DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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DIVERSITY OF *HELICOBACTER PYLORI* GENOTYPES IN ESTONIAN PATIENTS WITH CHRONIC INFLAMMATORY GASTRIC DISEASES

HELENA ANDRESON

TARTU UNIVERSITY
PRESS

Department of Microbiology, University of Tartu, Estonia

Dissertation is accepted for the commencement of the degree of Doctor of Medical Sciences on May 17, 2006 by the Council of the Faculty of Medicine, University of Tartu, Estonia

Opponent: Lars Engstrand MD, PhD, Professor of Clinical Bacteriology, Microbiology and Tumor Biology Center, Karolinska Institutet and Department of Bacteriology, Swedish Institute for Infectious Disease Control

Commencement: June 20, 2006

Publication of this dissertation is granted by University of Tartu

ISSN 1024–395X
ISBN 9949–11–370–9 (trükis)
ISBN 9949–11–371–7 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 313

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II. Sillakivi T, **Aro H***, Ustav M, Peetsalu M, Peetsalu A, Mikelsaar M. Diversity of *Helicobacter pylori* genotypes among Estonian and Russian patients with perforated peptic ulcer, living in Southern Estonia. FEMS Microbiology Letters 2001; 195:29–33.

III. Maaroos H-I, **Andreson H**, Lõivukene K, Hütt P, Kolk H, Kull I, Labotkin K, Mikelsaar M. The diagnostic value of endoscopy and *Helicobacter pylori* tests for peptic ulcer patients in late post-treatment setting. BMC Gastroenterology 2004; 4:27.

IV. **Andreson H**, Sillakivi T, Peetsalu M, Peetsalu A, Mikelsaar M. Persistence of *Helicobacter pylori* infection in patients with peptic ulcer perforation. Scandinavian Journal of Gastroenterology (submitted).

* Andreson Helena (born Aro)

ABBREVIATIONS

AGS	human gastric adenocarcinoma cell-line
BabA	blood group antigen binding adhesin
bp	base pairs
cagA	cytotoxin associated gene A
CagA	cytotoxin associated protein A
CG	chronic gastritis
Csk	C-terminal Src kinase
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
glmM	phosphoglucosamine mutase encoding gene
Grb2	growth factor receptor bound 2
H. pylori	*Helicobacter pylori*
iceA	induced-by-contact-with-epithelium gene A
Ig	immunoglobulin
IL	interleukin
kb	kilo base pairs
kDa	kilodalton
MALT	mucosa associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
PAI	pathogenicity island
PCR	polymerase chain reaction
PUD	peptic ulcer disease
PPU	perforated peptic ulcer
RFLP	restriction fragment length polymorphism
Scr	tyrosine kinase protein family
SHP-2	Src homology 2 domain
UBT	urea breath test
ureA	urease A gene
vacA	vacuolating cytotoxin gene A
VacA	vacuolating cytotoxin A

INTRODUCTION

In 1982 a new *Campylobacter*-like bacterium was successfully assessed in human gastric biopsies by two Australian scientists (Warren and Marshall, 1983). At first the bacterium was named *Campylobacter pyloridis* but in 1989 it was reclassified for *Helicobacter pylori* (Goodwin *et al.*, 1989). It was shown for the first time that bacterial infection can be associated with the wide-spread gastric diseases, as the newly discovered bacterium was found from practically all studied Australian patients with gastritis and duodenal ulcer (Marshall and Warren, 1984). Further, high prevalence of *Helicobacter pylori* infections was detected over the world.

In Estonia, in patients with different gastric diseases like chronic gastritis, gastric- and duodenal ulcer, perforated peptic ulcer and gastric cancer the prevalence of *H. pylori* has been assessed in children and adults by several work-groups (Maaroos *et al*., 1990, 1995, 1999; Mikelsaar *et al*. 1990, 1996; Peetsalu, A. *et al*., 1991; Vorobjova *et al*., 1991, 1994, 1998a, 1998b, 2000, 2001b; Soplepmann *et al*., 1997; Peetsalu, M. *et al*., 1998, 2005; Sillakivi, 2003; Oona *et al*., 2004). The assessment of *H. pylori* infection in Estonia has been based mostly on histological, bacteriological or serological methods. In the population of Estonia, the *H. pylori* antibodies have been detected in nearly 80% of inhabitants (Vorobjova *et al.*, 1994; Maaroos, 1995; Vorobjova *et al.*, 1998b). However, in clinical and epidemiological studies the application of more advanced modern molecular methods is mostly required for the specific characterization of *H. pylori* strains, especially in a population with high *H. pylori* prevalence.

Worldwide, due to the application of molecular methods in the investigations of *H. pylori* genetically different *H. pylori* strains in distinct host populations have been described (Miehlke *et al.*, 1996; Campbell *et al.*, 1997; Alm *et al.*, 1999; Vilaichone *et al.*, 2004). Several *H. pylori* genes (*vacA, cagA, iceA,* etc.) have been associated with specific gastric pathologies (Atherton *et al.*, 1997; Tham *et al.*, 2001; Ladeira *et al.*, 2004; Zagari and Bazzoli, 2004; Quintero *et al.*, 2005; Wu *et al.*, 2005). We have been using virulence genes, particularly the *cagA* and *vacA* genes, to characterize the genotype of *H. pylori* by its high prevalence among the patients in Estonia patients with gastritis and peptic ulcer (Lõivukene *et al.*, 2000). However, the question remained whether the *H. pylori* strains in the case of the most severe gastric disease like perforated peptic ulcer (PPU) could carry more specific sets of virulence markers than those with peptic ulcer. *H. pylori* strains with these molecular markers could worsen the outcome of peptic ulcer either due to the recrudescence of persistent strains or reinfection by new strains.

