and sequencing of these variable regions are shown in Table 2. Briefly, following an initial denaturation for 3 min at 94°C, amplification was done by 10 cycles of 94°C for 30 s, 59°C to 69°C for 30 s and 72°C for 90 s, followed by 25 or 30 cycles of 94°C for 30 s, 59°C to 69°C for 30 s and 72°C for 90 s with an additional 5 s elongation time for each cycle. A final extension step was performed at 72°C for 20 min. Purification of PCR products and sequencing were performed as described above. Based on the multiple alignment, using Kodon software, of the *ureAB* gene cluster of different *H. suis* strains (HS1, HS5, NCBI accession numbers AF 507995, AF 508001, AF508008, AF508009, AF508013), variable sites were detected in this gene cluster. Therefore, primers were designed for amplification and sequencing of the variable region of this gene cluster (Table 2).

Table 1. Oligonucleotide primers used for amplification and sequencing of complete coding sequences

primer	sequence (5'--3')	polarity	amplicon size(bp)	position	annealing temperature	Use of primers
atpA JunF1	TTGTTAAAATTAAAAGCAGAAGAAATTAGCG	Forward	1512	1 - 31	54°C	A*,S*
atpA JunR1	CTAGAGAGTTAGACTTAGTTTAAATTCTTCAATCGC	Reverse		1477-1512		A, S
ATPA-F	CCCCCCTATTCTCCCTTGCCC	Forward		1270-1290		S
ATPA-R	CAAGCCCCTATCGCCGGTATC	Reverse		169-189		S
AtpA_fw_in	TACCCGTAGGCGATGCGGTC	Forward		293-312		S
efp JunF1	ATGGCCATTGGAATGGGTGAAC	Forward	567	1 - 22	53°C	A,S
efp JunR1	TTAAACTTTGAGCTTTTCTAAATACTCC	Reverse		540 - 567		A,S
EFP-F	GCCTAATTTTAGAGGGGATAC	Forward		414 - 434		S
EFP-R bis	ATTAGGCTCTTCGCACTTATCC	Reverse		174 - 195		S
mutY JunF1	ATGTCCCAGCAAACCCAAATTGAGGTTG	Forward	873	1 - 28	58°C	A,S
mutY JunR1	CTACAAAATCCCCGTCTTTTCTAGTAATACC	Reverse		843 - 873		A,S
MUTY-F	AGTACCGCGTGCAAGTTTGGC	Forward		719 - 739		S
MUTY-R	CACGGGCATAATAACCTAGAC	Reverse		125 – 145		S
ppa JunF1	ATGAACATCTCAAAAATTCCAGTTAGTAACGC	Forward	528	1 - 32	56°C	A,S
ppa JunR1	TCAACCTTGGGCTTGTTGGTAATTTGCAAGTGC	Reverse		496 - 528		A,S
PPA-F2	CATTGTGCTACAAAAGATCAGAC	Forward		378- 400		S
PPA-R2	TAGCTGGATATACCATAGATG	Reverse		134 - 154		S
trpC JunF1	ATGCATGATTTTTTAACAACCATGTTAGAA C	Forward	1359	1 - 31	57°C	A,S
trpC JunR1	TCAATACTCCCGTAACATTTTTGCAATTTGTGC	Reverse		1327 - 1359		A,S
TRPC-F	GCTAGCTGGAGGGTTAAATGC	Forward		1209-1229		S
TRPC-R	GAGGGGGAGGCTTGTTTGCAC	Reverse		150 - 170		S
TRPC_rv_in	TTTAAGCGCAGTTGGCATGCA	Reverse		960 – 980		S
ureI JunF1	ATGCTAGGACTTGTGTTATTGTATGTTGCGATCG	Forward	588	1 - 34	55°C	A,S
ureI JunR1	TTACACCCAGTGTTCGATAAAGAGAAGCCAAGCAGG	Reverse		553 - 588		A,S
UREI-F	ACTGGTTGGATTGAGGAAGTGG	Forward		463 - 484		S
UREI-R	CAAACAATAGAGATCCCACCTAC	Reverse		106 - 128		S
yphC JunF1	ATGCTTAAAATTGCTATTTTGGGCAAGCC	Forward	1317	1 - 29	57°C	A,S
yphC JunR1	TCATGTTTGCAAAGTTTCTTCTTTGCCTCTGGC	Reverse		1285-1317		A,S
YPHC-F2	TTGCTTTGGTTATGAATCGCCCTAAG	Forward		1175- 1200		S
YPHC-R	AAAGCTCCACCCCATGGCCTGCTAG	Reverse		136- 160		S

* A: primers used for amplification; S: primers used for sequencing

Table 2. Oligonucleotide primers used for *H. suis* MLST

primers	sequence (5'--3')	polarity	amplicon size in bp	position	annealing temperatures	Use of primers
atpA-MLST-A	TTATGAGGTGGTTGAATTTGATACCGGC	forward	790 (732)**	150 - 177	63°C	A*,S*
atpA-MLST-B	AGAGCCTGCCCCTTTCTTATCACTCATTT	reverse		911 - 939		A,S
atpA-MLST-C	ATGATTGCATCAATGGCAACAGTGG	reverse		530 - 554		S
efp-MLST-A	TACAAGGCGTTCCTTATCGCATTGT	forward	470 (379)**	47 - 71	61°C	A,S
efp-MLST-B	CACCTCCCCCTCTAGCACATGG	reverse		495 - 516		A,S
efp_mlstAquinto[#]	GGCCTTTGTACGGGCTAAA	forward	379	105-123	58°C	A,S
efp_mlstBbis[#]	CACCACTGCCCCGGT	reverse		469-483		A,S
mutY-MLST-A	CGCCCCTTTAGACCGGGTTTTACTT	forward	650	90 - 114	61°C	A,S
mutY-MLST-B	GCCAAACTTGCACGCGGTACTTG	reverse		717 - 739		A,S
mutY-MLST-C	TTAGGCAAAATGTGGGCGTGCTAGA	forward		278 - 302		S
ppa-MLST-A	TGCCGTTATTGAAATCCCGTATGGA	forward	480	45 - 69	60°C	A,S
ppa-MLST-B	CCTTGGGCTTGTTGGTAATTTGCAA	reverse		500 - 524		A,S
trpC-MLST-A	TGTGGCCTTAAGCGGGTTAAAGATG	forward	450	769 -793	60°C	A,S
trpC-MLST-B	TCCAGCTAGCATAAAGCGATGGGAT	reverse		1194-1218		A,S
ureAB_mlstA	GTGCGCTTTGAACCTGGCG	forward	688 (676)**	523 - 541	69 °C	A
ureB_mlstB	CCTGTTCCGCCTCCAAGCAT	reverse		1191-1210		A,S
ureB_mlstA	ATGTATGGCCCCACTACAGGCG	forward		759 - 780		S
yphC-MLST-A	GGATACAGGCGGGTTTGATGCAG	forward	850 (717)**	162 - 184	59°C	A,S
yphC-MLST-B	TTTGATTTGGAGGATATGGCGCTTAGA	reverse		985 - 1011		A,S
yphc-MLST-C	AAATGCCCTGATAGAGCAAGAACGC	forward		579 - 603		S
yphC_mlstAtris[#]	AAAATCCCCCCACAAGATGAGGATAA	forward	717	268-293	62°C	A,S
yphC_mlstBtris[#]	GATAGCACTTGTTGTAAGAAGCG	reverse		962-984		A,S

* A: primers used for amplification; S: primers used for sequencing

****** The number between brackets represents the actual length, after trimming, of nucleotide fragments used for multiple alignment, determination of ST and generation of concatenated sequences.

[#] Primer pairs used for amplification and sequencing of internal *efp* and *yphC* genes from the human *H. suis* strain described in this study

Based on the nucleotide sequence of the internal gene fragments, allele numbers for each gene were assigned for isolates HS1 to HS10, cultured *in vitro*. The sequence type (ST) was defined by the combined allelic profile of the 7 alleles.

Validation of *Helicobacter suis* MLST

For validation of the developed MLST on tissue samples, gastric biopsies were collected from slaughter pigs from 4 different herds. For each stomach, three pieces of tissue (1cm^2; 1 from the corpus, 2 from the antrum) were collected using sterile biopsy punches. In addition, an antral gastric biopsy from a pig veterinarian, suffering from gastric complaints and diagnosed with *H. suis* infection (Joosten *et al.,* 2013), was included as well. DNA was extracted using the Isolate Genomic DNA MiniKit (Bioline, London, UK) according to the manufacturer's instructions. All DNA samples were screened for the presence of *H. suis* using a Taq polymerase-based species-specific PCR, as described by De Groote et al. (De Groote *et al.,* 2000). Three to six *H. suis* positive samples/herd as well as the human sample were selected for multilocus sequence typing of colonizing *H. suis* strains, as described above.

Amplification of the internal gene fragments for *efp* and *yphC* genes from the human *H. suis* strain, however, yielded no pure PCR products. Therefore, new primer pairs were designed, resulting in the amplification of a single PCR product of the expected size (Table 2). In order to enable comparison, internal *efp* and *yphC* gene fragments from *in vitro* cultured *H. suis* strains and the porcine and human gastric tissue samples, obtained as described above, were all trimmed to this new size (379 bp and 717 bp for *efp* and *yphC*, respectively). Contig assembly of sequences obtained using different primers was done using Kodon software. The obtained sequences of internal gene fragments (harbouring variable nucleotide sites) for individual genes were aligned using the CLUSTAL W program.

In order to assess the stability of allelic polymorphisms during *in vitro* culture of *H. suis*, strains HS1 and HS5 were cultured and passed onto new plates continuously for another 6 weeks. These long-term *in vitro* cultured strains were designated as HS1p17 and HS5p21, respectively. DNA was extracted and used for sequencing of the internal gene fragments as described above.

Based on the nucleotide sequence of the internal gene fragments, allele numbers for each gene were assigned for *H. suis* strains detected in porcine and human stomach tissues. The sequence type (ST) was defined by the combined allelic profile of the 7 alleles.

The relationship among the different *H. suis* strains was examined using the concatenated internal gene fragment sequences of the 7 MLST loci. A phylogenetic tree was constructed using the neighbor-joining method via the PHYLIP 3.69 package, using DNADIST for distance analysis (Eeckhaut *et al.,* 2008).

Results

Selection of Genes for Multilocus Sequence Typing

A total of 6744 bp, representing the complete coding sequences of 7 housekeeping genes (*atpA, efp, mutY, ppa, trpC, ureI, yphC*), were amplified and sequenced for all *in vitro H. suis* isolates available (HS1-HS10), revealing a unique concatenated sequence for all isolates. Fifty-six nucleotide sites (56/6744, 0.83%) were found to be polymorphic. The *mutY* gene was shown to have the highest discriminatory power, with 32 variable nucleotide sites. The *ppa* gene showed 9 variable nucleotide sites, whereas the *yphC* gene revealed 5, the *atpA* gene 4 and the *efp* and *trpC* gene 3 variable nucleotide sites. Based on the complete *ureI* coding sequence (strains HS1-HS10), this gene was shown to lack discriminatory power (0 variable nucleotide sites).

MLST analysis of *in vitro* isolated *H. suis* strains and *H. suis* present in porcine and human gastric tissue

Based on the complete gene sequences, as described above, internal gene fragments/variability regions (379-732 bp) were selected for all 7 loci except the *ureI* gene. Instead, the latter was replaced by part of the *ureAB* gene cluster. For porcine *H. suis* strains HS1-HS10, allele numbers and sequence type (ST) were assigned, as shown in Table 3. Determination of allele numbers for long-term *in vitro* cultured HS1p17 and HS5p21 showed no differences for HS1p17 compared to its parental strain HS1. For HS5p21, however, a new allele type was attributed for *mutY* and *trpC*, indicating that long-term *in vitro* culture, and possibly also *in vivo* colonization may result in limited genetic diversification.

In addition, direct *H. suis* MLST strain typing was performed on gastric tissue of 1 *H. suis*-infected human and 17 *H. suis*-positive pigs at slaughter age from 4 different herds, as determined by *H. suis*-specific PCR. Three stomachs were tested from Herd A, 4 from Herd B and C, and 6 stomachs from Herd D. For MLST analysis, selected variability regions were amplified and sequenced. Close examination of the electropherograms indicated that only 1 ST colonized each animal. For the human sample as well as all 17 porcine gastric tissue samples, a total of 4084bp was aligned to the same gene regions of *H. suis* strains HS1-HS10. In total, 48 nucleotide sites (48/4084, 1.18%) were shown to be polymorphic. The *mutY* gene revealed a total of 27 variable nucleotide sites, whereas 7, 4, 3, 3, 2 and 2 variable nucleotide sites were detected in the *ppa* gene, *yphC* gene, *atpA* gene, *efp* gene, *trpC* gene and *ureAB* gene, respectively. Twenty-five sequence types (STs) were assigned based on unique allele combinations (Table 3). In Herd A, 2 out of 3 strains shared the

same ST (ST-11), while a third strain displayed an unrelated ST, belonging to another group (Figure 1). In Herd C, three out of four strains shared the same ST. For Herds B and D, however, all strains showed a different ST.

Table 3. Determination of allele numbers and sequence types (ST) for 10 *H. suis* strains isolated *in vitro* and *H. suis* bacteria present in 1 human and 17 porcine gastric tissue samples.

ST	Sample*	Sample origin	Allele numbers						
			atpA	*efp*	*mutY*	*ppa*	*trpC*	*ureAB*	*yphC*
1	HS1	*in vitro culture*	1	1	1	1	1	1	1
1	HS1p17	*in vitro culture*	1	1	1	1	1	1	1
2	HS2	*in vitro* culture	2	1	2	1	2	1	2
3	HS3	*in vitro* culture	1	2	2	1	2	1	2
4	HS4	*in vitro* culture	3	2	3	1	3	1	3
5	HS5	*in vitro* culture	4	2	4	1	1	1	1
6	HS5p21	*in vitro* culture	4	2	5	1	2	1	1
7	HS6	*in vitro* culture	1	3	6	1	1	1	3
8	HS7	*in vitro* culture	1	3	7	1	1	1	3
9	HS8	*in vitro* culture	1	4	8	1	1	1	1
10	HS9	*in vitro* culture	4	2	9	1	1	1	1
11	HS10	*in vitro* culture	1	4	3	2	1	1	3
12	A-P29	Herd A	4	2	9	1	2	1	1
13	A-P49	Herd A	1	4	10	1	1	1	3
12	A-P63	Herd A	4	2	9	1	2	1	1
11	B-V16	Herd B	1	4	3	2	1	1	3
14	B-V37	Herd B	1	2	3	1	1	2	4
15	B-V57	Herd B	1	2	3	1	1	1	1
16	B-V85	Herd B	1	2	3	1	1	2	3
17	C-P1A1	Herd C	1	2	11	1	1	1	1
17	C-P3A1	Herd C	1	2	11	1	1	1	1
17	C-P8A1	Herd C	1	2	11	1	1	1	1
18	C-P9A1	Herd C	1	2	12	1	1	1	1
19	D-P21A1	Herd D	1	2	13	1	2	1	5
20	D-P26A1	Herd D	1	2	14	1	1	1	2
21	D-P27A1	Herd D	1	4	15	1	1	1	1
22	D-P28A1	Herd D	1	3	16	1	2	1	2
23	D-P29A1	Herd D	1	2	16	1	2	1	2
24	D-P30A1	Herd D	1	2	14	1	1	1	6
25	HA	Human	1	2	17	1	1	1	1

* HS1-10: *H. suis* isolates cultured *in vitro*; HS1p17, HS5p21: long-term (6 weeks) *in vitro* cultures originating from HS1 and HS5, respectively; A-P29: for porcine gastric tissue samples, the number of the stomach (P29-P63; V16-V85; P1A1-P30A1) is preceded by a letter designating the herd (A-D); HA: *H. suis* strain colonizing the antrum of a human patient suffering from reflux oesophagitis and dyspepsia.