In addition, there are several studies referring to the differences of *H. pylori* prevalence and genotypes among different ethnical groups in different geographic locations (Graham *et al.*, 1991; Blecker *et al.*, 1995; Lambert *et al.*, 1995). Still, the relationship between *H. pylori* genotypes and the ethnical status of the host is largely unknown.

The main purpose of the study was to investigate the prevalence of different genotypes of *H. pylori* in chronic inflammatory gastric diseases and in distinct ethnic groups. In the course of this study, over 600 DNA samples extracted from biopsy samples from patients with chronic gastritis, peptic ulcer disease and its complications have been analyzed with molecular and histological methods to find associations between prevalent genotypes and the clinical diagnosis.

The selection of patients, the collection of the clinical samples, analyzing and publishing of the data took place in good collaboration with the colleagues from the Department of Surgery and the Department of Polyclinic and Family Medicine of the University of Tartu, the United Laboratories of the Tartu University Hospital and the Institute of Molecular and Cell Biology of the University of Tartu.

REVIEW OF THE LITERATURE

1. Characterization of genus *Helicobacter*

Since the discovery of *H. pylori* about 20 years ago, the genus *Helicobacter* is rapidly growing, including 23 formally named species and numerous novel species awaiting for a formal acceptance (DSMZ, 2006; NCBI, 2006). According to their preferred niches in the gastrointestinal tract, the members of the genus *Helicobacter* can be divided into two groups: gastric (stomach) or enteric (colonize the lower bowel, liver and bile ducts) helicobacters. Besides the human, *Helicobacter* species have been isolated from various mammalian species. All helicobacters are Gram-negative, non-spore-forming, curved, spiral-shaped or fusiform motile bacteria with one single polar non-sheathed flagella described in *H. pullorum* up to bipolar tufts of up to 20 sheathed flagella observed in many species (Solnick and Schauer, 2001). All gastric *Helicobacter* species produce urease while it is variable among enteric species. Oxidase activity is present in all and catalase activity in most species.

Helicobacter pylori is microaerophilic, rod-like or spiral-shaped bacterium that under stress conditions may transform into coccoid forms (Sorberg *et al.*, 1996; Donelli *et al.*, 1998; Nakamura *et al.*, 2000). It has been proposed by some authors that the coccoid form of *H. pylori* represents a degenerative or dead form of the bacterium (Kusters *et al.*, 1997; Enroth *et al.*, 1999) while the others consider it as a metabolically active "viable but non-cultivable" state, corresponding to a temporary adaptation to an unsuitable environment (Benaissa *et al.*, 1996; Cellini *et al.*, 1998; Rabelo-Goncalves *et al.*, 2002; Wang and Wang, 2004).

H. pylori colonizes the gastric and duodenal mucosa of humans and non-human primates (Dunn *et al.*, 1997). Rarely, in addition to *H. pylori*, humans can also become infected by a group of gastric not culturable spiral-shaped bacteria (comprising species like "*Candidatus* Helicobacter suis", *H. felis, H. bizzozeronii*) provisionally named as "*H. heilmannii*", found also in dogs, cats, pigs and other nonhuman primates (Solnick and Schauer, 2001; Solnick, 2003; Van den Bulck *et al.*, 2005a). Although, the "*H. heilmannii*" infection in humans is described as a zoonosis (Svec *et al.*, 2000; Solnick, 2003; Van den Bulck *et al.*, 2005b), it can in most cases cause mild gastritis. The infection has also been found in association with mucosa-associated lymphoid-tissue (MALT) lymphoma (Morgner *et al.*, 2000). Additionally, the colonization with "*H. heilmannii*" in humans may interfere in the diagnosis of *H. pylori* infection, as it is likely that some percentage of positive urea breath tests indicate the infection with "*H. heilmannii*" instead of *H. pylori* (Solnick and Schauer, 2001).

H. pylori is generally considered an extracellular bacterium, although there are considerable data showing the intracellular location of the bacteria *in vivo* (Noach *et al.*, 1994; Engstrand *et al.*, 1997; Ko *et al.*, 1999; Su *et al.*, 1999). This has been as well supported by *in vitro* studies demonstrating the possibility of intracellular invasion (Björkholm *et al.*, 2000; Kwok *et al.*, 2002). However, the clinical relevance of intracellular or coccoid-form *H. pylori* remains unclear.

It might seriously affect the prevalence or virulence of *H. pylori* as well as the persistence of *H. pylori* strains after different anti-microbial treatment schemes.

2. Prevalence of *H. pylori* infection

2.1. World-wide geographical differences

H. pylori is widespread all over the world, but the prevalence varies greatly among countries (Figure 1). There is a high divergence in *H. pylori* prevalence between the developed and the non-developed countries derived from the socio-economic status (Graham *et al.*, 1991; Malaty and Graham, 1994; Lindkvist *et al.*, 1998), as the highest prevalence is seen in Africa and South-America. Interestingly, within different countries the prevalence of *H. pylori* is often quite variable depending on the ethnic backgrounds of the people living there. In Australia, 43% of Ethiopians, 40% of Salvadorians and 60% of Chinese-Australians compared with 0.5% Australian Aborigines and 31% Caucasians were *H. pylori* seropositive (Lambert *et al.*, 1995).

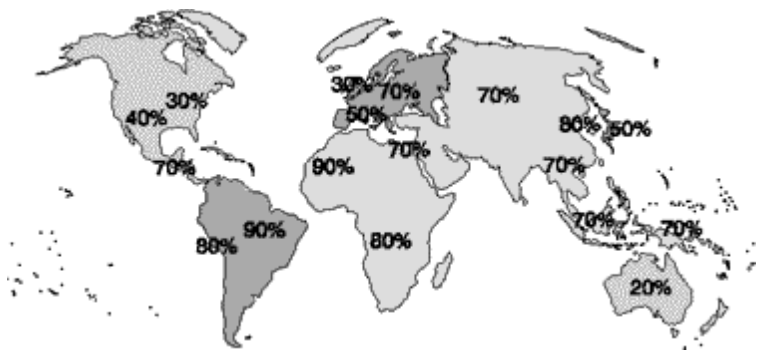

Figure 1. *H. pylori* world-wide prevalence (from http://www.helico.com/info/hpylori/hpylori-epidemiology.htm)

Although the exact mode of transmission is still unknown, it is suggested that *H. pylori* is mainly directly person-to-person transmitted, possibly through oral-oral or fecal-oral transfer (Brown, 2000). Moreover, it has been shown that person-to-person transmission occurs mainly within families, where the mother-child and sibling to sibling transmission as the most probable routes have been reported (Han *et al.*, 2000; Kivi *et al.*, 2003). Transmission through environmental sources such as drinking water has been also proposed (Enroth and Engstrand, 1995; Hulten *et al.*, 1996).

In the developing countries the prevalence in the adult population can reach to the level as high as 80–90% by their age 20 (Matysiak-Budnik and Megraud, 1994) while in the developed countries the prevalence of the infection is near 50% by the age of 50 (Glupczyski, 1996). The difference in prevalence among the young and the old populations in the developed countries is explained as a birth-cohort effect. Among adults, the primary acquisition of *H. pylori* infection is rare (Sipponen *et al.*, 1996) which means that the increased prevalence of *H. pylori* in older people is due to the poor socioeconomic conditions some 40–50 years ago in the most developed countries. Improved living conditions for children in recent years have decreased the prevalence of *H. pylori* (Roosendaal *et al.*, 1997) indicating the overall decrease in all age-groups in the coming years.

The low frequency of *H. pylori* infection in the developed countries compared to the developing countries has also been explained by the frequent use of antibiotics against *e.g.* nasopharyngeal infections during childhood (Brown, 2000), causing the extinction of *H. pylori.*

The overall prevalence of *H. pylori* in Estonia, in spite of the good industrial development, is still characteristic of the developing countries: around 80% of the adult population has been *H. pylori* positive by histological and serological methods (Vorobjova *et al.*, 1994; Maaroos, 1995; Vorobjova *et al.*, 1998b). In Estonia, among school-children the seroprevalence of *H. pylori* has been 56% (Vorobjova *et al.*, 2000) which is about 10 and 18.5 times higher than in Finland (Ashorn *et al.*, 1995) or Sweden (Granström *et al.*, 1997), respectively. However, recently it was shown that during the years 1991–2002 the infection among children has significantly decreased from 42.2% to 28.1%, expressing positive socioeconomic changes in Estonia during the past decade (Oona *et al.*, 2004).

The high prevalence of *H. pylori* is reflected in the high prevalence of severe gastric diseases in Estonia. Particularly, several epidemiological studies among two Estonian randomly selected adult populations in Kambja and in Saaremaa have been carried out since 1970s (Villako *et al*., 1976, 1982, 1990, 1991, 1995; Maaroos *et al*., 1999; Vorobjova *et al*. 2001a, 2001b), revealing the high occurrence of chronic gastritis (over 60%).

A significant increase in the perforated peptic ulcer has been noted since 1991, reaching the level 2 to10 times higher than in the developed countries (Sillakivi *et al.*, 2002). It was associated with increased social stress, smoking and the lack of modern antiulcer therapy for the patients with ulcer disease (Sillakivi, 2000). Therefore, several efforts have been made and are ahead to find the most effective treatment means to eliminate *H. pylori* infection. However, the specific type of *H. pylori* strains in Estonia has to be assessed by molecular studies.

2.2. Prevalence of *H. pylori* strains

The phylogenetic analyses, based on the sequence comparisons of 7 house-keeping genes and one virulence-associated gene (*vacA*), have shown that the distinct alleles of *H. pylori* genes exist in different geographical regions. In addition, *H. pylori* strains can be divided into the seven microbial populations and the subpopulations that originate from the ancestral populations in Africa, Central Asia and East Asia (Achtman *et al.*, 1999; Falush *et al.*, 2003). Estonian *H. pylori* strains belonged to the highly diverse European group (hpEurope) (Falush *et al.*, 2003).

Campbell *et al.* demonstrated that the *H. pylori* strains isolated from the Polynesian and European descendant populations of New Zealand were genetically distinct (Campbell *et al.*, 1997), suggesting even the race differences of bacterial colonization. The same was noted in the United States, where the prevalence of *H. pylori* was higher in the blacks (70%) than in the whites (34%) (Graham *et al.*, 1991). However, in the study of *H. pylori* infection among minority children in the United States, the prevalence was similar in white Hispanic and black children (Malaty *et al.*, 2001). A significantly higher prevalence was observed in the non-Caucasian patients when compared to the Caucasian subjects, even if both groups were born in Belgium and had been living in the same area ever since (Blecker *et al.*, 1995).

Recently, two separate research-groups have compared Malaysian *H. pylori* strains and found distinctive differences in the strain distribution among Chinese, Malaysian and Indian patients (Ramelah *et al.*, 2005; Tan *et al.*, 2005). The variation of *H. pylori* genotypes was also observed among Thai, Thai-Chinese and Chinese ethnic groups in Thailand (Vilaichone *et al.*, 2004). However, in Kazakhstan, the prevalence of *H. pylori* infection was almost identical between the two ethnic groups e.g. for Russians 79% and Kazakhs 80% (Nurgalieva *et al.*, 2002). Therefore, it is not clear if there is a true ethnic diversity or in the closed populations the circulation of specific *H. pylori* strains is apparent. The better knowledge about the global diversity of *H. pylori* and its transmission pathways would be important in the development of antibiotic therapy, vaccines for prophylactics or even diagnostic tests for the detection of *H. pylori* infection. It is possible that Estonia with its two main ethnic populations (Estonians and Russians) could provide an excellent basis for study.