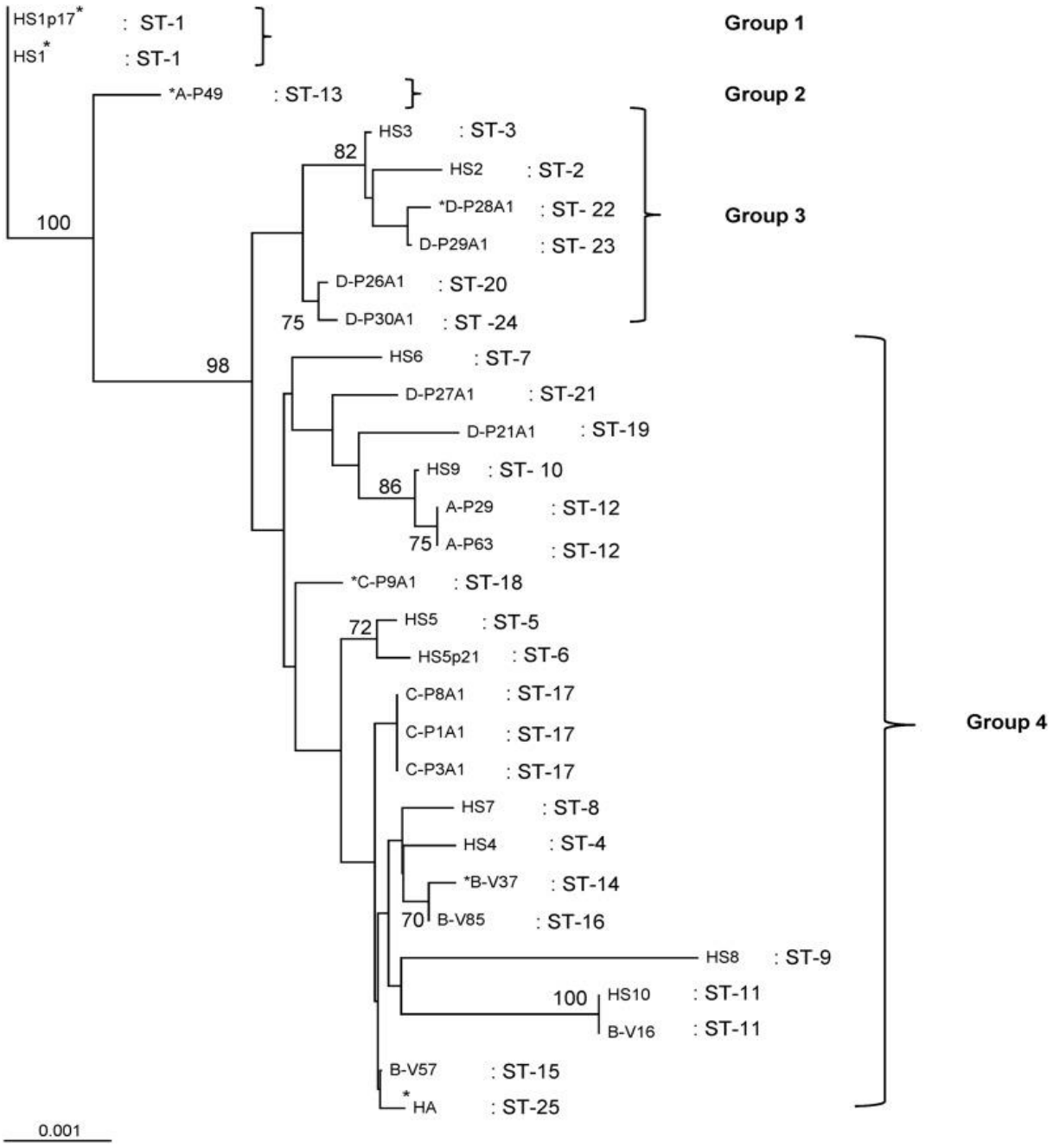

Figure 1. A Neighbor-joining phylogenetic tree (unrooted), constructed using the concatenated sequences (4084 nucleotides) of 7 *H. suis* gene fragments from 27 *H. suis* strains tested. Bootstrap values equal or greater than 70 are presented at the nodes of the phylogenetic tree. The topology shows all sequence types cluster into four groups. * HS1-10: *H. suis* isolates cultured *in vitro*; HS1p17, HS5p21: long-term (6 weeks) *in vitro* cultures originating from HS1 and HS5, respectively; A-P29: for gastric tissue samples, the number of the stomach (P29-P63; V16-V85; P1A1-P30A1) is preceded by a letter designating the herd (A-D); HA: *H. suis* strain colonizing the antrum of a human patient suffering from reflux oesophagitis and dyspepsia.

Relationships between the isolates

A neighbor-joining tree (unrooted) was constructed, based on the concatenated sequences of all 7 MLST gene fragments (4084bp), indicating the genetic relationship between all strains tested (Figure 1). In general, calculated bootstrap values were low (< 70), resulting in the assignment of 4 groups, of which groups 1 and 2 were composed of a singleton. Although most strains detected in porcine gastric tissue belonged to the same group with low internal bootstrap values (group 4), strains belonging to a particular herd tended to cluster, indicating a lower genetic distance between strains present in the same herd. Figure 1 clearly shows that the human *H. suis* strain is closely related to porcine *H. suis* strains.

Discussion

Until now, very little is known on transmission of *H. suis* between pigs and from pigs to humans. It is thought that pigs are the most important source of infection for humans through direct contact with the animals or through the consumption of raw or undercooked pig meat, contaminated with *H. suis* (Meining *et al.*, 1998; De Cooman *et al.*, 2011). In addition, few data are available on strain-related differences in virulence or the immune response evoked in its animal or human host. So undoubtedly, there is a strong need for a robust *H. suis* strain typing method, which is essential to provide answers to some of the above-mentioned questions.

Baele et al. (2008), describing the first successful *in vitro* isolation of *H. suis*, performed genomic fingerprinting of isolates HS1, HS2 and HS3 by amplified fragment length polymorphism, revealing distinct AFLP patterns for these 3 isolates. Although this typing method has proven its value for strain typing of isolates cultured *in vitro* of several bacterial species (Hagen *et al.*, 2012; Overdevest *et al.*, 2011), this can not be used for typing of *H. suis* strains in a complex matrix such as biological tissue samples. Keeping in mind that *H. suis* isolation remains extremely difficult and time-consuming, we decided to develop an MLST technique, which can be applied on samples, without the need for *in vitro* cultivation. As a genotyping method and technique, MLST has often been used in strain typing studies and can be considered to be a golden standard (Chen *et al.*, 2006; Rimbara *et al.*, 2012; Kilian *et al.*, 2012; Laukkanen-Ninios *et al.*, 2011).

For MLST analysis of *H. pylori* strains, a closely related, major human gastric pathogen, 7 housekeeping genes are used (*atpA, efp, mutY, ppa, trpC, yphC, ureI*) (Devi *et al.*, 2007; Yamaoka *et al.*, 2009). The whole-genome sequence of *H. suis* strains 1 and 5 revealed the presence of a homologue for all of these genes (Vermoote *et al.*, 2011). Therefore, these genes were also

investigated as MLST candidates in our preliminary study. Each gene was successfully amplified and sequenced from all *H. suis* strains. The *mutY* gene was found to have, by far, the most discriminatory power, revealing 17 alleles for 30 samples tested. The remaining 6 genes revealed a significantly lower number of alleles, compared to the *mutY* gene. The *ureI* gene was even found to have no variable nucleotide sites and this locus was therefore replaced by part of the *ureAB* gene cluster, in the final MLST scheme.

For *H. pylori*, all 7 loci used for MLST show a similar discriminatory ability, in contrast to what we found for *H. suis* (http://pubmlst.org/hsuis/). Nevertheless, assessment of the allelic profiles for the 10 available *H. suis* isolates cultured *in vitro* enabled us to discriminate 10 distinct sequence types (ST), underlining the suitability of the proposed MLST scheme to identify different *H. suis* strains. In addition, this scheme was applied to gastric biopsies from 1 human patient and 17 slaughter pigs, originating from 4 different herds. We were able to distinguish 14 additional ST's and all tissue samples revealed the presence of only 1 ST, based on the analysis of electropherograms. Because isolation and purification of colonizing *H. suis* strains was not attempted on these samples, we can not exclude the presence of additional sequence types in 1 individual animal, at a lower colonization rate compared to the dominant strain. For *H. pylori*, it has been shown that mixed infections with more than 1 strain in single individuals is indeed common in certain geographic regions. These mixed infections are in fact important for genetic diversification, since *H. pylori* genomes seem more stable in the absence of a mixed infection in one individual (Kennemann *et al.*, 2011). On the other hand, long-term *in vitro* culture of *H. suis* strain HS5 resulted in the appearance of new alleles for a number of genes, indicating changes or mutations do occur during *in vitro* culture, and most likely also during *in vivo* colonization, as has been described for *H. pylori* in the absence of mixed infection (Farnbacher *et al.*, 2010). Recombination events in the presence of other *H. suis* strains can be excluded, since *H. suis* strains HS1 and HS5 were purified, before the onset of the present study, from a single colony on 1% Brucella agar plates after initial isolation from slaughterhouse pigs by biphasic culture methods (Baele *et al.*, 2008).

Based on the Neighbor-joining tree and calculated bootstraps of concatenated sequences from all 7 housekeeping genes, four groups could be distinguished among the isolates tested in this study, of which 2 (groups 1 and 2) were composed of a singleton. Most *H. suis* strains belonged to a large group (group 4) with low internal bootstrap values, although strains belonging to a particular pig herd showed a tendency to cluster. For 2 out of 4 herds (B and D) tested in the present study, all

samples revealed the presence of a different ST, indicating a substantial genetic heterogeneity among *H. suis* bacteria present at the herd level. The human *H. suis* strain clearly showed a close relationship to porcine *H. suis* strains, indicating the patient, a pig veterinarian, most likely contracted the infection through its close contact with pigs (Joosten *et al.*, 2013). At this point, no conclusions can be drawn on possible correlations between STs and pathologies in pigs and humans. For the slaughterhouse samples, no detailed histopathology was performed. *H. suis* strains HS1-9 have all been shown to generate an immune response and gastritis *in vivo* (Haesebrouck *et al.*, 2009; Flahou *et al.*, 2010; Flahou *et al.*, 2012). Strain HS1 generates an immune response that is somewhat different from most other *in vitro* cultured *H. suis* strains, with regards to the expression of IL-4 and IL-6 (Flahou *et al.*, 2012) and interestingly, this strain constitutes a distinct singleton in the present study. Future studies should try and elucidate whether additional correlations can be made between the ST and the clinical significance of the strain.

In conclusion, we developed a Multilocus Sequence Typing scheme and method for identification of *H. suis* strain differences, both in pure *in vitro* cultures and in biological samples. Most likely, this technique will further prove its usefulness since until today, *H. suis* isolation and cultivation remains a challenge. In the future, this scheme can be used for strain typing, not only of porcine *H. suis* strains, but also of human strains and those present in contaminated pork (De Cooman *et al.*, 2011). In the end, this should provide better insights into the epidemiology of *H. suis* infections worldwide.

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CHAPTER 2

Purification of *Helicobacter suis* strains from biphasic cultures by single colony isolation: influence on strain characteristics

Jungang Liang, Ellen De Bruyne, Richard Ducatelle, Annemieke Smet, Freddy Haesebrouck, Bram Flahou

Department of Pathology , Bacteriology and Avian Disease, Faculty of Veterinary Medicine, Ghent University,

Salisburylaan 133, 9820 Merelbeke, Belgium

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Abstract

Background: *Helicobacter* (*H.) suis* causes gastritis and decreased weight gain in pigs. It is also the most prevalent non-*H. pylori Helicobacter* species in humans with gastric disease. *H. suis* is extremely fastidious and so far, biphasic culture conditions were essential for isolation and culture, making it impossible to obtain single colonies. Hence, cultures obtained from an individual animal may contain multiple *H. suis* strains, which is undesirable for experiments aiming for instance at investigating *H. suis* strain differences. **Materials and methods:** Pure cultures of *H. suis* were established by growing bacteria as colonies on 1% brucella agar plates, followed by purification and enrichment by biphasic subculture. Characteristics of these single colony-derived strains were compared with those of their parent strains using multilocus sequence typing (MLST), and by studying bacterium-host interactions using a gastric epithelial cell line and Mongolian gerbil model. **Results:** The purification/enrichment procedure required a non-stop culture of several weeks. For 4 out of 17 *H. suis* strains, MLST revealed differences between parental and single colony-derived strains. For 3 out of 4 single colony-derived strains tested, the cell death-inducing capacity was higher than for the parental strain. One single colony-derived strain lost its capacity to colonize Mongolian gerbils. For the 4 other strains tested, colonization capacity and histopathological changes were similar to what has been described when using strains with only a history of limited biphasic culture. **Conclusions**: A method was developed to obtain single colony-derived *H. suis* strains, but this procedure may affect the bacterial genotype and phenotype.

Introduction

Helicobacter suis (*H. suis*) is a Gram-negative, motile, spiral-shaped microaerophilic bacterium that colonizes the gastric mucosa of the majority of pigs worldwide (Grasso *et al*., 1996; Park *et al*., 2004; Hellemans *et al*., 2007). Its reported prevalence often exceeds 60% of pigs at slaughter age (Grasso *et al*., 1996; Park *et al*., 2004; Hellemans *et al*., 2007; Haesebrouck *et al*., 2009). In pigs, *H. suis* infection is associated with chronic gastritis and decreased daily weight gain (De Brruyne *et al*., 2012). In addition, *H. suis* infection has been associated with ulceration of the *pars oesophagea* of the stomach (De Brruyne *et al*., 2012; barbosa *et al*., 1995; De Groote *et al*., 2000; Roosendaal *et al*., 2000). *H. suis* is also the most prevalent gastric non-*H. pylori Helicobacter* (NHPH) species in humans (Haesebrouck *et al*., 2009; Van den Bulck *et al*., 2005). Pigs are considered to be a source of infection for humans (Meining *et al*., 1998; Joosten *et al*., 2013). Recently, pork has been identified as a possible vector for transmission of the infection to humans (De Cooman *et al*., 2013).

H. suis is an extremely fastidious micro-organism. For its isolation by cultivation as well as for subculture, strict biphasic conditions are required (Baele *et al*.,2008; Flahou *et al*., 2010). These consist of a solid medium with on top of it a liquid medium, making it impossible to obtain single colonies. A high density of viable bacteria (> 1-5 x 10^7 viable bacteria/ml) is necessary to maintain the cultures and to enable subculture. In general, dilutions during subculture exceeding 5-10-fold compromise the viability of *H. suis* bacteria. Recently, multilocus sequence typing (MLST) (Liang *et al*., 2013) performed on stomach biopsies of slaughterhouse pigs has revealed that several *H. suis* strains may occasionally be present in the same animal (unpublished results). Hence, an apparently pure biphasic culture obtained from an individual animal may contain more than one *H. suis* strain, which is undesirable for experimental studies aiming for instance at investigating *H. suis* strain differences. Also, to construct and isolate deletion mutants for genes of interest, culture on dry agar plates as single colonies is preferred.

Therefore, the aim of the present study was to purify *H. suis* strains from biphasic cultures, by growing them as colonies on dry agar plates, followed by purification of a single colony and subsequent enrichment by biphasic subculture. This procedure however requires a non-stop culture of several weeks, possibly influencing strain characteristics, including virulence. Genotypic and phenotypic changes have indeed been described under certain *in vitro* or *in vivo* settings for various bacterial species including *Mycobacterium bovis*, *Helicobacter pylori* and *Escherichia coli* (Ram

et al., 1992; Crabtree *et al*., 2002; Domenech *et al*., 2009). Colony-derived, subcultured *H. suis* strains were therefore typed by MLST and compared to the corresponding parental strains. In addition, to analyse possible differences in virulence, these purified and subcultured strains were tested in previously developed *in vivo* and *in vitro* models (Flahou *et al*., 2010, 2011).

Methods

Purification of single *H. suis* colonies and subsequent enrichment

Frozen (-70°C) biphasic cultures of 18 *H. suis* strains (HS1LP, HS2LP, HS3LP, HS4LP, HS5aLP, HS6LP, HS7LP, HS8LP, HS9LP, HS10LP, P13/04LP, P13/24LP, P13/26LP, P13/28LP, P13/32LP, P13/35LP, P13/36LP and P13/37LP) were thawed and grown for 2 days as a biphasic culture under microaerobic conditions, as described previously (Flahou *et al*., 2010). These parental strains have undergone a low number of *in vitro* passages (LP = low number of passages) and have a history of biphasic *in vitro* culture of no more than 10-14 days in total. They were all isolated from the stomach of slaughtered fattening pigs or sows, using the method described by Baele and colleagues (2008).

Subsequently, a 100 µl aliquot was plated onto a 1% brucella agar plate with a pH adjusted to 5 and supplemented with 20% fetal calf serum (HyClone, Logan, UT, USA) and Vitox supplement (Oxoid, Basingstoke, UK). The plates were left to dry, incubated under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2; 37°C) and after 5-14 days, depending on the strain, growth was visible as tiny pin-point colonies. For each strain, a single colony was picked and purified on 1% brucella agar plates. When growth was clearly visible (after 5-10 days, depending on the strain), these subcultures were harvested with a cotton swab, resuspended in brucella broth (pH 5) and cultured for another 20-25 days (approximately 8 subcultures on fresh plates) under biphasic microaerobic conditions (Baele *et al*., 2008; Flahou *et al*., 2010) before aliquots were frozen at -70°C until further use. *H. suis* strains purified and enriched in this way were designated as single colony-derived *H. suis* strains HS1kol-HS10kol, P13/04kol, P13/24kol, P13/26kol, P13/28kol, P13/32kol, P13/35kol and P13/36kol.

Multilocus sequence typing

For molecular strain typing, bacterial genomic DNA of single colony-derived *H. suis* strains and their corresponding parental strains with a low number of *in vitro* passages was extracted as described by Wilson (Wilson *et al*., 1994). Subsequently, strains were subjected to multilocus sequence typing (MLST) using 7 partial gene fragments from housekeeping genes (*atpA, efp, mutY, ppa, trpC, ureAB, yphC*), as described previously (Liang *et al*., 2013). Allele numbers, designated on the basis of exact nucleotide sequences, were compared between single colony-derived and parental *H. suis* strains.

In addition, the sequence type was determined, as described above, for *H. suis* strains HS5aLP, HS5bLP and HS5cLP, which are subpopulations with a low number of *in vitro* passages obtained by separate biphasic subculture early after the initial biphasic isolation of *H. suis* strain HS5.