3. Virulence factors of *H. pylori*

3.1. Flagella and urease

Flagella provide microbe motility to approach and invade the gastric and duodenal mucosa. The flagellar filaments of *H. pylori* comprise two protein subunits, the flagellins FlaA and FlaB (Kostrzynska *et al.*, 1991; Josenhans *et*

al., 1995). Over 60 genes are involved in the biogenesis of flagella, the assembly of the flagellar motor and the chemotaxis system (Tomb *et al.*, 1997).

The production of urease is another essential factor of *H. pylori* for the colonization of the acidic environment of the stomach. Urease hydrolyses the urea secreted by gastric cells to produce ammonia and CO_2 in order to buffer the gastric acid. Produced ammonia is a nutrient for the bacteria and at the same time toxic to human gastric epithelial cells (Smoot *et al.*, 1990). *H. pylori* urease enzyme consists of six copies of each of the structural subunits, UreA and UreB, and two nickel ions reside in each of the six active sites. For the expression of active urease, accessory urease proteins UreE-H are necessary (Mobley *et al.*, 1995). In addition, UreI is needed as a pH dependent urea membrane channel that increases the access of gastric juice urea to intrabacterial urease (Sachs *et al.*, 2000; Prinz *et al.*, 2003). The produced ammonia buffers the cytosol, periplasm and creates a neutral layer around the bacterial surface (Weeks *et al.*, 2000; Bury-Mone *et al.*, 2001; Montecucco and Rappuoli, 2001).

3.2. Vacuolating cytotoxin (VacA)

All *H. pylori* strains possess *vacA* gene that encodes the vacuolating cytotoxin (VacA), although strains vary considerably in the production of toxin (Forsyth *et al.*, 1998). VacA is an autotransporter that is synthesized as a 140-kDa precursor, which is then processed to the monomeric 95-kDa mature form that is secreted from the bacterium by the two-step process involving an amino-terminal 33 amino-acid signal peptide and a 45-kDa C-terminal region (Telford *et al.*, 1994; Lupetti *et al.*, 1996; Fischer *et al.*, 2001; Montecucco and Rappuoli, 2001).

The secreted VacA toxin has a strong tendency to assemble into flower-shaped oligomers consisting of seven monomers, each of which can be cleaved into two fragments of p37 (37-kDa) and p58 (58-kDa) (Lupetti *et al.*, 1996). The p37 has been proposed to correspond to the enzymatically active portion of VacA while p58 domain mediates cell binding and the interaction with the membrane hydrophobic core in the process of p37 translocation (Moll *et al.*, 1995; de Bernard *et al.*, 1998). However, when exposed to low pH, the VacA oligomers dissociate into the monomers that bind to epithelial cells and form hexameric ring-like channels allowing egress of anions and urea (Papini *et al.*, 1998; Czajkowsky *et al.*, 1999; Iwamoto *et al.*, 1999; Szabo *et al.*, 1999; Tombola *et al.*, 1999; Tombola *et al.*, 2001). These channels are then endocytosed and transferred to the late endosomes, activating V-ATPase by allowing the influx of Cl^-, causing NH_4^+ accumulation and therefore inducing the osmotic swelling of the late endosomes, resulting in vacuole formation (Szabo *et al.*, 1999; Boquet *et al.*, 2003).

Recently it has been shown, that VacA arrests phagosome maturation in macrophages (Zheng and Jones, 2003), selectively inhibiting the antigen presentation to T cells (Molinari *et al.*, 1998), and blocks T cell proliferation

(Gebert *et al.*, 2003), altogether resulting in the specific immune suppression necessary for *H. pylori* persistence.

Among *H. pylori* strains there is a difference in VacA cytotoxins that is caused by genotypic variation in *vacA* gene (Figure 2). One of the divergent regions is the signal region that encodes the signal peptide and the N terminus of the mature toxin. Two main signal sequence (s) types have been identified, namely, s1 (subtyped as s1a, s1b, s1c) and s2 (Atherton *et al.*, 1995; Atherton *et al.*, 1997; van Doorn *et al.*, 1998b). Type s1 VacA has a hydrophobic N terminus required for the toxin to be fully active explaining why s1 allele is correlated to more severe disease outcome, while type s2 VacA has an N-terminal extension that blocks the vacuole formation, making the s2 allele rarely to be detected in patients with PUD or gastric adenocarcinoma (Atherton *et al.*, 1997; Letley and Atherton, 2000; Letley *et al.*, 2003). Secondly, *vacA* gene varies in its mid-region, which encodes the part of the toxin-cell binding domain. Two types of mid-regions (m) are mainly detected (m1 and m2), but additional variants like m1a, m1T, m1Tm2, m1b, m1b-m2, m2a and m2b have been described (Atherton *et al.*, 1995; Pan *et al.*, 1998; Strobel *et al.*, 1998; van Doorn *et al.*, 1998b; Wang *et al.*, 1998).

The particular combination of the s and m genotype of the *vacA* gene is suggested to determine the cytotoxic activity. For example, the s1m1 alleles containing strains are strongly associated with the increased gastric epithelial damage, enhanced gastric inflammation, duodenal ulceration and gastric carcinoma (Atherton *et al.*, 1997; Miehlke *et al.*, 2000) (see pages 19–20).

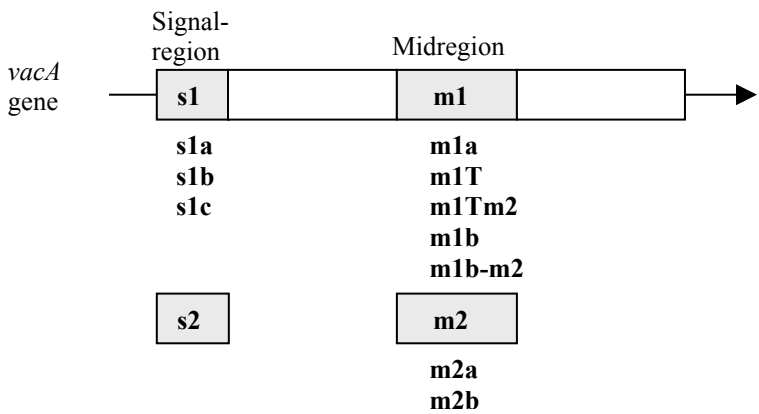

Figure 2. The genotypic variation of *H. pylori vacA* gene comprising two possible signal regions (s1 and s2) and mid-regions (m1 and m2) with presently found subtypes. The scheme has been modified from Atherton et al., 1995 and Blaser and Atherton, 2004.

3.3. Cag-pathogenicity island (cag-PAI)

The cytotoxin-associated gene A (*cagA*) is a historical name of the gene that encodes 128–145 kDa protein CagA. It has been shown that *cagA* gene is not chromosomally linked to the *vacA* gene (being hundreds of kb apart) nor needed for the expression of VacA (Tomb *et al.*, 1997; Akopyants *et al.*, 1998). However, a strong statistical linkage exists between these two genes in *H. pylori* strains (Atherton *et al.*, 1995).

The *cagA* gene is a marker for a cag-pathogenicity island (cag-PAI) of about 40 kb, that is flanked by 31 bp direct DNA repeats allowing the loss or the gain of the whole locus through homologous recombination (Censini *et al.*, 1996; Akopyants *et al.*, 1998; Kersulyte *et al.*, 1999), yet partially deleted cag-PAIs are also found in clinical isolates (Nilsson *et al.*, 2003).

The cag-PAI contains genes encoding a type IV secretion system that delivers CagA into gastric epithelial cells (Odenbreit *et al.*, 2000; Yamazaki *et al.*, 2003). Injected CagA associates with the cell membrane and becomes phosphorylated on the tyrosine-phosphorylation sites (known as EPIYA motifs) by the host cell kinases belonging to the Src (Asahi *et al.*, 2000; Selbach *et al.*, 2002; Stein *et al.*, 2002). Once phosphorylated, CagA activates an eukaryotic tyrosine phosphatase SHP-2 that affects spreading, migration and adhesion of epithelial cells (Higashi *et al.*, 2002b; Yamazaki *et al.*, 2003), causing the "hummingbird" phenotype seen *in vitro* (Segal *et al.*, 1999). Besides of the SHP-2, tyrosine-phosphorylated CagA is able to bind and activate C-terminal Src kinase (Csk) via its SH2 domain, leading to the inactivation of the Src family of protein-tyrosine kinases. Since Src family kinases are responsible for CagA phosphorylation, CagA-Csk interaction down-regulates the SHP-2 signaling pathway (that induces apoptosis in AGS cells) thus preventing the excess cell damage (Tsutsumi *et al.*, 2003). Csk inactivation causes the tyrosine dephosphorylation of the actin binding protein cortactin, inducing the rearrangements of the actin cytoskeleton (Selbach *et al.*, 2003). Moreover, independently from the tyrosine phosphorylation, CagA is able to interact with the growth factor receptor bound 2 (Grb2) both *in vitro* and *in vivo*, which results in the activation of the Ras/MEK/ERK pathway and leads to cell scattering as well as proliferation similarly to the above mentioned pathways (Mimuro *et al.*, 2002).

In addition, independently from CagA, *H. pylori* cag-PAI-mediated contact with the epithelial cell seem to induce the production and the secretion of interleukin 8 (IL-8), cytokine that plays an important role in the pathogenesis of *H. pylori* gastritis (Rieder *et al.*, 2001).

3.4. Other virulence factors

Besides most intensively studied *vacA* and *cagA* genes there are several other virulence factors that may influence *H. pylori* pathogenecity. Adherence is one of the factors that is relevant to the persistence of *H. pylori* infection. Several *H.*

pylori outer membrane proteins have been more or less described to be involved in adhesion to the host cells:

BabA

The most studied adhesin is the outer-membrane bound Bab (blood group antigen-binding protein) that binds to the fucosylated Lewis b (Le^b) histo-blood group antigen on gastric epithelial cells (Ilver *et al.*, 1998). The strains with BabA adhesin encoded by *babA2* gene appear to be clinically important (Gerhard *et al.*, 1999; Gatti *et al.*, 2005; Olfat *et al.*, 2005). The patients infected with BabA positive strains express higher levels of IL-8 and colonization densities, indicating the important role of BabA for colonization (Rad *et al.*, 2002), although not all *H. pylori* strains express BabA adhesin.

SabA

The other adhesin is *sabA* encoded SabA (sialic acid binding adhesin A) that binds to sialylated and fucosylated glycoconjugates such as sialyl-dimeric-Lewis x (Mahdavi *et al.*, 2002). *H. pylori* infection induces inflammation in the host gastric epithelium leading to an upregulation of sialyl Lewis x expression that promotes colonization through increased appropriate adhesion targets in the host tissue (Mahdavi *et al.*, 2002).

AlpA and AlpB

The adherence-associated lipoproteins AlpA and AlpB encoded by *alpA* and *alpB*, are the outer membrane proteins that may be necessary for the proper adhesion of *H. pylori* to gastric tissue *in vitro* (Odenbreit *et al.*, 1999; Odenbreit *et al.*, 2002). However, the function of AlpA and AlpB as a receptor binding adhesins is not yet proved.