Effect of *H. suis* lysates on viability of human gastric adenocarcinoma-derived AGS cells

Whole bacterial cell lysate was prepared from single colony-derived *H. suis* strains HS1kol, HS5akol, HS8kol and HS10kol as well as from their corresponding parental strains HS1LP, HS5aLP, HS8LP and HS10LP. Bacteria were harvested by centrifugation, washed 2 times with HBSS and resuspended in HBSS. The bacterial suspension was sonicated 8 times for 30 seconds on ice and centrifuged (15000 g, 5 min, 4°C) to remove cellular debris. The supernatant was filtered through a 0.22-µm pore filter (Schleicher and Schuell, Gent, Belgium) and stored for a maximum of 1 month at -80°C until further use.

Human gastric adenocarcinoma-derived AGS cells were cultured in Ham's F12 (Invitrogen, Carlsbad, CA, USA), supplemented with 0.5 mM glutamine, 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), penicillin and streptomycin (Invitrogen). Cells were seeded at a density of 10^4 cells per well of a flat-bottomed 96-well cell culture plate (Greiner Bio One, Frickenhausen, Germany) and allowed to adhere overnight. Subsequently, cells were incubated in 100 µl culture medium supplemented with 100 µl HBSS (serving as negative control cells) or 100 µl culture medium supplemented with 100 µl bacterial lysate from different *H. suis* strains, prepared in HBSS as described above, to reach a final protein concentration of 100 µg/ml. After 24 hours of incubation, the supernatant fluid was carefully removed. After 3 mild washing steps in HBSS, 200 µl of neutral red working solution (33 µg/ml neutral red in RPMI) was added to each well and incubation was continued at 37°C. After 3 hours, cells were washed 3 times with

HBSS, 150 µl of neutral red destaining solution (glacial acetic acid, ethanol, H_2O; 1/50/49 v/v/v) was added to each well and plates were shaken for 10 minutes in the dark. Finally, 100 µl was transferred to a new plate and absorbances were read at 540 nm using a Labsystems Multiskan MS microplate reader. Cells treated for 2 min with 0.1% Triton-X 100 served as positive controls. Three independent experiments were carried out, with three replications for each treatment in a single experiment.

Experimental infection of Mongolian gerbils

The animal infection study was conducted at the Faculty of Veterinary Medicine (University of Ghent, Belgium). The animal maintenance and experimental procedures described here were evaluated and approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2013-120).

Helicobacter suis strains HS1kol, HS5akol, HS6kol, HS8kol and HS10kol, were used for experimental infection of Mongolian gerbils. Thirty female Mongolian gerbils (Charles River, France) arrived at our animal housing units at the age of 4 weeks. They were randomly divided in 6 groups, with 5 animals each. Animals were kept in filter-top cages, all held in the same room under the same controlled environmental conditions. At the age of 6 weeks, the animals were briefly anaesthetized with 3% isoflurane and inoculated intragastrically twice, with a two-day interval. Twenty-five animals received 8 x 10^7 viable *H. suis* bacteria/dose (1 strain per 5 animals). The remaining 5 animals were sham-inoculated with the culture medium of *H. suis*: brucella broth with a pH of 5.

Nine weeks after the first infection, animals were anaesthetized with 5% (vol/vol) isoflurane followed by cervical dislocation. The stomach was opened along the greater curvature. A longitudinal strip was taken from the forestomach to the duodenum, fixed in 4% phosphate buffered formaldehyde and embedded in paraffin for histopathological examination. A biopsy of both the antral and fundic region was taken for DNA and RNA extraction. Biopsies for DNA extraction were frozen at -20°C and samples for RNA extraction were immediately submerged in RNA*later* (Qiagen, Hilden, Germany) and stored at −70°C until further processing.

DNA extraction and determination of *H. suis* colonization rates

The ISOLATE Genomic DNA kit (Bioline, London, UK) was used to extract DNA from biopsy samples of the mucosa of the antrum and fundic region according to the instructions of the

manufacturer. DNA extractions were frozen at -20°C until further analysis. Quantitative-PCR was performed on the DNA samples as described previously (Flahou *et al*., 2012).

RNA extraction and cytokine expression analysis

After removal of RNA*later*, samples were weighed and 1 ml of Tri-reagent was added. Subsequently, the stomach tissue was homogenized using a MagnaLyser device (Roche Applied Science, Indianapolis, USA) following the manufactures recommendations. Bromoanisole (BAN) (50 µl) was added to the samples for optimal RNA and DNA separation. The fraction containing RNA then was further purified using the RNeasy Mini kit (Qiagen) following the suppliers instructions. Total RNA was quantified using a Nanodrop spectrometer (Nanodrop Technologies, USA). Synthesis of cDNA was done by using the iScript^{TM} cDNA synthesis kit (Bio-rad, Hercules, USA) as described by the manufacturer. mRNA expression levels of various cytokines (IL-1β, IL-5, IL-6, IL-12p40, IL-10, IL-17, IFN-γ and TNF-α) were quantified by Real-Time PCR, using IQ^{TM} SYBR$^{®}$ Green Supermix (Biorad), performed on a CFX96 RT-PCR System with a C1000 Thermal Cycler (Biorad). The primer sequences are summarized in Table 1.

Table 1: Forward (F) and reverse (R) primers used for analysis of cytokine expression levels

Gene	Primers Sequence (5' ➔ 3')	Reference
IL-17	F: AGC TCC AGA GGC CCT CGG AC	(Sugimoto et al.,2009)
	R: AGG ACC AGG ATC TCT TGC TG	
TNF-α	F: GCT CCC CCA GAA GTC GGC G	(Sugimoto et al.,2009)
	R: CTT GGT GGT TGG GTA CGA CA	
IL-10	F: GGT TGC CAA GCC TTA TCA GA	(Joosten et al., 2013)
	R: GCT GCA TTC TGA GGG TCT TC	
IL-1β	F: GGC AGG TGG TAT CGC TCA TC	(Sugimoto et al.,2009)
	R: CAC CTT GGA TTT GAC TTC TA	
IL-5	F: AGA GAA GTG TGG CGA GGA GAG ACG	This study
	R: ACA GGG CAA TCC CTT CAT CGG	
IL-6	F: CAA AGC CAG AGC CAT TCA GAG	(Joosten et al.,2013)
	R: GCC ATT CCG TCT GTG ACT CCA GTT TCT CC	
IL-12p40	F: GAC ACG ACC TCC ACC AAA GT	(Joosten et al.,2013)
	R: CAT TCT GGG ACT GGA CCC TA	
IFN-γ	F: CCA TGA ACG CTA CAC ACT GCA TC	(Crabtree et al.,2004)
	R: GAA GTA GAA AGA GAC AAT CTG G	
B-actin	F: TCCTCCCTGGAGAAGAGCTA	(Sugimoto et al.,2011)
	R: CCAGACAGCACTGTGTTGGC	
HPRT	F: GCCCCAAAATGGTTAAGGTTGCA	This study
	R: TCAAGGGCATATCCAACAACAAAC	
GAPDH	F: AACGGGAAGCTCACTGGCATG	This study
	R: CTGCTTCACCACCTTCTTGATGTCA	
RPS18	F: CGAGTACTCAACACCAACATCGATGG	This study
	R: ATGTCTGCTTTCCTCAACACCACATG	

Histopathology

A section of 5 µm was sliced from the paraffin-embedded stomach tissue. This section was used for hematoxylin and eosin (HE) staining to score the intensity of infiltration of mononuclear and polymorphonuclear cells, as follows: 0 = no infiltration with mononuclear and/or polymorphonuclear cells; 1 = mild diffuse infiltration or presence of one small (50-200 cells) aggregate of inflammatory cells; 2 = moderate diffuse infiltration and/or the presence of 2-4 inflammatory aggregates; 3 = marked diffuse infiltration and/or the presence of at least 5 inflammatory aggregates; 4: diffuse infiltration of large regions with large aggregates of mononuclear and/or polymorphonuclear cells.

A second section was used for determining the influence of infection with different colony-derived *H. suis* strains on the epithelial proliferation rate, by staining this section as described previously (Flahou *et al*., 2010) using a mouse monoclonal anti-Ki67 antibody (1/25; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). Ki67-positive cells were counted in six randomly chosen High Power Fields (magnification: x400), both in fundus and antrum.

Statistical analysis

All data are expressed as mean ± standard deviation. The *in vitro* effect of *H. suis* lysates on AGS cells was analyzed using One-way ANOVA and a Bonferroni post hoc test. Gastritis scores in Mongolian gerbils and relative gene expression were analyzed using a non-parametric Mann-Whitney U test (SPSS 21, IBM, US) and a Bonferroni correction was applied to correct for multiple pair-wise comparisons. Results of Ki-67 counting were analyzed using one-way ANOVA with a Bonferroni post hoc test. P-values ≤ 0.05 were considered statistically significant.

Results

Purification and subsequent enrichment by biphasic subculture

For all parental *H. suis* strains except P13/37LP, single colonies were purified and subcultured with success.

MLST analysis

Results of MLST analysis of parental strains as well as single colony-derived, subcultured strains are shown in table 2. For 4 strains (HS2, HS3, HS5a and P13/35), changes in allele numbers were observed for the single colony-derived strain, when compared to the original parental strains.

The three biphasically cultured subpopulations HS5aLP, HS5bLP and HS5cLP, that have never undergone the purification and enrichment procedure described in this study, belonged to different sequence types (Table 2).

Table 2: Allele numbers assigned after multilocus sequence typing of parental and single colony-derived, enriched *H. suis* strains

	atpA	*efp*	*mutY*	*ppa*	*trpC*	*ureAB*	*yphC*
HS1LP	1	1	1	1	1	1	1
HS1kol	1	1	1	1	1	1	1
HS2LP	2	1	2	1	2	1	2
HS2kol	1	1	1	1	1	1	1
HS3LP	1	2	2	1	2	1	2
HS3kol	1	1	1	1	1	1	1
HS4LP	3	2	3	1	3	1	3
HS4kol	3	2	3	1	3	1	3
HS5aLP	4	2	4	1	1	1	1
HS5bLP	4	2	11	1	1	1	1
HS5cLP	1	2	11	1	1	1	1
HS5akol	4	2	5	1	2	1	1
HS6LP	1	3	6	1	1	1	3
HS6kol	1	3	6	1	1	1	3
HS7LP	1	3	7	1	1	1	3
HS7kol	1	3	7	1	1	1	3
HS8LP	1	4	8	1	1	1	1
HS8kol	1	4	8	1	1	1	1
HS9LP	4	2	9	1	1	1	1
HS9kol	4	2	9	1	1	1	1
HS10LP	1	4	3	2	1	1	3
HS10kol	1	4	3	2	1	1	3
P13/04LP	1	2	18	1	1	1	3
P13/04kol	1	2	18	1	1	1	3
P13/24LP	4	4	19	1	2	1	7
P13/24kol	4	4	19	1	2	1	7
P13/26LP	1	3	5	1	1	1	3
P13/26kol	1	3	5	1	1	1	3
P13/28LP	1	3	3	1	1	1	3
P13/28kol	1	3	3	1	1	1	3
P13/32LP	4	1	20	1	1	1	1
P13/32kol	4	1	20	1	1	1	1
P13/35LP	1	4	3	1	1	1	1
P13/35kol	1	4	7	1	1	1	1
P13/36LP	1	3	11	1	2	1	1
P13/36kol	1	3	11	1	2	1	1

Differences in allele numbers of colony-derived, subcultured strains compared to parental strains are indicated as a grey box

Effect of *H. suis* lysates on viability of AGS cells

The cell death-inducing capacity of whole bacterial cell lysate (100 µg/ml) from single colony-derived, subcultured strains HS1kol, HS8kol and HS10kol was higher than for parental strains HS1LP, HS8LP and HS10LP, which in fact did not cause a marked cell death under experimental conditions used in this study (Figure 1). Only for HS5a, a similar cell death-inducing capacity was observed for the single colony-derived and parental strain.

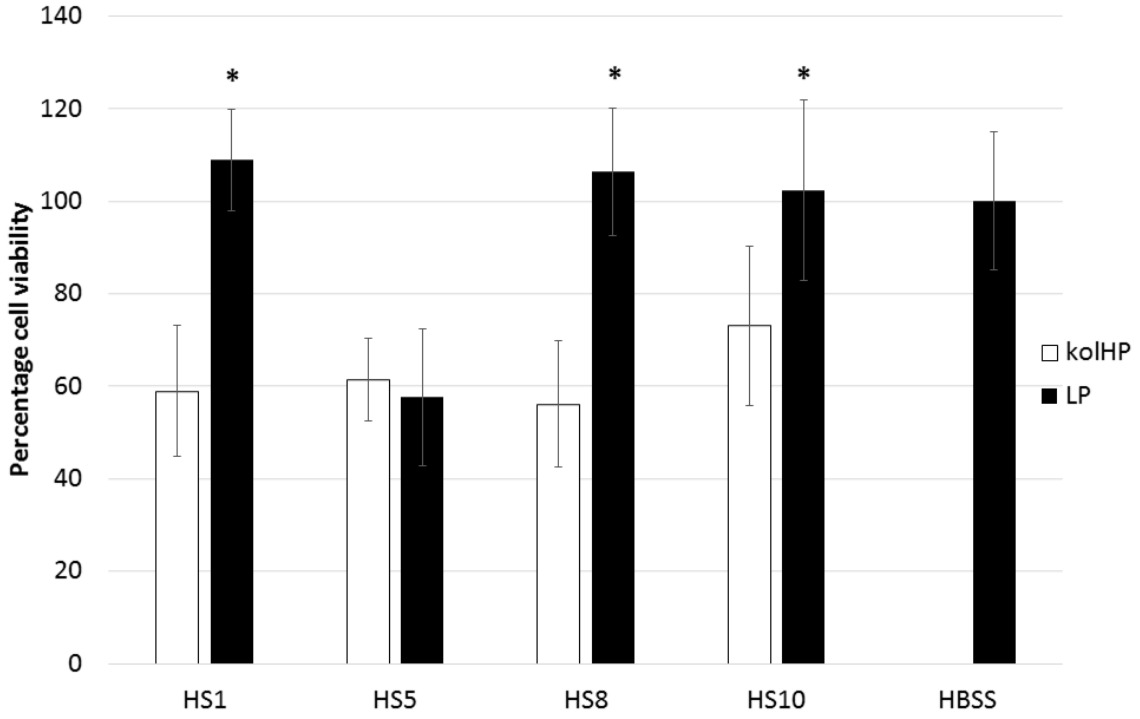

Figure 1. Cell death-inducing capacity of *H. suis* lysate. Shown is the percentage of viable cells compared to the number of viable cells in HBSS-treated control cultures. Cell cultures were treated with 100 µg/ml lysate of indicated *H. suis* strains for 24 hours. An * denotes a statistically significant difference between cell viability in cell cultures treated with lysate from corresponding parental and single colony-derived, subcultured strains. kolHP: single colony-derived, subcultured *H. suis* strains; LP: biphasically cultured parental *H. suis* strains with a history of limited culture *in vitro*.

Colonization capacity of single colony-derived *H. suis* strains

Surprisingly, *H. suis* strain HS5akol was shown not to be able to colonize the stomach of Mongolian gerbils. In contrast, strains HS1kol, HS6kol, HS8kol and HS10kol were able to persist in the stomach. All these strains colonized both the fundus and antrum, but the colonization density

was highest in antral samples (Figures 2A&B). No statistically significant differences were observed between colonization rates of different strains.

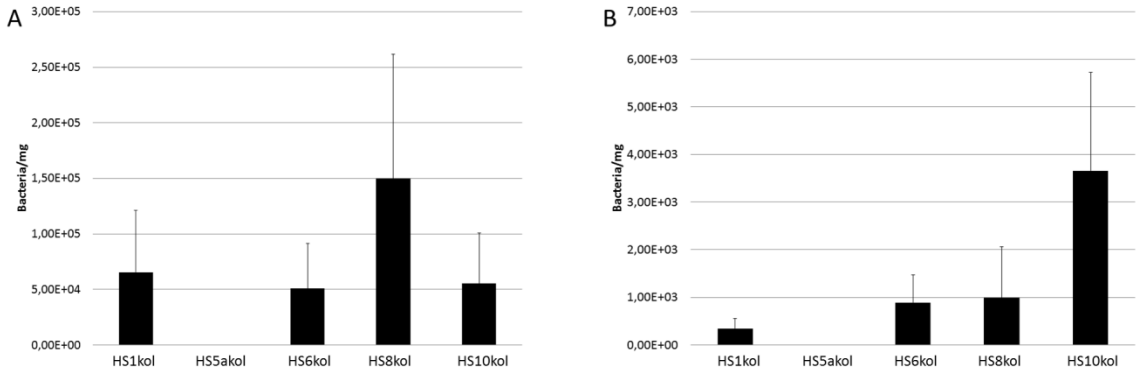

Figure 2. *Helicobacter suis* colonization rates. Shown are the average numbers of *H. suis* bacteria/milligram tissue in the antral (A) and fundic region (B) of the stomach of Mongolian gerbils after experimental infection with indicated single colony-derived, enriched *H. suis* strains.