HopQ

Another *H. pylori* adherence-related outer membrane protein is HopQ encoded by diverse *hopQ* gene. *H. pylori hopQ* alleles belong to different families, designated type I and type II (Cao and Cover, 2002). It was shown by Cao and Cover that type I *hopQ* alleles were present in *cag* positive strains significantly more commonly than in *cag*-negative strains, suggesting that the variance in *hopQ* genotypes may have an important role in *H. pylori* virulence.

OipA

The *oipA* (outer inflammatory protein) gene is related to the more severe clinical outcome and associated with enhanced IL-8 secretion, higher *H. pylori* density and increased inflammation (Yamaoka *et al.*, 2000; Yamaoka *et al.*, 2002).

In addition to outer membrane proteins, one of the two alleles of *iceA* (induced by contact with epithelium) gene, *iceA1* is associated with peptic ulcer evolution and increased mucosal concentrations of IL-8 (Peek *et al.*, 1998; van Doorn *et al.*, 1998c).

4. *H. pylori* strain divergences

Differences among *H. pylori* strains have been noted concerning the phenotypic markers like in vacuolating cytotoxin activity (Forsyth *et al.*, 1998), lectin reaction patterns (Hynes *et al.*, 2002) and IL-8 induction in gastric epithelial cells (Owen *et al.*, 2003).

In addition to the phenotypic divergence, the genotypic variance of *H. pylori* strains has been detected. In 1997, the first complete genome sequence of *H. pylori* (laboratory strain *H. pylori* 26695) was released by the TIGR Institute (Tomb *et al.*, 1997) and the second genome description based on the clinical isolate of *H. pylori* J99 was published 2 years later (Alm *et al.*, 1999). The comparison of the genomes of the two strains revealed that the overall genomic organization of the two strains was very similar, although, about 7% of the genes were specific to each strain (Alm *et al.*, 1999). This finding was further examined by using a whole-genome microarray analysis of 15 *H. pylori* strains, showing that each strain may possess the strain-specific genes that compose up to 18% of the genome. It was suggested that the strain-specific genes might encode adaptations to genetically diverse hosts or to the factors contributing different disease outcomes (Salama *et al.*, 2000).

In about 10% of the population the peptic ulcer disease develops during their lifetime (Schlesinger *et al.*, 1992), while the incidence of perforated peptic ulcer between 2.3 and 10.0 per 100 000/year is reported from the developed countries in the recent decades (Aeberhard *et al.*, 1990; Mäkelä *et al.*, 1992; Hermansson *et al.*, 1997).

From multiple epidemiologic studies, it has become obvious that the persons carrying *H. pylori cagA*-positive strains are at enhanced risk of developing both peptic ulcer disease and non-cardia gastric adenocarcinoma (Blaser *et al.*, 1995; Parsonnet *et al.*, 1997; Nomura *et al.*, 2002a; Nomura *et al.*, 2002b). According to Atherton *et al.*, the *cagA*-positive *H. pylori* strains are usually with *vacA* s1/m1 genotype, while *cagA*-negative are of s2/m2 type. Isolates with *vacA* s1/m2 genotype are mostly, but not definitely *cagA*-positive (Atherton *et al.*, 1995). In addition, the patients with *H. pylori vacA* s1a strains are more associated with enhanced gastric inflammation and duodenal ulceration than s1b strains while s2 strains were no more likely to have the ulcer disease than the uninfected patients (Atherton *et al.*, 1997).

However, there is a global variance in the distribution of *vacA* alleles in different ethnic populations (Van Doorn *et al.*, 1999) which might be the reason why different geographic regions give diverse results when *vacA* genotypes are tried to be linked with specific clinical diseases. The biggest differences have been noted between Asian and Western countries. The prevalence of s1a and s1c strains is high in Asia while s1b is frequent in Southern Europe, South-America, South-Africa and the United States (Ito *et al.*, 1997; Shimoyama *et al.*, 1998; Van Doorn *et al.*, 1999; Yamaoka *et al.*, 1999b; Wang *et al.*, 2003). In Asia, where s1 alleles predominate, *cagA*-positive s1a or s1c strains have not been associated with the more severe clinical outcome (Miehlke *et al.*, 1996; Pan *et al.*, 1998; Yamaoka *et al.*, 1998b; Wang *et al.*, 2003).

In a recent review it was shown (Tovey *et al.*, 2006) that there is a difference in virulence factors between the developed and the developing countries with low and high prevalence of *H. pylori*, respectively. In the countries with the low prevalence of *H. pylori* infection the *vacA* subtypes s1 and s1m1 as well as *iceA1* and *babA2* genes are strongly associated with duodenal ulceration. On the contrary, in the countries with high *H. pylori* prevalence (around 70–90%), the *cagA* and *vacA* genes have shown no relationship between these factors and the clinical diagnosis.

What kind of impacts express the different genotypes of *H. pylori* in Estonia, with its high prevalence of microbe and severe gastric diseases, needs to be elucidated.

5. Persistent infection with *H. pylori*

Long-term studies of the Estonian duodenal ulcer patients have shown that vagotomy which is an effective method in ulcer treatment, lowers acid production temporarily but does not eliminate *H. pylori* colonization in the stomach and the number of recurrent ulcers is increasing in time after the operation (Peetsalu *et al.*, 1998). Consequently, different antimicrobial treatment schemes are under a scope in order to avoid ulcer recurrences (Sillakivi *et al.*, 2001a, b). Despite the low prevalence of metronidazole and clarithromycin resistance in Estonia (Lõivukene *et al.*, 2000), the antibiotic treatment has often been unsuccessful in the complicated peptic ulcer patients (Sillakivi *et al.*, 2001a, b, c). For this reason, it is necessary to investigate other microbe or host-related reasons for the treatment failures.

Both, the wide genetic diversity of *H. pylori*, as well as clonal characteristics of *H. pylori* strains, related to their geographical origin, are evidently associated with their prolonged persistence in specific population groups. The mechanisms that are applied in establishing this high diversity of *H. pylori* include point mutations, insertions or deletions, chromosomal rearrangements, strain specific restriction-modification systems, horizontal gene transfer between strains, impaired DNA repair mechanisms, and an exceedingly high frequency of recombination (Blaser and Berg, 2001). Obviously, these continuous genetic changes must have a role in the ability of *H. pylori* to colonize, persist and cause a disease.

To establish persistent colonization, *H. pylori* possess still poorly understood features that help to evade the host immune and inflammatory responses. From the known factors, the urease production and motility, as well as multiple bacterial-surface components are essential for primary colonization (see pages 14–18).

Apparently, some phenotypical properties of *H. pylori* favor the persistence in the human host. The example of this is lipopolysaccharide (LPS) that is present in the cell wall of Gram-negative bacteria, consisting of the lipid A, an oligosaccharide core and the variable antigenic O-polysaccharide chain (Wang *et al.*, 2000). Interestingly, most *H. pylori* strains express the Lewis blood group

antigens (structures similar to those occurring in the human gastric mucosa) in their surface-expressed LPS O-antigen (Simoons-Smit *et al.*, 1996; Monteiro *et al.*, 1998; Monteiro *et al.*, 2000a; Monteiro *et al.*, 2000b). The exact role of these Lewis antigenic structures during *H. pylori* infection is unclear. However, it is suggested that *H. pylori* Lewis antigens have a role in adhesion, colonization and immune evasion through mimicking the host blood group antigens expressed on the gastric mucosa (Sherburne and Taylor, 1995; Moran *et al.*, 1996; Wirth *et al.*, 1997; Heneghan *et al.*, 2000).

Clinical relevance of persistent H. pylori infection

Although a majority of *H. pylori*-infected people are asymptomatic, *H. pylori* infection is strongly associated with chronic gastritis and the peptic ulcer disease. The increasing number of studies concentrate on *H. pylori* role in development of gastric cancer and MALT-lymphoma (Graham, 1997; Parsonnet, 1998; Ikeno *et al.*, 1999; Blaser and Berg, 2001; Peek and Blaser, 2002). According to the Maastricht Consensus Report 1 from the year 1996 and updated Maastricht Consensus Report 2 from 2000, *H. pylori* eradication therapy is strongly recommended for all the ulcer patients with confirmed infection (Malfertheiner *et al.*, 2002).

In Western populations the rate of recurrent infection with *H. pylori* after successful eradication is very low (0.5% to 2.0% per year) in adults (Bell *et al.*, 1993; Berstad *et al.*, 1995; Bell and Powell, 1996; van der Hulst *et al.*, 1997). At the same time, in the developing countries with a high prevalence of *H. pylori* it is ranging between 1% in China and 73% in Peru (Coelho *et al.*, 1992; Ramirez-Ramos *et al.*, 1997; Kim *et al.*, 1998; Mitchell *et al.*, 1998; Gunaid *et al.*, 2004). The latter could have been predicted in the communities with high *H. pylori* prevalence.

Unfortunately, the methods used in most of this type of studies (usually urease tests) to detect recurrent *H. pylori* infection do not exactly allow the discriminating recrudescence of the original strains from the true reinfection. Accurate and sensitive genomic fingerprinting techniques are required to help determine if the most common cause of the treatment failure is the insufficient treatment or reinfection with a new strain. At the same time, there is not much known about the influence of the persistence or replacement of *H. pylori* strains with different virulence to the clinical outcome of gastric diseases during the long-lasting infection.

6. Diagnosis of *H. pylori* infection

For the diagnostics of *H. pylori* infection the invasive and non-invasive tests can be chosen. Invasive methods require endoscopy and sampling of the biopsy specimens that can be used for histological examinations, the culture of the bacterium or the direct molecular examination. Non-invasive methods include antigen/antibody tests, the urea breath test (UBT) or the molecular detection from non-invasively acquired samples. There is no single gold standard

(although histological examination is still considered as one of the best) method without limitations, making it in certain clinical situations important to select the most reliable result providing one single test or the complex of two or more tests.

6.1. Histological examination

Different techniques of staining (hematoxyllin-eosin, Giemsa, silver staining) are routinely available for the detection of spiral or curve-shaped *H. pylori* in the tissue sections of the formalin processed and paraffin embedded gastric mucosa specimens (Rotimi *et al.*, 2000).

The disadvantage of this technique is the need for endoscopy to obtain tissue samples. In addition, the accuracy of histology for the detection of *H. pylori* may depend on the adequate number of gastric biopsies, rightly chosen biopsy sites or on skills of pathologist. The advantage of histology contains besides the detection of *H. pylori* and its colonization density the information about morphological changes in the gastric mucosa demonstrating gastritis, atrophy, intestinal metaplasia, dysplasia or malignancies (Megraud, 1997).

6.2. Culture

A large number of different agar media for recovering *H. pylori* have been described, all suitable for the cultivation and the isolation of the pathogen (Goodwin and Armstrong, 1990; Ansorg *et al.*, 1991; Glupczyski, 1996). Still, culturing of *H. pylori* is difficult, time consuming, expensive and it is seldom required for the routine clinical practice, although helpful in determining the antimicrobial susceptibility of *H. pylori* for the planned treatment. The sensitivity of this method is dependent on bacterial density, transport conditions, culture medium, incubation conditions, and the skill of laboratory (Lõivukene *et al.*, 2000; Krogfelt *et al.*, 2005). Experienced laboratories are able to culture *H. pylori* from the fresh or appropriately frozen tissue nearly 100% of the time (Graham *et al.*, 2005). In addition, culturing is often considered the mandatory step for further molecular investigations in the research field.