Histopathology

The stomach of all control animals as well as animals inoculated with HS5akol showed a normal histomorphology (= score 0).

The most severe inflammation was observed in the antrum of animals inoculated with *H. suis* strain HS1kol (2.9±0.55) and *H. suis* strain HS10kol (2.9±0.42). In the antrum of gerbils infected with *H. suis* strain HS8kol, a significantly lower gastritis score (1.8±0.45) was seen compared to animals inoculated with strains HS1kol ($p=0.018$) and HS10kol ($p=0.007$). Gastritis scores in animals infected experimentally with *H. suis* strain HS6kol did not differ significantly from those in the other *H.* suis-infected groups. An overview of the average gastritis scores in the antrum is shown in figure 3 and representative images are depicted in figure 4.

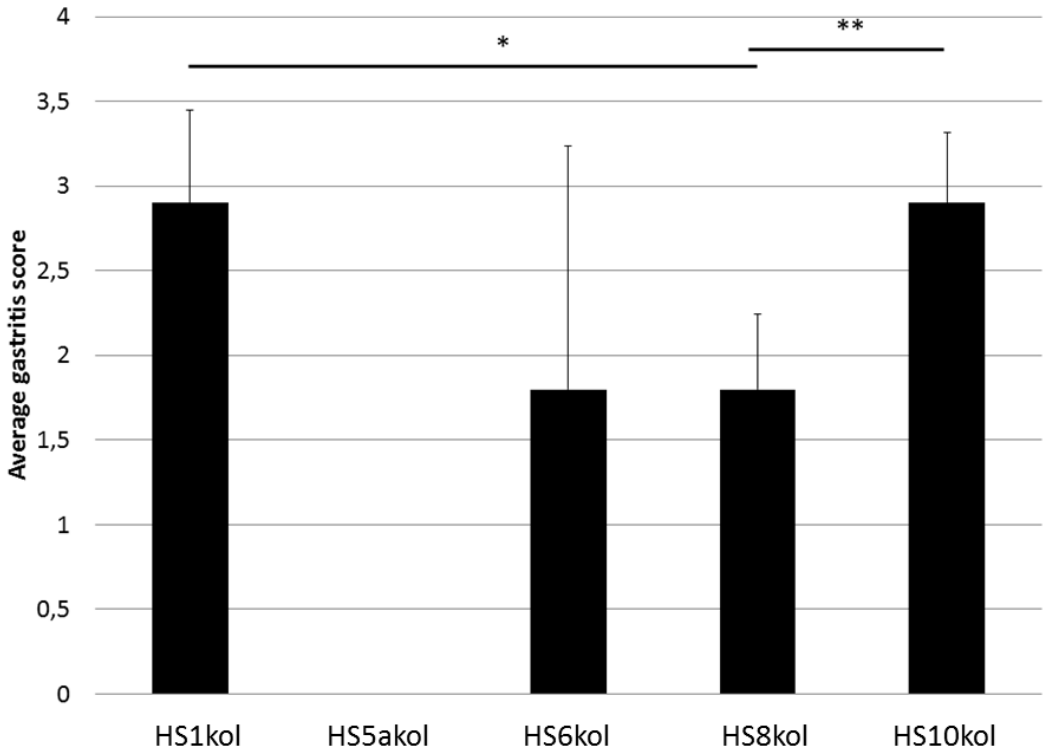

Figure 3. Inflammation in the antral mucosa. Shown are the average gastritis scores in the antrum of gerbils inoculated with single colony-derived, enriched *H. suis* strains. Relevant statistically significant differences between groups are indicated by * and **.

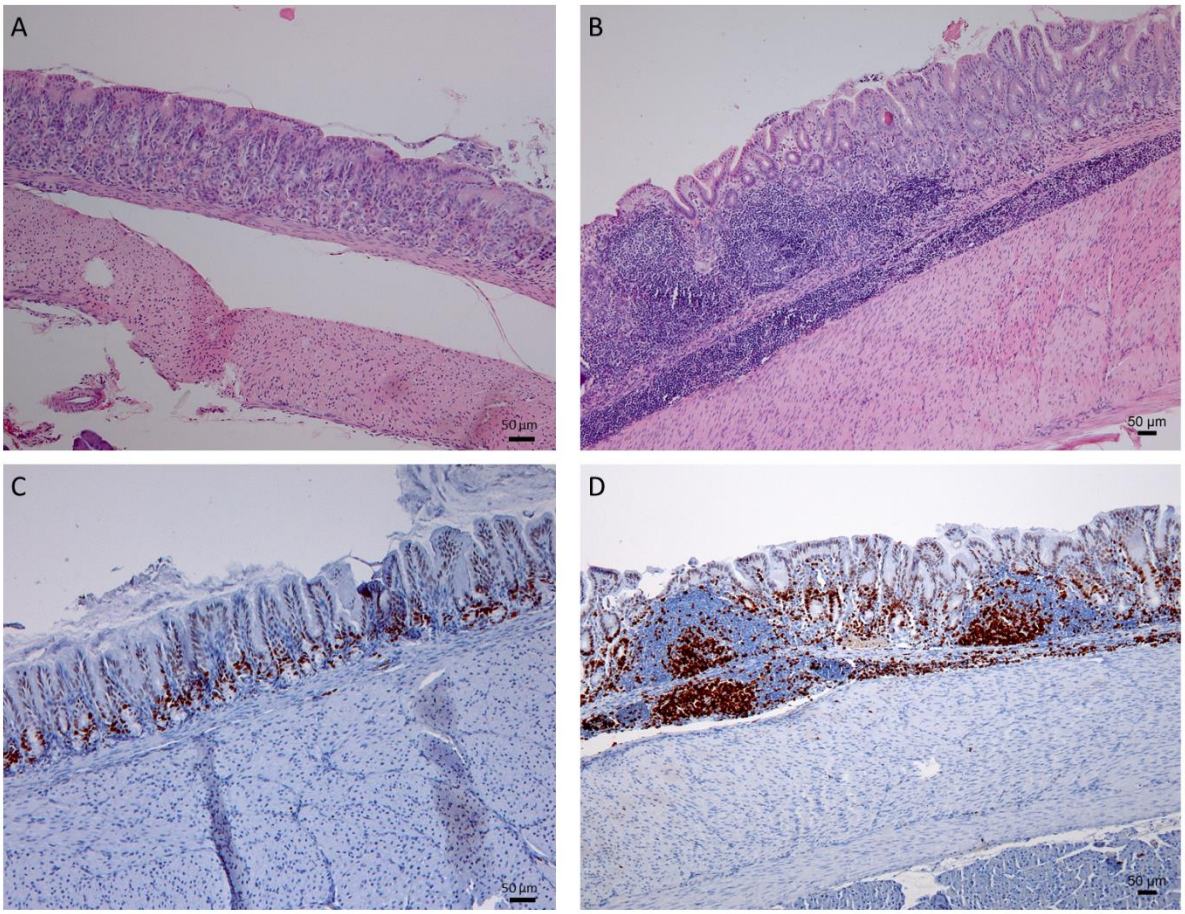

Figure 4. Inflammation evoked by experimental infection of gerbils with single colony-derived *H. suis* strains. H&E (A) and Ki-67 (C) staining of the antrum of a control gerbil; H&E (B) and Ki-67 (D) staining of the antrum of a gerbil infected with *H. suis* strain HS6kol for 9 weeks showing lymphocytic infiltration of the lamina propria, lamina muscularis mucosae and submucosa, proliferating germinal centres and an increased proliferation rate of the epithelium. Original magnification: 400x.

In general, only little inflammation was observed in the fundus of experimentally infected animals. In two animals inoculated with HS8kol and HS10kol, a mild infiltration of lymphocytes was observed in the transition zone between the forestomach and the glandular zone.

Compared to control animals, an increased proliferation rate of the epithelium was demonstrated in the antrum of animals infected with *H. suis* strains HS1kol, HS6kol and HS10kol (Figure 5). In the fundic region, no differences in expression of Ki-67 were observed.

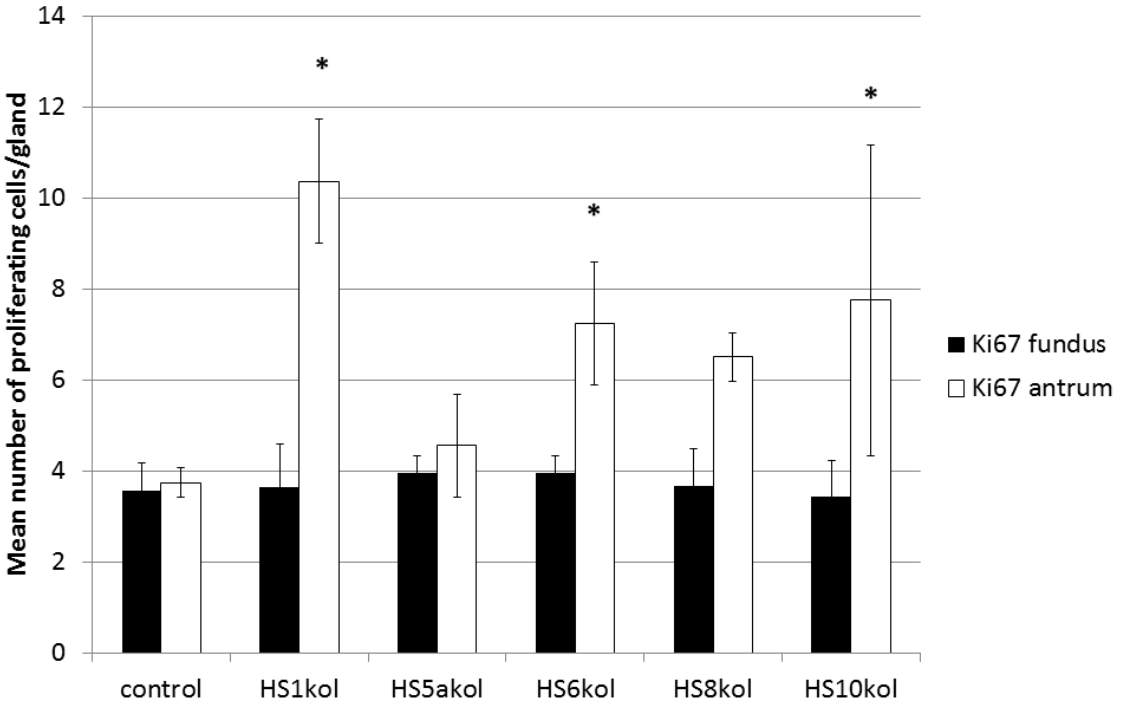

Figure 5. Proliferation rate of gastric epithelial cells. Shown are the mean numbers of Ki67-positive epithelial cells per gland in the antrum of Mongolian gerbils after experimental infection with indicated single colony-derived, enriched *H. suis* strains. An * shows statistically significant differences compared to control animals.

Cytokine-expression

Compared to uninfected controls, an increased expression of IL-1β, IL-10, IL-17, TNF-α and INF-ɣ was observed in the gastric mucosa of animals inoculated with *H. suis* strains HS1kol, HS6kol, HS8kol and HS10kol. No changes were observed in animals inoculated with *H. suis* strain HS5akol. Figure 6 presents the expression levels of these cytokines. No significant differences in expression were observed between uninfected controls and single colony-derived *H. suis*-infected animals for IL-5, IL-6 and IL-12p40.

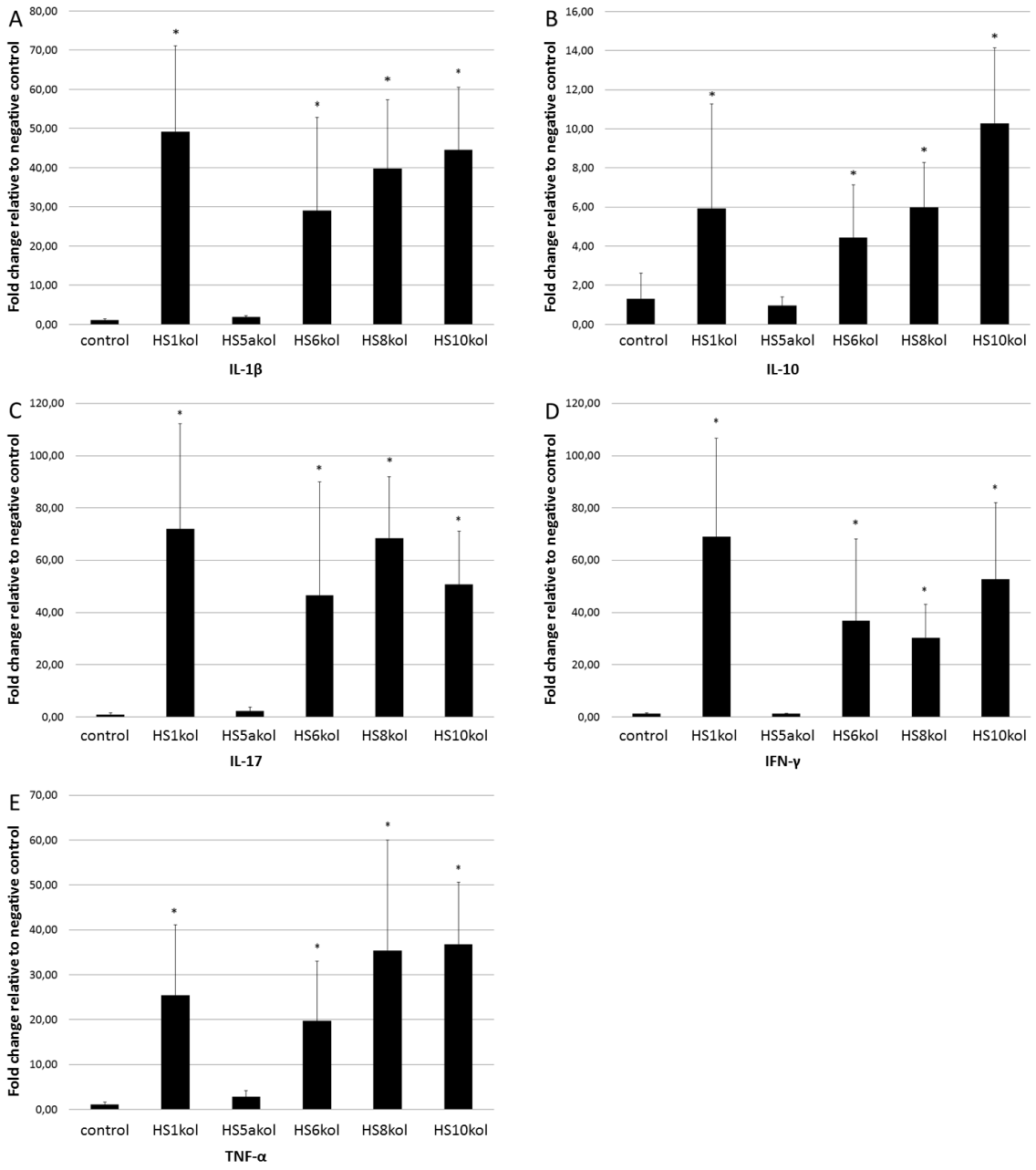

Figure 6. Cytokine expression analysis. Shown are mRNA expression levels of IL-1β (A), IL-10 (B), IL-17 (C), INF-ɣ (D) and TNF-α (E) in the antrum of the stomach of Mongolian gerbils after experimental infection with indicated single colony-derived, enriched *H. suis* strains. Significant differences between inoculated groups and the negative control group are indicated by an * ($p < 0.05$).

Discussion

In previous studies, isolation and culture of *H. suis* was always performed under biphasic culture conditions starting from mucus and mucosal scrapings of a large surface area of pig stomachs obtained at the slaughterhouse (Baele et al., 2008). For *H. pylori*, it has been shown that individuals can be infected with multiple related or unrelated *H. pylori* strains, resulting in frequent recombination (Falush *et al*., 2001; Patra *et al*., 2012). In a few cases, we have observed the presence of double nucleotide peaks in electropherograms after sequencing of MLST genes of *H. suis* bacteria present in a single porcine gastric biopsy (non-published results). In addition, we have determined the sequence type for *H. suis* strains HS5aLP, HS5bLP and HS5cLP, which are subpopulations obtained by separate biphasic subculture early after the initial isolation of *H. suis* strain HS5. All three biphasically cultured subpopulations revealed a different sequence type. These data strongly suggest that, similar to *H. pylori* in humans, multiple *H. suis* strains may be present in the same individual animal as well as in initial biphasic *H. suis* cultures obtained from a single animal. The possible presence of multiple strains in some initial biphasic cultures is also supported by the fact that occasionally, growth of *H. suis* bacteria on two identically inoculated plates starting from the same mother plate differs markedly. So in any case, there is an obvious need for obtaining pure cultures of *H. suis* strains, for instance when studying strain differences in bacterium-host interactions and for isolation of mutants deleted in putative virulence factors.

In the present study, we describe a technique to grow *H. suis* as colonies on dry agar, which can then be used for purification of single colonies and subsequent subculture/enrichment. Initial attempts failed, but by using agar concentrations reduced to 1% and by increasing the time of the initial dry culture step, we succeeded in obtaining colonies for 16 out of 17 biphasic isolates available, in general after 7-14 days of culture. When subculturing single picked colonies on new dry 1% agar plates, most strains lost their capacity to grow in dry culture after only a few passages. Therefore, when growth was visible after the first dry subculture from a single picked colony, bacteria were harvested and transferred to biphasic culture conditions, which in general enabled us to increase bacterial numbers after repeated biphasic subculture, sufficient to create a mother stock of approximately $5 \times 10^9 - 1 \times 10^{10}$ bacteria per colony-derived strain. In general, however, this process took 25-50 days, depending on the strain.

For the majority of the colony-derived and subcultured strains, MLST analysis revealed a sequence type identical to that of the parental strain. However, changes of allele numbers were observed for

the colony-derived strains in 4 cases, suggesting that mutations may have occurred in housekeeping genes, as has been previously described for *H. pylori* and other gastric helicobacters, both *in vitro* and *in vivo* (Kondadi *et al*., 2013; Linz *et al*., 2014). Although in none of these 4 cases sequencing of MLST genes of the low passage parental strains revealed the presence of double nucleotide peaks in electropherograms, it can not be excluded that in some original biphasic parental cultures more than one *H. suis* strain was present, with different capacities to grow on dry agar plates. It can be hypothesized that if in some original biphasic cultures from the gastric mucosa of pigs, multiple strains with different capacities to grow *in vitro* were present, during subculture a strain better adapted to dry agar plate conditions will outgrow a strain with a lower capacity to grow in these conditions. If in the stomach and original biphasic culture the strain with the higher capacity to grow on a dry agar plate was present in only low numbers, it would not have been detected in the MLST assay. Purification on dry agar plates of tenfold dilutions of original biphasic cultures of the strains with changes in allele numbers may help to shed light on this matter.

The capacity to induce gastric epithelial cell death *in vitro* was shown to be lower for whole bacterial cell lysate of parental strains HS1LP, HS8LP and HS10LP, compared to lysate of their corresponding single colony-derived subcultured strains. Since *H. suis* bacteria die within hours under culture conditions required for eukaryotic cell culture, whole bacterial cell lysates had to be used. Changes in virulence of *in vitro* cultured strains or *in vivo* passaged strains have already been described for several bacterial species, including *Mycobacterium bovis*, *Helicobacter pylori* and *Escherichia coli* (Ram *et al*., 1992; Crabtree *et al*., 2002; Domenech *et al*., 2009). Schott and colleagues (2011) described that regulatory mechanisms such as slipped-strand mispairing may respond to different environmental conditions, resulting in phase variation. In addition, it has been described for *H. pylori* that high rates of mutation, for instance in outer membrane protein genes or virulence-related genes, occur under specific environmental conditions (Linz *et al*., 2014; Farnbacher *et al*., 2010). In the latter 2 studies, colonization in and adaptation to the gerbil stomach and acute human infection were the specific changes in environmental conditions. It remains to be determined if long term culture *in vitro*, under suboptimal conditions such as culture on dry agar plates, may also result in higher mutation rates. It has indeed been described that bacteria exposed to sub lethal stress factors show increased mutation rates (Couce *et al*., 2009) which might generate heterogeneous populations with clones able to adapt and to survive adverse conditions. For *H. pylori*, it has been reported that stressful conditions resulting in DNA damage favour genetic exchange by transformation also leading to genetic diversification (Dorer *et al*., 2010). The *H. suis*

genome contains homologs to *H. pylori* genes involved in the Com type IV secretion system which plays a role in genetic transformation (Vermoote *et al*., 2011).

Unexpectedly, strain HS5akol was shown not to be able to persistently colonize the stomach of Mongolian gerbils, which contrasts to the results of previous studies showing that *H. suis* easily colonizes the stomach of mice and Mongolian gerbils, at colonization densities exceeding those described for *H. pylori* (Flahou *et al*., 2010, 2012; O'Rourke *et al*., 2004). In order to confirm this, inoculation of Mongolian gerbils with strain HS5akol was repeated in an independent experiment. Again, this strain failed to colonize the stomach of these animals (results not shown). Possibly, long-term, suboptimal *in vitro* culture may have led to an altered expression of factors essential for colonization. For several pathogens, loss of virulence due to altered expression of virulence factors has indeed been described after *in vitro* culture (Ram *et al*., 1992; Somerville *et al*., 2002). Alternatively, multiple *H. suis* strains may have been present in the biphasic HS5 inoculum used in previous studies. In any case, the present study is the first to report the failure of an *H. suis* strain to colonize in a rodent model widely used for *Helicobacter* research. However, the loss of the capacity of single colony-derived strains of *H. suis* to colonize the stomach of Mongolian gerbils seems to be an exception rather than the rule. For the other colony-derived strains used to inoculate Mongolian gerbils, only mild differences in virulence were observed and differences were often not statistically significant. The colonization capacity, inflammation scores, gastric histopathological changes and levels of epithelial hyperproliferation, were indeed very similar to what has been described when using biphasically cultured strains (HS5aLP and HS5cLP, respectively) with a history of limited culture *in vitro* (Flahou *et al*., 2010; Zhang *et al*., 2015). In general, an upregulated expression of pro-inflammatory IFN-γ, IL-17 and IL-1β as well as anti-inflammatory/regulatory IL-10 was observed in all animals infected with colony-derived *H. suis* strains HS1kol, HS6kol, HS8kol and HS10kol, which also corresponds to what has been observed in Mongolian gerbils infected with low-passaged parental non-colony-derived *H. suis* strain HS5cLP, grown as a biphasic culture (Zhang *et al*., 2015). This indicates that, for most strains, the purification and subculture/enrichment procedure used to obtain pure single colony-derived strains has little or no impact on the outcome of an experimental *H. suis* infection.

In conclusion, we developed a protocol for establishing pure *in vitro* cultures of *H. suis*, by growing the bacteria as colonies on dry agar plates, followed by enrichment through long-term biphasic subculture. This procedure was shown to require a non-stop culture of several weeks, which may possibly influence strain characteristics. For 4 out of 17 *H. suis* strains, genotyping indeed revealed

alterations of the sequence type when comparing parental and colony-derived, subcultured strains. Although the cell-death inducing capacity of the latter was shown to be increased, the histopathological changes observed upon *H. suis* infection in a Mongolian gerbil model were similar to what has been described when using parental strains with a history of limited culture *in vitro*. Nevertheless and surprisingly, colony-derived strain HS5akol was shown to be no longer able to colonize the Mongolian gerbil stomach, in contrast to the parental strain, suggesting that the developed purification protocol can affect the bacterial phenotype. Caution is therefore advisable when comparing results from experiments using these different types of *H. suis* cultures.

Acknowledgements

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CHAPTER 3

Multilocus Sequence Typing of *Helicobacter heilmannii* sensu stricto and the putative new species "*Helicobacter ailurogastricus*"

Jungang Liang, Bram Flahou, Annemieke Smet, Richard Ducatelle, Frank Pasmans, Freddy Haesebrouck

Department of Pathology, Bacteriology and Avian Disease, Faculty of Veterinary Medicine. Ghent University, Merelbeke, Belgium

Abstract

Nine spiral-shaped *Helicobacter* strains were recently isolated in our laboratory from feline gastric tissue. Strains were designated as ASB1, ASB2, ASB3, ASB6, ASB7, ASB9, ASB11, ASB13, and ASB14. Unpublished genomes of strains ASB1 and ASB7 showed that, in contrast to what was previously described, these strains most likely belong to 2 different species. Subsequent investigation has revealed that ASB1, ASB2, ASB3, ASB6 and ASB14 should be referred to as *Helicobacter heilmannii* sensu stricto, whereas ASB7, ASB9, ASB11 and ASB13 most likely belong to a novel *Helicobacter* species, for which the name "*H. ailurogastricus"* has been proposed. Eight complete housekeeping genes (*atpA, efp, ppa, mutY, trpC, ureI, ureAB, yphC)* of 5 *H. heilmannii* s.s and 4 "*H. ailurogastricus"* strains were amplified by PCR and sequenced. For *H. heilmannii* s.s., a total number of 9,131 bp represented the complete coding sequences of these 8 housekeeping genes. Six hundred and fifteen nucleotide sites (615/9,131; 6.7%) were found to be polymorphic and the *atpA* gene was shown the most powerful for discrimination of the strains. For "*H. ailurogastricus"*, a total of 9,314 bp represented the complete coding sequences of the same 8 housekeeping genes. Eighty six nucleotide sites (86/9,314; 0.92%) were found to be polymorphic and the *atpA* gene was shown to be the most powerful for discrimination. The *ppa* gene was shown to lack discriminatory power (0 variable nucleotide sites). The MLST schemes described here are useful to obtain insight into the epidemiology and population structure of the bacteria, both in humans and pet animals.

Introduction

Besides *H. pylori*, several gastric non-*H. pylori Helicobacter* (NHPH) species have been described to cause gastric disease in humans. The nomenclature of gastric NHPH, including *H. heilmannii* s.s., has always been confusing. In studies on human NHPH infection, the term '*H. heilmannii'* infection has often been used. Subsequent research has, however, revealed that several bacterial species can be involved in these *'H. heilmannii'*-infected humans, including *H. suis*, naturally colonizing the stomach of pigs worldwide, and several dog- and cat-associated gastric *Helicobacter* species, including *H. felis, H. bizzozeronii, H. salomonis* and the true *H. heilmannii*: *H. heilmannii* s.s. (Hanninen *et al.*, 1996; Jalava *et al.*, 1997; Baele *et al.*, 2008b; Haesebrouck *et al.*,2011; Smet *et al.*, 2012).

Helicobacter heilmannii sensu stricto (s.s) is a Gram-negative, long, spiral-shaped bacterium which naturally colonizes the stomach of dogs and cats. Infection by this gastric pathogen is also of zoonotic importance. Like all other gastric non-*H. pylori Helicobacter* species from animal origin, human infection with *H. heilmannii* s.s. is associated with the development of gastritis, peptic ulceration and mucosa associated lymphoid tissue (MALT) lymphoma (Haesebrouck *et al.*, 2009; Trebesius *et al.*, 2001; Van den Bulck *et al.*, 2005; Baele *et al.*, 2008a; O'Rourke *et al.*, 2004a). Although many attempts have been made in the past, the first succesful isolation of this bacterium was done only in 2012, from the stomach of cats (Smet *et al.*, 2012) .

Due to the confusion in nomenclature, the exact prevalence of *Helicobacter heilmannii* s.s. infection in cats and large felines remains to be further investigated, but it seems that infection with this bacterium is rather common in cats (O'Rourke *et al.*, 2004b; Van den Bulck *et al.*, 2005; Smet *et al.*, 2013). It was shown to vary from 20 ~100% in cats and dogs depending on the geography and authors (Haesebrouck *et al.*, 2009; O'Rourke *et al.*, 2004b; Neiger *et al.*, 1998; Van den Bulck *et al.*, 2005; Hwang *et al.*, 2002). In humans, *H. heilmannii* s.s. has been described in 8~19% of human gastric biopsies with histological evidence of NHPH infection (Haesebrouck *et al.*, 2009; Trebesius *et al.*, 2001; Van den Bulck *et al.*, 2005; Joosten *et al.*, 2013).

Since the first description of *H. heilmannii* s.s. in 2012, nine putative *H. heilmannii* strains (ASB1, ASB2, ASB3, ASB6, ASB7, ASB9, ASB11, ASB13 and ASB14) have been isolated from feline gastric tissues, as detemined by sequencing of the *ureAB* gene cluster (unpublished results). Unpublished genomes of strains ASB1 and ASB7, however, showed that, in contrast to what was previously described (Smet *et al.*, 2013), these strains most likely belong to 2 different species.

Based on data from *in vivo* experiments in Mongolian gerbils, we have provisionally divided these strains into 2 groups: *H. heilmannii* s.s. strains (ASB1, ASB2, ASB3, ASB6, ASB14) and strains most likely belonging to a new species, for which the name "*H. ailurogastricus"* has been proposed (ASB7, ASB9, ASB11, ASB13) (Joosten *et al.*, 2014).

Since no information is available on the epidemiology of infection with these 2 *Helicobacter* species and in order to confirm that the ASB isolates indeed belong to 1 of the 2 species mentioned above, the primary aim of this study was to develop an unambiguous and robust discriminatory typing scheme for *H. heilmannii* sensu stricto and "*H. ailurogastricus"*, which can subsequently be applied to biological tissue without the need for cultivation. This will allow us to gain insight into the epidemiology and population structure of these bacteria.

Materials and methods

Bacterial strains and DNA extraction

Nine *Helicobacter* strains were isolated from the gastric mucosa of cats. Strains ASB1, ASB6, ASB7, ASB9, ASB11, ASB13 and ASB14 were obtained from animals housed in a shelter for homeless cats in Flanders, Belgium. All animals were euthanized for reasons unrelated to this project. Strains ASB2 and ASB3 were isolated from animals captured in the framework of a sterilization project for stray cats, but euthanized because they suffered from sever viral disease. cultured *in vitro* on biphasic Brucella agar plates (Oxoid, Basingstoke, UK), with a pH adjusted to 5 and supplemented with 20% (v/v) fetal calf serum (HyClone, Logan, UT, USA) and Vitox supplement (Oxoid) as described previously (Smet *et al.*, 2012; Joosten *et al.*, 2013). These bacteria were grown microaerobically at 37°C in an atmosphere containing 85% N_2, 10% CO_2 and 5% O_2. Genomic DNA of these strains was extracted as described by Wilson (Wilson K, 1994) and used for the development of MLST.

Development of multilocus sequence typing for *Helicobacter heilmannii* senso stricto (s.s.)

Based on their use in MLST schemes for other gastric helicobacters and the available whole genomic DNA sequence of strain ASB1, eight housekeeping genes (*atpA, efp, mutY, ppa, trpC, ureAB, ureI, yphC*) were selected and the primers were designed for amplification and sequencing (Table 1). The complete coding region of each gene (or near-complete for the *ppa* of *H. heilmannii*)

was amplified by PCR assay using a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). Briefly, PCR assays were started with an initial 3-min denaturation step at 95°C, followed by 30 cycles of 94°C for 60 s, 56°C~60°C for 60 s, 72°C for 60 s and a final extension at 72°C for 5 min. All PCR products and the primers needed for sequencing were sent to GATC Biotech AG (Headquarter, European Genome and Diagnostics Centre, Germany) for sanger sequencing (Table 1). The obtained sequences were aligned and curated using Kodon software (Applied Maths, Sint-Martens-Latem, Belgium). After multiple sequence alignment, using Kodon software, variable nucleotide sites were investigated and recorded manually for all genes.

Development of multilocus sequence typing for "*Helicobacter ailurogastricus*"

Eight housekeeping genes (*atpA, efp, mutY, ppa, trpC, ureAB, ureI, yphC*) were selected and all the primers used for PCR assays and nucleotide sequencing were designed based on the whole genomic DNA sequence of ASB7 (Table 2). The protocols used for PCR are similar to those described above for *H. heilmannii* s.s. and annealing temperatures were chosen based on the theoretical melting temperatures of the primers (Table 2). PCR products and the primers for nucleotide sequencing were sent to GATC Biotech AG. The obtained sequences were aligned and curated using Kodon software (Applied Maths, Sint-Martens-Latem, Belgium). After multiple sequence alignment, using Kodon software, variable nucleotide sites were investigated and recorded manually for all genes.

Analysis of clonality

ClonalFrame software version 1.1 (Didelot and Falush, 2007) was used for preliminary analysis of the clonal relationship between and evolution of strains, which could provide a first idea of the population structure. The sequences of all 8 housekeeping genes were first trimmed and aligned using MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/) for strains ASB1, ASB2, ASB3, ASB6, ASB14, ASB7, ASB9, ASB11 and ASB13, as well as 10 *in vitro* isolated strains (HS1-HS10) from a closely related, but distinct bacterial species: *Helicobacter suis*. The output from 3 independently run MCMC chains (100 000 iterations) was compared using the tree comparison tool embedded in the ClonalFrame software. Recombination/mutation rates were visualized for the different nodes of the consensus tree.

Table 1. Oligonucleotide primers used for amplification and sequencing of complete coding regions of 8 *Helicobacter heilmannii* sensu stricto housekeeping genes

primer	sequence (5'--3')	polarity	amplicon size(bp)	position	annealing temperature	Use of primers[a]
ATPAHHF1	ATGCTAAAACTCAAAGCCGAAGAAATCAGCG	Forward	1512	1 - 31	62°C	A,S
ATPAHHR1	CTAGAGTGAAAAGCCTAGCTTAAATTCTTCAATGGC	Reverse		1477-1512		A, S
ATPAHHF2	CCTATTCGCCCCTACCCATTG	Forward		1274-1294		S
ATPAHHR2	AAGTCCGCGATCGCCCGTGTC	Reverse		169-189		S
EFPHHF1	ATGGCCATTGGCATGGGCGAG	Forward	564	1-21	64°C	A, S
EFPHHR1	TTATTTCACCTTTTCTAGGTATTCACC	Reverse		530 -564		A,S
MUTYHHF1	ATGTCCCAACAAACCCAAATCGG	Forward	888	1-23	56°C	A,S
MUTYHHR1	TTACAGGGTATTTTGGTGATCAAGAG	Reverse		836-888		A, S
MUTYHHF2	AAATCCACCAAGCCCTTTTGG	Forward		740-760		S
MUTYHHR2	TAGCCTAACCCCTGCCATAGC	Reverse		114-134		S
PPAHHF3	GCCGTGATTGAAATCCCCTACC	Forward	483	46-67	59°C	A,S
PPAHHR1	TTAACATGCTTTGCTGTTGTAGTTGGC	Reverse		502-528		A,S
PPAHHR2	ACGACCTACCCCCCATTGTGCTC	Reverse		365-387		S
TRPCHHF1	TTGCTAAAAACCATGGTAGAACAC	Forward	1347	1 - 24	56°C	A,S
TRPCHHR1	TCAATACTCCCGTAACATTTTCGC	Reverse		1324-1347		A,S
TRPCHHF2	TAGAAAAGGCAGCCGCCATAGG	Forward		1226-1247		S
TRPCHHR2	TAGAGGGGGAGGCCTGCTTGC	Reverse		143-164		S
TRPCHHF3	AATTAGCCACCCTTGCGCAGTC	Forward		425-446		S
UREABHHF1	ATGAAGCTTACCCCTAAAGAGC	Forward	2429	1-22	65°C	A,S
UREABHHR1	CTAGAATAGGTTGTAGAGTTGTGCC	Reverse		2404-2429		A,S
UREABHHF2	CATCAAAGGCGGTTTCATCGCGC	Forward		2066-2088		S
UREABHHR2	GCCTTCTTGCATCAAATCTGCCACGC	Reverse		158-183		S
UREABHHF3	AGTGGATCGACAAGCCGATCACG	Forward		606-628		S
UREABHHF4	AGATGGCACCAACGCCACCACCATC	Forward		1211 - 1235		S
UREIHHF1	ATGTTAGGACTTGTGTTATTGTATGTTGCG	Forward	588	1-30	58°C	A,S
UREIHHR1	TTATGCCCAGTGTTGGATAAAGAGC	Reverse		564-588		A,S
UREIHHF2	CTGGCTGGATTGAAGAAGTGG	Forward		464-485		S
UREIHHR2	ATAGGGGTGGCGTGGAAAGTG	Reverse		153-173		S
YPHCHHF1	GTGGTGCTTAAAATCGC	Forward	1320	1-17	62°C	A,S
YPHCHHR1	TCATGGATTGATTGCGTCTTCCTTGCC	Reverse		1294-1320		A,S
YPHCHHF2	ACACCCCTTACCTAGTGATCATGG	Forwrd		1101-1124		S
YPHCHHR2	AGATATGGGTGGGGTCAAAGC	Reverse		176-196		S
YPHCHHF3	GTCTCTGCAAGCCATAATCGGGGAC	Forward		424 - 448		S

[a] A, primers used for amplification; S: primers used for sequencing

Table2. Oligonucleotide primers used for amplification and sequencing of complete coding reegions of 8 housekeeping genes from "*Helicobacter ailurogastricus"*

primer	sequence (5'--3')	polarity	amplicon size(bp)	position	annealing temperature	Use of primers[a]
atpA-asb7-F1	TTGTTAAAATTAAAAGCCGAAG	Forward	1512	1 - 22	50°C	A,S
atpA-asb7-R1	CTAGAGAGAAAAGCCTAG	Reverse		1495-1512		A, S
atpA-asb7-F2	CCCCCTATTCGCCCCTACC	Forward		1271-1289		S
atpA-asb7-R2	GTCTTTGCCCCCACCCAGC	Reverse		228-246		S
atpA-asb7-F3	CCG GTA AAA CGA CCG	Forward		521-535		S
efp-asb7-F1	ATGGCCATTGGCATGGGCG	Forward	567	1 - 19	64°C	A,S
efp –asb7-R1	CTAAGCCTTCACTTTTTCTAAATACTCTCCCG	Reverse		536 - 567		A,S
efp-asb7-F2	TCGCCCAAGTGGTGGCGC	Forward		377-394		S
efp-asb7-R2	CTTCGCACTTGTCGCCC	Reverse		171-187		S
mutY-asb7 -F1	ATGTTTAAACCCCTACACAATGCCC	Forward	1017	1 - 25	60°C	A,S
mutY-asb7-R1	CTACGAATCTAGACTTTGATTTGCCCC	Reverse		991- 1017		A,S
mutY-asb7-F2	CAGCTACACCAAATACCGCC	Forward		831-850		S
mutY-asb7-R2	GCCCAGTCCCTGCCAC	Reverse		237-252		S
ppa-asb7-F1	ATGGACTTGTCAAAAATTAAAGCGGGCGAGGC	Forward	528	1 - 32	66°C	A,S
ppa –asb7-R1	TTAACATGCCTTGCTGTTGTAGCTGGC	Reverse		502- 528		A,S
ppa –asb7-F2	GATCTGCACGACTTGCCC	Forward		358-375		S
ppa –asb7-R2	TCGACCACAACCGCCCCG	Reverse		102-119		S
trpC-asb7-F1	ATGCATGACTTGCTAAAAACCATG	Forward	1356	1-24	62°C	A,S
trpC-asb7-R1	TCAATACTCCCGTAACATTTTCGCCAC	Reverse		1330-1356		A,S
trpC-asb7-F2	AAGCGGTTGGCACGGGGG	Forward		1241-1258		S
trpC-asb7-R2	TCAAACCCTTAGAGGGGG	Reverse		164-181		S
trpC-asb7-F3	TGTGCCACAAAGTGCAGG	Forward		668-685		S
ureI –asb7-F1	ATGTTAGGACTCGTGTTATTGTATGTTGCG	Forward	588	1 - 30	66°C	A,S
ureI –asb7-R1	TTACGCCCAGTGTTGGATGAAAAGAAGCC	Reverse		560 - 588		A,S
ureI –asb7-F2	TGAGGGCGACTGGTGGGC	Forward		394-428		S
ureI –asb7-R2	CACACAATGGAGAGCGCG	Reverse		111-128		S
ureAB-asb7-F1	ATGAAGCTTACCCCTAAAGAGCTAG	Forward	2429	1 -25	58°C	A,S
ureAB-asb7-R1	CTAGAATAGGTTATAGAGTTGTGCC	Reverse		2405-2429		A,S
ureAB-asb7-F2	TTTGGCCACCACGGCAAAGCC	Forward		2154-2173		S
ureAB-asb7-R2	ATGTGCGCCACGCCAGGC	Reverse		213-230		S
ureAB-asb7-F3	TTTGATCGACATTGGCGGC	Forwrd		558-576		S
ureAB-asb7-F4	ATCCACGAAGACTGGGGCAGC	Forward		1377-1397		S
yphC- asb7-F1	ATGCTTAAAATCGTCATTTTAGGCATGCC	Forward	1317	1 - 29	62°C	A,S
yphC- asb7-R1	TCATGGATTAAGCGAGTCTTCTTTGCCC	Reverse		1290-1317		A,S
yphC- asb7-F2	GACCATGGCAAGATTGTGCG	Forward		1114-1133		S
yphC- asb7-R2	GCAGGCTTCAACCGCTCC	Reverse		223-240		S
yphC- asb7-F4	GCACAAGAACGCTCTTTGGTC	Forward		592-612		S

[a] A, primers used for amplification; S, primers used for sequencing

Results

Eight (near-) complete housekeeping genes were amplified by PCR assay and sequenced from 5 *H. heilmannii* s.s and 4 "*H. ailurogastricus"* strains, respectively. For *H. heilmannii* s.s., a total number of 9,131 bp was sequenced for each strain. Six hundred and fifteen nucleotide sites (615/9,131; 6.7%) were found to be polymorphic and the *atpA* gene was shown to be the most powerful gene for discrimination of the strains. For "*H. ailurogastricus"*, a total of 9,314 bp were sequenced for each strain, and eighty six nucleotide sites (86/9,314; 0.92%) were found to be polymorphic. As for *H. heilmannii* s.s., the *atpA* gene was shown to be the most powerful for discrimination and the *ppa* gene was shown to lack discriminatory power (0 variable nucleotide sites). Data are summarized in Table 3.

Table 3. Variable nucleotide sites of eight housekeeping genes of *H. heilmannii* s. s. as well as "*H. ailurogastricus"*

	atpA	*efp*	*ppa*	*mutY*	*trpC*	*ureI*	*ureAB*	*yphC*
H.heilmannii s.s.	229 (1512bp)	19 (564bp)	14 (483bp)	44 (888bp)	67 (1347bp)	49 (588bp)	188 (2429bp)	5 (1320bp)
"H.ailurogastricus"	28 (1512bp)	9 (567bp)	0 (528bp)	2 (1017bp)	12 (1356bp)	1 (588bp)	25 (2429bp)	9 (1317bp)

When analyzing the consensus tree, generated using ClonalFrame 1.1 software, for all ASB isolates and some *H. suis* strains, a clear distinction could be made between the 3 different *Helicobacter* species (Figure 1). Analysis of the recombination/mutation rates at the nodes representing the most recent common ancestor of ASB strains, revealed a greater diversification for *H. heilmannii* s.s. and "*H. ailurogastricus"*, compared to *H. suis.* Especially for *H. heilmannii* s.s., recombination events, as opposed to mutation, seemed to contribute largely to the diversification (Figure 2).

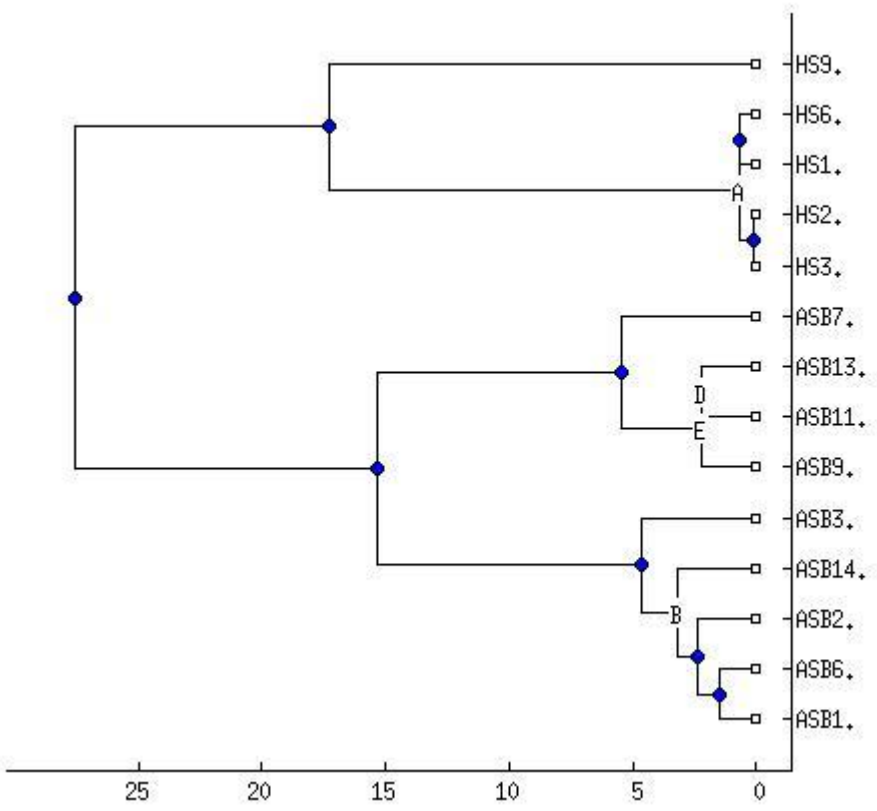

Figure 1 ClonalFrame consensus tree. This tree was generated using ClonalFrame 1.1 software. A clear distinction can be made between the *H. suis* (HS1-HS9), *H. heilmannii* s.s. (ASB1, ASB2, ASB3, ASB6, ASB14) and "*H. ailurogastricus"* (ASB7, ASB9, ASB11, ASB13).

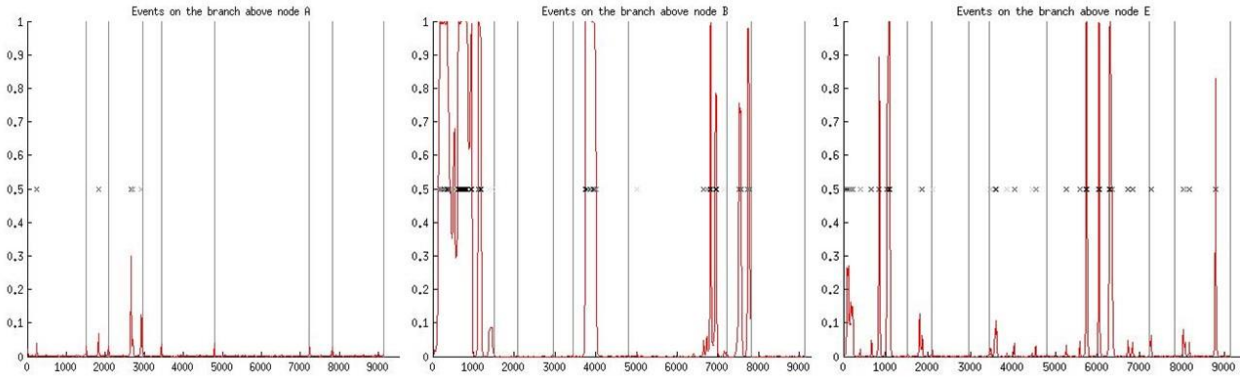

Figure 2 Analysis of recombination/mutation rates. Shown are the estimates of recombination and mutation at nodes A, B and E from the tree depicted in figure 1**.** All eight blocks (representing the 8 housekeeping genes included in this study) are separated by vertical lines. Each inferred substitution is indicated by a cross. The red line indicates, at each locus, the probablilty of an import (recombination) on a scale from 0 (bottom of the axis) to 1 (top of the axis). This indicates that mainly for *H. heilmannii s.s.* (node B), recombination is involved in the diversification.

Discussion

Similar to the high prevalence of *H. pylori* infection in human beings and *H.suis* infection in pigs (Haesebrouck *et al*., 2009), the prevalence of *Helicobacter heilmannii* sensu stricto in cats (and dogs) is also relatively high, although more research is needed to determine the exact prevalence rates of *H. heilmannii* sensu stricto, as well as the closely related, putative new species "*H. ailurogastricus"*, in animals and humans (Joosten *et al*., 2013; Joosten et al., 2014). In any case, no information is available on the strain diversity and transmission of both cat-associated gastric *Helicobacter* species between animals and from animals to humans. To provide answers to some of these questions, there is a clear need for a robust strain typing method for these newly isolated and characterized feline *Helicobacter* species.

One of the main characteristics of all gastric NHPH bacteria is their fastidious nature, which makes it difficult to obtain *in vitro* isolated strains (Baele *et al*., 2008b). Since multilocus sequence typing can be applied to biological tissue without the need for cultivation, we decided to use this technique

to develop a strain typing method for *H. heilmannii* s.s. and "*H. ailurogastricus"*. In order to facilitate inter-species comparison, we decided to use the same genes that were used for *H. pylori* and *H. suis* strain typing (Achtman *et al*., 1999; Devi *et al*., 2006; Liang *et al*., 2013). Both for *H. heilmannii* s.s. and "*H. ailurogastricus"* and in contrast to what we observed for *H. suis*, all loci revealed a (very) large number of variable nucleotide sites. ClonalFrame analysis of the events at the nodes revealed, especially for *H. heilmannii* s.s., that mainly recombination, as opposed to mutation, was responsible for the diversification of strains. Possibly, the fact that most cats, as opposed to purpose-bred pigs, have free access to the outdoor environment and other cats, sometimes from a completely different geographical origin, could explain this remarkable difference. Indeed, it can be assumed that the chances for recombination between feline *Helicobacter* strains are higher compared to porcine *Helicobacter* strains, due to the completely different lifestyle of the respective hosts.

In the future, these developed MLST tests should be further optimized, for instance by shortening the polymorphic sites subjected to nucleotide sequencing, with the aim of increasing the simplicity and cost. In addition, a larger number of strains and/or gastric biopsies from *H. heilmannii* s.s. and "*H. ailurogastricus"*-positive animals and humans should be analyzed. In the end, this should allow us to obtain better insights into the epidemiology of infections with both gastric non-*H. pylori Helicobacter* species worldwide.

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GENERAL DISCUSSION

During the past 4 years, most experiments performed within the framework of this PhD project have been around genotypic and phenotypic strain typing of existing and new isolates of gastric non-*H. pylori Helicobacter* species in general and *H. suis* in particular. One of the main goals was to develop an unambiguous and discriminatory typing scheme for *H. suis*, *H. heilmannii* s.s. and the putative new species "*H. ailurogastricus"*, which should allow us to increase our understanding of the epidemiology of *H. suis* infection in pigs and humans, *Helicobacter heilmannii* sensu stricto and "*H. ailurogastricus"* infection in animals and humans, as well as the population structure of these bacteria.

Since the first successful isolation of *H. suis* in 2008, several studies have revealed that this bacterium causes a variety of gastric disorders in pigs and humans. The ability to accurately identify the strains of infectious agents that cause disease and the ability to correlate the presence of a certain strain with the absence or presence of disease is crucial for epidemiological surveillance and public health decisions. Molecular typing (genotyping) or comparative sequence analysis are used to resolve very different kinds of problems. For instance, do the isolates recovered from a localized outbreak belong to an already identified strain causing disease (short term or local epidemiology) and how are strains causing disease in one geographic area related to those strains isolated world-wide (long term or global epidemiology)? Different methods may be appropriate for investigating local and global epidemiology, but in both cases they should be highly discriminatory so that isolates assigned to the same molecular type are likely to have descended from a recent common ancestor, and isolates that share a more distant common ancestor are not assigned to the same type (Maiden *et al*., 1998).

In the last decades, numerous techniques and methods have been developed for bacterial strain typing, based both on phenotypic and genotyic approaches. The majority of these methods created are based on differences in the genetic content of the pathogen, but some of these methods suffer from significant drawbacks, including inadequate discrimination, limited availability of reagents, poor reproducibility within and between laboratories, and an inability to quantitate the genetic relationships between isolates. Different typing methods are often used for the same pathogens in different laboratories, and even when a uniform method is used, the data are sometimes difficult to compare between laboratories and are often unsuitable for evolutionary, phylogenetic, or population genetics studies (Maiden *et al*., 1998; Coffey *et al*., 2006; Platonov *et al*., 2000).

Phenotypic characterization of bacteria has been used as a typing tool, and this includes serotyping, phage typing, biotyping, bacteriocin–like inhibitor typing schemes, and antibiotic susceptibility testing (Hill AW and Brady CA, 1989; Lammler C, 1991; Tagg JR and Vugler LG, 1986; Qi *et al*., 2014). However, these methods are unsuitable for the analysis of closely related strains (Coffey *et al*., 2006) and these techniques are tedious and have other important drawbacks. The discriminatory ability of these approaches are not optimal and they frequently fail to discriminate between epidemiologically related and unrelated strains (Hickman-Brenner *et al*., 1991; Stubbs *et al*., 1994; Lopes *et al*., 2004; Borrego *et al*., 1992; Threlfall *et al*., 1990; Kotetishvili *et al*., 2002). DNA-based methods show more potential, with DNA restriction analysis by pulsed-field gel electrophoresis (PFGE) appearing to be a reliable and highly discriminatory method (Baseggio *et al*., 1997; Douglas *et al*., 2000; Jayarao *et al*., 1993; Phuektes *et al*., 2001). However, techniques that reflect slowly accumulating variation, such as multilocus enzyme electrophoresis (MLEE) (Selander *et al*., 1986), are more appropriate for long-term epidemiological understanding.

Indeed, high levels of discrimination can be achieved in two quite different ways. In one approach, individual loci, or uncharacterized regions of the genome, that are highly variable within the bacterial population are identified. For bacterial pathogens, several methods based on this approach are currently popular, e.g. ribotyping, pulsed-field gel electrophoresis (PFGE), and PCR with repetitive element primers. In these methods, restriction enzymes (or PCR primers) are chosen that give maximal variation within the population. As a consequence, the variation that is indexed is evolving very rapidly, usually for unknown reasons (Maiden *et al*., 1998). Another approach, typified by multilocus enzyme electrophoresis (MLEE), is to use variation that is accumulating very slowly in the population and that is likely to be selectively neutral. The major disadvantage of MLEE, as with the other typing methods mentioned, is the relatively poor portability between laboratories, thereby preventing comparison of results (Maiden *et al*., 1998; Coffey *et al*., 2006).

Multilocus sequence typing (MLST) was first proposed by Maiden in 1998 as an improvement of multilocus enzyme electrophoresis (MLEE) (Maiden *et al*., 1998). The evolution of the selected housekeeping genes is constrained by their requirement to encode functional products and is not affected by the rapid evolution that may be detected within genes encoding proteins that influence survival in a particular niche. The motivation underpinning the original development of MLST for bacteria was not only to design a method to simply type bacterial strains, but also to provide a tool to investigate populations and population dynamics on a global scale (Coffey *et al*., 2006). When

using data generated by MLST, phylogenetic and clonal relationships can indeed be investigated relatively easy, for instance by constructing a phylogenetic tree based on concatenated sequences from all the alleles or by using the data for ClonalFrame analysis.

One of the main advantages of MLST is the ease by which sequence data can be compared readily between laboratories, which means that a typing method based on the sequences of gene fragments from a number of different housekeeping loci is fully portable and data stored in a single expanding central multilocus sequence database can be interrogated electronically on the internet to produce a powerful resource for global epidemiology (Platonov *et al.*, 2000; Bilhere *et al.*, 2009). Also the newly developed *H. suis* MLST has been made publicly available (http://pubmlst.org/hsuis/). Another major advantage of MLST, in particular when working with fastidious microorganisms such as *H. suis, H. heilmannii* s.s. and "*H. ailurogastricus"*, is that no pure bacterial cultures are needed, as the gene fragments are amplified directly from biological samples and clinical material (e.g. blood, cerebrospinal fluid or tissues).

In chapter 1, the MLST typing scheme which we developed for *H. suis* was shown to have a good resolution on the level it was tested. It remains, however, to be validated in the setting of the presence or absence of gastric disease in different pig herds. This might allow to investigate whether some *H. suis* strains are more frequently associated with disease in pigs than others. Eventually it may help to detect virulence markers in *H. suis*, for instance, by sequencing the genome of *H. suis* strains belonging to different sequence types. In addition, the typing scheme may be applied to examine the population structure of this human and animal pathogen. Currently, additional samples from *H. suis*-infected pigs, humans and nonhuman primates are being analyzed using the developed technique. These samples are being collected from various continents (Europe, Asia, Africa, North and South America and Oceania), which should help us to resolve the population structure of this widespread gastric pathogen, by applying ClonalFrame and Structure Analysis on the obtained sequences for the different MLST loci.

In the studies described in chapters 1 and 2, the MLST scheme developed for *H. suis* was shown to have an acceptable resolution. Indeed, different strains could be detected at the herd level and changes of the genotype due to prolonged subculture were detected on a few occasions. On the level of the herd, however (eg. Herd C from chapter 1), the same strains were sometimes detected in the majority of the animals. This may very well indeed be the case. Alternatively, however, the strains or clones circulating in a certain herd may be very closely related and only separated by

microevolutionary events, to such an extent that the seven loci-based *H. suis* MLST is not able of discriminating these different strains/clones. This could be overcome by extending the numbers of loci included in the MLST scheme, or by developing an alternative typing scheme with an even higher resolution, such as ribosomal MLST (rMLST), which includes 53 ribosomal protein loci (Jolley *et al.*, 2012) or even whole-genome MLST (wgMLST). An important limitation in the case of *H. suis* typing, however, is the fact that most strains were and most probably will be analyzed in stomach tissue of infected animals, without *in vitro* isolates being available, due to the very fastidious nature of the organisms. For rMLST and wgMLST, however, pure cultures of the isolate are preferred and essential, respectively (Maiden *et al*., 2013; Jolley and Maiden, 2014).

In a very recent study, applying the *H. suis* multilocus sequence typing scheme (chapter 1) on additional stomach biopsies of slaughterhouse pigs, several *H. suis* strains were occasionally detected in the same animal (unpublished results). Hence, an apparently pure biphasic culture obtained from an individual animal may contain more than one *H. suis* strain, which is of course undesirable for experimental studies aiming for instance at investigating *H. suis* strain differences. We therefore described in chapter 2, a method for single colony isolation and subsequent purification of *H. suis* strains, which was shown not to be so straightforward due to the fastidous nature of this bacterium. In some cases, changes of the genotype and the phenotype were observed after this long-term purification process. Most likely, mutation is involved in this process. Also for other bacterial species, microevolution of a standard strain during storage and *in vitro* serial passage has been shown to alter the phenotype or genotype (Franzot *et al*., 1998; Somerville *et al*., 2002; Hanel *et al*., 2009; Wang *et al*., 2007; Domenech *et al*., 2009). The ability of *Staphylococcus aureus* to evade the host immune response and cause disease is due to an extensive repertoire of known and putative virulence factors and the production of these virulence factors is regulated by the accessory gene regulatory (*agr*) operon and several other global regulatory loci (Morfeldt *et al*., 1988; Peng *et al*., 1988). *S. aureus* strains newly isolated from patients revealed two-fold higher aconitase activity (strongly correlated to beta-hemolytic phenotype) than a strain passaged extensively *in vitro* and aconitase specific activity decreased over time during *in vitro* passage. The loss of the beta-hemolytic phenotype also coincided with the occurrence of mutations in the *agrC* coding region or the intergenic region between *agrC* and *agrA* in the derivative strains. Serial passage of *S. aureus* caused significant phenotypic changes and alteration of the global regulator *agr* (Somerville *et al*., 2002).

Another example of the effect of subculture on strain characteristics is provided by mycobacteria. *Mycobacterium tuberculosis* strain H37Rv is highly prone to losing the ability to synthesize the cell wall lipid phthiocerol dimycocerosate (PDIM) during extended periods of *in vitro* culture. Domenech and Reed (2009) demonstrated that after weekly subculture of PDIM-positive isolates over a period of 20 weeks, the proportion of PDIM-negative cells rises above 30%. That PDIM biosynthesis is negatively selected *in vitro* is evident from the broad range of mutation types within cultures originating from a single PDIM-positive parental clone. Moreover, the appearance of these multiple mutation types coupled with an enhanced growth rate of PDIM-negative bacteria ensures that "PDIM-less" clones rapidly dominate in *in vitro* cultures. It has been known for almost a decade that strains of *M. tuberculosis* that lack PDIM are severely attenuated during *in vivo* infection. Two factors seem to contribute to the rapid emergence of PDIM-negative clones *in vitro*. Firstly, multiple independent mutations appear to be occurring simultaneously that result in the loss of PDIM. Secondly, the fact that these mutants are no longer synthesizing PDIM affords them a competitive growth advange within a heterogenous population. Genomic deletions and point mutation result in the loss of PDIM synthesis (Domenech and Reed, 2009).

For *H. bizzozeronii*, a canine gastric *Helicobacter* species related to *H. suis*, it has been postulated that a high plasticity of the genome may be (in part) responsible for the zoonotic nature of this bacterium (Schott *et al*., 2011). In addition, *H. pylori*, a close human-adapted relative of *H. suis*, is known for its remarkably high level of genetic diversity creating a dynamic pool of genetic variants. As an example, the Mongolian gerbil-adapted *H. pylori* strain B8 was completely sequenced, annotated and compared to genomes of the original parental human isolate B128. Compared to its parental strain B128, the gerbil-adapted strain B8 was shown to have the potential to build, possible by a high rate of mutation and recombination, a dynamic pool of genetic variants (e.g. fragmented genes and repetitive regions) required for the adaptation processes. These variants are supposed to be essential for the colonization and persistence of strain B8 in the gerbil stomach during inflammation (Farnbacher *et al*., 2010).

Similar to *H. pylori*, a high rate of recombination may occur in the *H. suis* population or even in laboratory *H. suis* isolates harbouring multiple different strains. *H. suis* has been shown to harbour most members of the *comB* cluster, which has been described to be a DNA transformation competence system in *H. pylori* (Vermoote *et al*., 2011), enabling the uptake of foreign DNA. Interestingly, data described in chapter 3 suggest that recombination events in *H. suis* may not be

so frequent compared to those observed in *H. heilmannii* s.s. or "*H. ailurogastricus"*. The analysis of additional samples from different geographic regions is, however, essential to provide conclusive answers to these questions. Besides mutation (or recombination), other events may be responsible for the changes in *H. suis* phenotype and/or genotype described in chapter 2. For instance, alterations of gene expression may be involved, which has indeed also been described for *H. pylori* after adhesion to gastric epithelial cells *in vitro* (Kim *et al*., 2004). In this species, regulation of gene expression has been shown to frequently occur, turning certain genes on or off, giving it a selective advantage during host-pathogen interaction by simple phenotypic variation (phase variation) (Wang *et al*., 1999). In addition, genomic rearrangement in strains with an apparently stable genome may occur in *H. suis*, as has been described for *Campylobacter jejuni* strains. Possibly, storage of *H. suis* bacteria in frozen state after the single colony isolation and purification may have selected for variants which are more resistant to low temperatures compared to others (Rosas *et al*., 1997). Although storage in frozen (or lyophilized) state is likely to be the best option to preserve the original characteristics of the strain, this is not necessarily a guarantee of stability of all genotypic and phenotypic markers.

Future studies on *H. suis* should aim at investigating which effects contribute to the changes in phenotype and genotype. Currently, whole genome sequencing of *H. suis* strain HS5aKOL, which was shown no longer to be able to colonize the stomach of Mongolian gerbils (chapter 2), is being performed and the genome will be compared to that from the parental strain, for instance by looking at genetic rearrangements or the presence of Single Nucleotide Poymorphisms. In addition, adhesion experiments are being performed on gastric tissue from Mongolian gerbils, and preliminary results indicate that strain HS5aKOL indeed shows an impaired adhesion capacity, suggesting that alterations in outer membrane proteins may be involved. In addition, the influence of long-term host adaptation on the pathogenesis of *H. suis* infection will be studied. Indeed, several other research groups using *H. suis* strains with a history of mouse passage of years, or even decades (Cinque *et al*., 2006; Nakamura *et al*., 2008), have found that experimental infection causes a Th1 response in several mouse models. Interestingly, a similar Th1 response was in general absent in mouse studies performed in the laboratory of the candidate (Flahou *et al*., 2012). In addition, much more pronounced lesions resembling gastric MALT lymphoma have been described by others using these *in vivo* passaged *H. suis* 'strains' in C57BL/6 mice after only 6 months of infection, which contrasts to C57BL/6 mice infected experimentally with *in vitro*

isolated *H. suis* strains, showing only mild changes in the stomach of these animals, even after 8 months of infection (Flahou *et al.*, 2010)

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SUMMARY

Besides the well-known gastric pathogen *Helicobacter pylori* (*H. pylori)*, human patients suffering from gastric disease can also be infected with gastric non-*H. pylori Helicobacter* (NHPH) species. This group comprises highly related bacteria with a very similar spiral-shaped morphology. The natural hosts of these bacteria are domesticated animals, such as pigs (*H. suis*), dogs and cats (including *H. heilmannii* sensu stricto and the putative new species "*H. ailurogastricus"*). Especially in pigs, *H. suis* infection is considered to have a substantial impact, since infection in this economically important animal species has been shown to cause gastritis and a decrease of the daily weight gain. The development of a successful isolation method for some of the most fastidious gastric NHPH, has opened new doors for investigating these bacteria and the diseases they evoke. Indeed, the first successful *in vitro* isolation of *H. suis* from pigs in 2008 and *H. heilmannii* sensu stricto as well as the newly proposed *H. ailurogastricus* from cats in 2012 can be considered important events in the history of *Helicobacter* research. When we started our studies, no information was available on the epidemiology of *H. suis* infection in pigs and humans and *H. heilmannii* s.s. and "*H. ailurogastricus"* infection in carnivores and humans. In addition, little information was present on the strain diversity, both on the genotypic and phenotypic level, in these populations.

In **chapter 1**, we describe the development of a Multilocus Sequence Typing (MLST) method, in order to gain insight into the genetic diversity of porcine and human *H. suis* strains. In a preliminary study, 7 housekeeping genes (*atpA, efp, mutY, ppa, trpC, ureI, yphC*) of 10 *H. suis* isolates cultured *in vitro* were investigated as MLST candidates. All genes displayed several variable nucleotide sites, except the *ureI* gene, which was replaced by part of the *ureAB* gene cluster of *H. suis*. The *mutY* gene was found to have the most discriminatory power. Subsequently, internal gene fragments, ranging from 379-732 bp and comprising several variable nucleotide sites, were selected. For validation of the developed MLST technique, gastric tissue from 17 *H. suis*-positive pigs from 4 different herds and from 1 *H. suis*-infected human patient was used for direct, culture-independent strain typing of *H. suis*. The latter is extremely important, since still now, isolation of *H. suis* remains difficult and time-consuming. In addition to the 10 unique sequence types (STs) among the 10 isolates grown *in vitro*, 15 additional STs could be assigned. Individual animals were shown to be apparently colonized by only 1 *H. suis* strain, as shown by the presence of clean nucleotide peaks in electropherograms. In general, multiple *H. suis* strains were present in all herds tested, revealing *H. suis* is a genetically diverse bacterial species. The human *H. suis* strain showed a very close relationship to porcine strains, supporting the zoonotic nature of human

H. suis infections. To investigate the genetic stability of strains when subjected to long-term biphasic subculture, low and high passage numbers of strains HS1 and HS5 (HS1 and HS1p17, HS5 and HS5p21), were included as well. No differences were revealed for HS1p17 compared to its parental strain HS1. For HS5p21, however, a new allele type was attributed for *mutY* and *trpC*, indicating that long-term *in vitro* biphasic culture, may result in genetic diversification. In conclusion, the developed MLST scheme may prove useful for direct, culture-independent typing of porcine and human *H. suis* strains. The *H. suis* MLST homepage is available for the public at http://pubmlst.org/hsuis/.

Although not detected in our first study, colonization of the same animal by several different *H. suis* strains, has nevertheless been detected in a number of cases. As mentioned above, *H. suis* is extremely fastidious and at the onset of the present studies, biphasic culture conditions were essential for isolation and culture, making it impossible to obtain single colonies. Hence, cultures obtained from an individual animal may contain multiple *H. suis* strains, which is undesirable for experiments aiming for instance at investigating *H. suis* strain differences. In addition, to construct and isolate *H. suis* deletion mutants for genes of interest, culture on dry agar plates as single colonies is preferred. For these reasons, we developed a technique to purify *H. suis* from biphasic cultures by single colony isolation (**chapter 2**). Pure cultures of *H. suis* were established by growing bacteria as colonies on 1% brucella agar plates, followed by purification and enrichment by biphasic subculture. Characteristics of these single colony-derived strains were compared with those of their parent strains using multilocus sequence typing (MLST), and by studying bacterium-host interactions using a gastric epithelial cell line and Mongolian gerbil model. The purification/enrichment procedure required a non-stop culture of several weeks. For 4 out of 17 *H. suis* strains, analysis by MLST revealed differences between parental and single colony-derived strains. For 3 out of 4 single colony-derived strains tested, the cell death-inducing capacity was higher than for the parental strain. One single colony-derived strain lost its capacity to colonize Mongolian gerbils. For the 4 other strains tested, colonization capacity and histopathological changes were similar to what has been described when using strains with only a history of limited biphasic culture. So, although we successfully developed a method to obtain pure *H. suis* cultures, the elaborate purification and enrichment procedure was shown to be able to affect the the bacterial genotype and phenotype.

The *Helicobacter* isolation protocol described by our group in 2008 was shown to be not only effective for isolation of *H. suis* from pigs, but also of gastric helicobacters from the stomach of cats. Nine newly isolated feline gastric *Helicobacter* strains were designated as ASB1, ASB2, ASB3, ASB6, ASB7, ASB9, ASB11, ASB13, and ASB14. Unpublished genomes of strains ASB1 and ASB7 showed that, in contrast to what was previously described and assumed, these strains most likely belong to 2 different species. Subsequent investigation has revealed that strains ASB1, ASB2, ASB3, ASB6 and ASB14 should be referred to as *H. heilmannii* sensu stricto, whereas strains ASB7, ASB9, ASB11 and ASB13 most likely belong to a novel *Helicobacter* species, for which the name "*H. ailurogastricus"* has been proposed. As for *H. suis*, little information is present on the strain diversity of these *Helicobacter* species, both on the genotypic and phenotypic level. Therefore, as described in **chapter 3,** we developed an unambiguous and discriminatory typing scheme for *H. heilmannii* sensu stricto and "*H. ailurogastricus",* which should allow us to increase our understanding of the epidemiology and population structure of these bacteria. Eight complete housekeeping genes (*atpA, efp, ppa, mutY, trpC, ureI, ureAB, yphC*) of 5 *H. heilmannii* s.s and 4 "*H. ailurogastricus"* strains were amplified by PCR and sequenced. For *H. heilmannii* s.s., a total number of 9,131 bp represented the complete coding sequences of these 8 housekeeping genes. Six hundred and fifteen nucleotide sites (615/9,131; 6.7%) were found to be polymorphic and the *atpA* gene was shown the most powerful for discrimination of the strains. For *H. "ailurogastricus",* a total of 9,314 bp represented the complete coding sequences of the same 8 housekeeping genes. Eighty six nucleotide sites (86/9,314; 0.92%) were found to be polymorphic and the *atpA* gene was shown to be the most powerful for discrimination. The *ppa* gene was shown to lack discriminatory power (0 variable nucleotide sites). The MLST schemes described here will be further optimized and validated for use in human or feline gastric biopsies.

In conclusion, the succesful development of a multilocus sequence typing method for *H. suis*, *H. heilmannii* sensu stricto, as well as "*H. ailurogastricus"* will allow researchers to apply these techniques for direct strain typing without the need for cultivation. Due to the unambiguousness and portability of nucleotide sequence data, for instance obtained directly from gastric biopsies of *Helicobacter*-infected animals or humans, data can be easily compared between laboratories without the need of exchanging strains. The developed *H. suis* MLST technique, along with results from *in vitro* and *in vivo* experiments, has shown that a method we developed to obtain pure *H. suis* cultures, sometimes affects *H. suis* strain characteristics.

SAMENVATTING

Behalve *Helicobacter pylori*, kunnen ook de zogenaamde niet-*H. pylori* helicobacters (NHPH) maagaandoeningen veroorzaken bij mensen. Deze groep bevat heel moeilijk cultiveerbare bacteriën met een gelijkaardige morfologie, namelijk een spiraalvorm. De natuurlijke gastheren van deze bacteriën zijn gedomesticeerde dieren, zoals varkens (*H. suis*), honden en katten (onder andere *H. heilmannii* sensu stricto en een mogelijk nieuwe species waarvoor de naam "*H. ailurogastricus*" is voorgesteld). Bij varkens kunnen *H. suis* infecties leiden tot aanzienlijke economische verliezen. Deze zijn het gevolg van de *H. suis*-gerelateerde maagontsteking en een daling van de dagelijkse gewichtsaanzet. De pathogene betekenis van *H. heilmannii* sensu stricto en *H. ailurogastricus* voor katten en honden is nog onduidelijk. De eerste succesvolle *in vitro* isolatie van *H. suis* uit een varkensmaag in 2008 en de isolatie van zowel *H. heilmannii* sensu stricto als de recent ontdekte soort "*H. ailurogastricus"* uit de magen van katten in 2012 mogen als belangrijke mijlpalen in de geschiedenis van het NHPH onderzoek gezien worden. Dit heeft het mogelijk gemaakt om de eigenschappen van deze bacteriën en de interacties met hun gastheren beter te bestuderen.

Bij aanvang van dit doctoraatsonderzoek waren heel weinig gegevens beschikbaar over de epidemiologie van *H. suis, H. heilmannii* sensu stricto en "*H. ailurogastricus"* infecties bij mensen en dieren. Bovendien was er zo goed als geen informatie beschikbaar over mogelijke stamverschillen binnen deze bacteriële species, zowel op genotypisch als fenotypisch niveau.

In **hoofdstuk 1** beschrijven we de ontwikkeling van een Multilocus Sequence Typing (MLST) methode. Deze liet ons toe nieuwe inzichten te verwerven in de genetische diversiteit van *H. suis* stammen die voorkomen bij varkens en bij mensen. In een eerste studie werden 7 referentiegenen (*atpA, efp, mutY, ppa, trpC, ureI, yphC*) van 10 *H. suis* isolaten onderzocht als mogelijke MLST kandidaten. Alle genen vertoonden verschillen op nucleotideniveau, met uitzondering van het *ureI* gen. Daarom werd dit gen vervangen door de *ureAB* genencluster van *H. suis*. Het *mutY* gen bleek het grootste onderscheidende vermogen te bezitten. Vervolgens werden interne genfragmenten, variërend van 379 tot 732 bp, geselecteerd. Voor validatie van de ontwikkelde MLST methode werd een directe, cultuur-onafhankelijke stamtypering van *H. suis* toegepast op maagweefsel van 17 met *H. suis*-geïnfecteerde varkens (afkomstig van 4 verschillende bedrijven) en op één maagbiopt van een humane patiënt, geïnfecteerd met *H. suis*. Een cultuur-onafhankelijke typering is in deze context van groot belang aangezien isolatie van *H. suis* nog steeds een erg moeilijk en tijdrovend proces is. Naast de 10 unieke 'sequentie types' (STs) van de 10 *in vitro* geïsoleerde stammen, konden, na analyse van bovengenoemd maagweefsel, 15 extra STs toegekend worden. De aanwezigheid van een zuiver nucleotidenpatroon in het elektroferogram van de maag van individuele dieren, was een aanwijzing dat ze besmet waren met één enkele *H. suis* stam. Over het algemeen waren wel meerdere stammen aanwezig binnen een groep dieren afkomstig van één bedrijf, wat aantoont dat *H. suis* een genetisch diverse bacteriële soort is. De humane *H. suis* stam die werd onderzocht in

hoofdstuk 1 vertoonde een nauwe verwantschap met de varkensstammen, wat het zoönotisch karakter van *H. suis* ondersteunt.

Om na te gaan of *H. suis* stammen al dan niet genetisch stabiel blijven wanneer ze gekweekt worden in een bifasisch milieu, werden culturen van de stammen HS1 en HS5 die slechts voor korte tijd (HS1LP en HS5bLP) óf langdurig (HS1p17 en HS5p21) werden gesubcultiveerd, onderzocht met de MLST methode. Er werden geen verschillen waargenomen tussen stam HS1p17 en de oorspronkelijke stam HS1LP. Voor HS5p21 werd evenwel een nieuw allelnummer toegekend voor de *mutY* en *trpC* genen, wat aantoont dat *in vitro* cultivatie genetische diversificatie kan veroorzaken. De ontwikkelde *H. suis* MLST techniek kan nuttig zijn voor directe, cultuur-onafhankelijke typering van varkens-geassocieerde en humane *H. suis* stammen. De *H. suis* MLST webpagina is beschikbaar voor het publiek op http://pubmlst.org/hsuis/.

Hoewel dit niet aangetoond werd in onze eerste studie, is kolonisatie van hetzelfde dier door verschillende *H. suis* stammen mogelijk, zoals later onderzoek heeft aangetoond in een aantal gevallen. Zoals hierboven beschreven, is *H. suis* zeer moeilijk cultiveerbaar. Bovendien waren bij aanvang van deze studies bifasische cultuurcondities essentieel voor de isolatie en het opgroeien van *H. suis*, wat het onmogelijk maakt om de bacteriën als afzonderlijke kolonies op te groeien. Vandaar dat *H. suis* culturen, afkomstig van één en hetzelfde dier toch meerdere *H. suis* stammen kunnen bevatten. Dit is vanzelfsprekend niet wenselijk in experimenten die als doel hebben om stamverschillen van *H. suis* te onderzoeken. Om *H. suis* mutanten aan te maken waarin bepaalde genen zijn uitgeschakeld, zijn eveneens bacteriële culturen afkomstig van afzonderlijke kolonies op droge agarplaten wenselijk. In **hoofdstuk 2** beschrijven we de ontwikkeling van een techniek om *H. suis* bacteriën vanuit bifasische culturen als afzonderlijke kolonies op te groeien en verder op te zuiveren. Hierbij werd gebruik gemaakt van petri platen met Brucella medium met een verlaagde agarconcentratie. Dit liet toe om *H. suis* als afzonderlijke kolonies op te groeien, die daarna werden aangerijkt door middel van bifasische subcultuur. De eigenschappen van deze opgezuiverde en aangerijkte stammen werden vergeleken met deze van de oorspronkelijke stam. Hierbij werd niet alleen gebruik gemaakt van MLST, maar werden ook de bacterie-gastheerinteracties bestudeerd in zowel celculturen als Mongoolse gerbils. De opzuiverings/aanrijkingsprocedure nam voor elke stam verschillende weken in beslag. MLST analyse toonde verschillen aan tussen de ouderstam en de als kolonievorm opgegroeide stam voor 4 van de 17 geteste *H. suis* stammen. Voor 3 van de 4 als afzonderlijke kolonies opgegroeide en aangerijkte stammen, was de capaciteit om epitheliale celdood te veroorzaken hoger dan deze van de ouderstam. Eén van de kolonie-afgeleide stammen bleek niet meer in staat om de maag van Mongoolse gerbils te koloniseren. De overige kolonie-afgeleide *H. suis* stammen veroorzaakten histopathologische veranderingen die gelijkaardig waren aan deze die worden veroorzaakt door ouderstammen die slechts gedurende korte tijd *in vitro* waren opgegroeid. Hoewel we er dus in geslaagd

zijn om een methode te ontwikkelen om reinculturen van *H. suis* te bekomen, werd ook aangetoond dat de opzuiverings- en aanrijkingsmethode het bacteriële genotype en fenotype kan beïnvloeden.

De methode die in vroeger onderzoek op punt gesteld werd voor isolatie van *H. suis* uit de maag van varkens, bleek ook efficiënt te zijn voor de isolatie van gastrale helicobacters uit de maag van katten. Negen nieuwe kat-geassocieerde *Helicobacter* stammen werden op korte tijd geïsoleerd: ASB1, ASB2, ASB3, ASB6, ASB7, ASB9, ASB11, ASB13 en ASB14. Ongepubliceerde genoomsequenties van stammen ASB1 en ASB7 toonden aan dat het hier, in tegenstelling tot wat oorspronkelijk werd beschreven, wellicht om 2 verschillende species gaat. Verder onderzoek wees uit dat stammen ASB1, ASB2, ASB3, ASB6 en ASB14 tot de species *H. heilmannii* sensu stricto behoren. De stammen ASB7, ASB9, ASB11 en ASB13 daarentegen mogen hoogstwaarschijnlijk beschouwd worden als een nieuwe *Helicobacter* species, waarvoor de naam "*H. ailurogastricus"* werd voorgesteld. Net zoals bij *H. suis* is er weinig informatie beschikbaar omtrent de stamdiversiteit van deze helicobacters, zowel op genotypisch als op fenotypisch niveau. Daarom werd in **hoofdstuk 3** een ondubbelzinnig en discriminerend typeringsschema ontwikkeld voor *H. heilmannii* sensu stricto en "*H. ailurogastricus"*. Dit moet ons toelaten om de epidemiologie en de populatiestructuur van deze bacteriën beter te begrijpen. Acht referentiegenen (*atpA*, *efp*, *ppa*, *mutY*, *trpC*, *ureI*, *ureAB*, *yphC*) werden geamplifieerd en gesequeneerd voor 5 *H. heilmannii* sensu stricto en 4 "*H. ailurogastricus"* stammen. Voor *H. heilmannii* sensu stricto werden 615 polymorfe nucleotideplaatsen gedetecteerd voor een totaal van 9131 gesequeneerde baseparen per stam (6.7 %). Het *atpA* gen vertoonde het grootste onderscheidend vermogen. Voor *H. ailurogastricus* werden 86 polymorfe nucleotideplaatsen gedetecteerd voor een totaal van 9314 gesequeneerde baseparen per stam (0.92 %). Ook hier had het *atpA* gen het grootste onderscheidend vermogen, terwijl voor het *ppa* gen geen variabele nucleotide posities werden gedetecteerd. Het MLST schema zal voor beide *Helicobacter* soorten verder geoptimaliseerd en gevalideerd worden voor toekomstig gebruik in maagbiopten van geïnfecteerde mensen en katten.

Tot besluit kunnen we stellen dat de ontwikkeling van een multilocus sequence typeringsmethode voor zowel *H. suis*, *H. heilmannii* sensu stricto als "*H. ailurogastricus"* onderzoekers in staat zal stellen om een directe typering van *Helicobacter* stammen uit te voeren op maagweefsel, zonder voorafgaande cultivatie. Deze methode laat bovendien toe dat de gegevens gemakkelijk vergeleken kunnen worden tussen verschillende laboratoria, zonder uitwisseling van de stammen. De ontwikkelde *H. suis* typeringsmethode en de resultaten van de *in vitro* en *in vivo* experimenten beschreven in studie 2, toonden aan dat opzuivering en *in vitro* aanrijking van *H. suis* in sommige gevallen de karakteristieken van de *H. suis* stammen kan beïnvloeden.

CURRICULUM VITAE

Jungang Liang was born on November 21, 1962, in Jiaohe, Jilin, China. When he was 1 year old, his whole family moved to his homeland, Hefei, Anhui, China. He grew up there and completed his primary school and high school. In 1979, he was enrolled to be an undergraduate student at the Anhui State University of Medical Science (ASUMS, 5 academic years). He graduated from ASUMS, and received his bachelor science degree in 1984. He was enrolled as a graduate student in the graduate school from the China Center for Disease Control and Prevention (China CDC, 3 academic years) in 1992. He completed his research there and received his master science degree based on two publications as first author in 1995. All the data derived from his laboratory work on Lyme disease in China, coupled with additional data from his colleagues won the top award (collective) in 1993 issued by the Ministry of Science and Technical Progress, China Central Government. He was offered an academic title of assistant professor by China CDC (Beijing, China) in June 1996 and as a further promotion, a position of associate professor was offered by the National Vaccine & Serum Institute in October, 2000 (Beijing, China).

As a visiting scholar and scientist, he has been working abroad from January 2001 through June 2008, at the Université de la Méditerranée (Marseille, France), North Carolina State University (Raleigh, NC, USA), University of Florida (Gainesville, FL, USA) and University of Federico II (Naples, Italy).

In October 2010, he started his PhD studies at the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Belgium. His doctoral research was funded by the Special Research Fund (BOF08/GOA/004 and 01W00310) of Ghent University, under the guidance of Prof. Freddy Haesebrouck and Dr. Bram Flahou. He developed a strain typing method for *Helicobacter suis*, *Helicobacter heilmannii sensu stricto* and a putative new *Helicobacter* species ("*Helicobacter ailurogastricus"*), as well as a method allowing purification of *Helicobacter suis* strains. He also investigated the (genetic) stability of this very fastidious species during subculture. His research generated several scientific pubications in international journals.

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I wish to express my appreciation and my sincerest thanks to Professor Freddy Haesebrouck. Frankly speaking, I come here only for his brilliant achievements devoted to human science and techniques, as well as the first isolation of *Helicobacter suis*. Many thanks for his kind invitation to join his team. I consider myself lucky to be a part of it.

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Yours sincerely

Jungang Liang

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