6.3. Urease tests

Urease tests depend on the *H. pylori* production of enzyme urease to break down urea for the production of CO_2 and ammonia. This test is able to detect only the presence or the absence of the *H. pylori* infection without any additional information about the bacterial properties.

In case of the invasive rapid urease test, the biopsy specimen is placed in a media containing urea and a pH indicator. The presence of urease is detectable by the color change of the pH indicator dye. The test is quick, simple and with

the sensitivity around 90% and the specificity near 100% (Malfertheiner *et al.*, 1996) with the only limitation of its need for endoscopy.

The non-invasive UBT applies either non-radioactive ^{13}C- or radioactive ^{14}C-labeled urea that is given to a patient. In the presence of *H. pylori* the $^{13}CO_2$ or $^{14}CO_2$ will be released from urea and detected in breath samples. The UBT is recommended for the diagnosis of the infection in primary care as well as for the confirmation of *H. pylori* eradication after treatment (Malfertheiner *et al.*, 2002). The first disadvantage of the UBT is the poor accessibility due to the expensive equipment and diagnostic probes, especially in the developing countries. The second problem is concerned with false-positivity with the urea producing strains of "*H. heilmannii*" (Solnick, 2003).

6.4. Antigen/antibody tests

Serum antibodies to *H. pylori* are usually detected by the enzyme-linked immunosorbent assay (ELISA) or latex agglutination tests (Williams, 1997). These tests are generally simple, reproducible, inexpensive, and can be done on stored samples. They are widely used in epidemiological studies. However, as antibody titers fall slowly after successful eradication, serology cannot be used to evaluate the eradication success or to measure reinfection rates. Yet, there are several new tests available that are providing fast and accurate diagnostics like detecting IgG antibodies in urine with better results than serum ELISA (Krogfelt *et al.*, 2005).

Recently, the stool antigen tests have come to use for the primary diagnosis of *H. pylori* infection with relatively high sensitivity and specificity (Andreson *et al.*, 2003; Li *et al.*, 2004; Krogfelt *et al.*, 2005). Similarly to the UBT, stool antigen tests can only indicate the presence or the absence of the infection without any additional information about possible morphological changes in gastric mucosa and characterization of the particular *H. pylori* strain.

6.5. Molecular methods

Molecular methods, primarily PCR (polymerase chain reaction) based, allow detecting *H. pylori* from invasively or non-invasively obtained clinical materials, e.g. directly from gastric biopsies (Hammar et al 1992, Owen et al 1994, Mapstone 1997), from saliva (Ferguson et al 1993), dental plaque and faeces (Makristathis et al 1998). The PCR method allows *H. pylori* detection from small clinical samples with a low density of bacteria and it is relatively cheap. Moreover, PCR can be performed to the detect virulence markers of *H. pylori* such as the *vacA* s and m subtypes and *cagA* gene, allowing a rapid determination of hypothetically high-risk patients for developing a peptic ulcer (Peek et al 1995, Atherton et al 1995, van Doorn et al 2000). However, the validity of such investigations in the countries with a high prevalence of *H. pylori* has not been clearly assessed.

Different DNA fingerprinting methods for typing and discriminating *H. pylori* strains are available and widely used in both epidemiological and clinical studies. The ribotyping, the PCR-based restriction fragment length polymorphism (PCR-RFLP) and the random amplified polymorphism DNA (RAPD) analysis, PCR-DNA sequencing, PFGE (pulsed-field gel electrophoresis), etc. have been applied and validated in different studies (Ge and Taylor, 1998; Burucoa *et al.*, 1999). Recently, a new real-time PCR assay was developed that allows rapid detection of *H. pylori* with mutations to clarithromycin susceptibility directly from stool and biopsy specimens showing nearly 100% sensitivity and specificity (Schabereiter-Gurtner *et al.*, 2004). All these molecular methods are developing rapidly providing more accurate information for the diagnostics of *H. pylori* infection.

The application of DNA, extracted directly from gastric biopsy specimens for genotyping, has shown similar results to bacterial DNA from cultures (Park *et al.*, 2003). However, it is not yet known how well the molecular methods work in the direct tissue samples of gastric mucosa from the patients with a late post-treatment setting.

* * *

Accordingly, there are unsolved questions concerning the presence of different genotypes of *H. pylori.* Whether the genotypic diversity of *H. pylori* is more related to the geographic distribution of the strains, the ethnicity of the host or the specific clinical diagnose, needs to be elucidated. Particularly, it is not clear yet how well the common virulence markers like *cagA* and *vacA* genes of *H. pylori* can be associated with chronic inflammatory gastric diseases, especially in the countries with a high prevalence of the infection.

In addition, although it is well known that the colonization of *H. pylori* could be long lasting and even life-long and that the eradication of the infection is often unsuccessful, it is not clear if the persistent infection is caused by the reinfection or by the persistent strains. In the latter case it needs to be resolved what kind of virulence markers the *H. pylori* strains carry.

AIMS OF THE STUDY

The main goal of the study was to assess if there are specific strains of *H. pylori* circulating in Estonia that are responsible for the different chronic inflammatory gastric diseases.

Accordingly, the aims of the present study were:

1. To compare the presence and the distribution of *H. pylori* virulence markers (*cagA* and *vacA*) in the patients suffering from the chronic gastritis, the peptic ulcer disease and its complication — the perforated peptic ulcer.

2. To study the diversity of *H. pylori* strains by comparing the genomic variation of *H. pylori* in the gastric mucosa samples obtained from the Estonian and the Russian perforated peptic ulcer patients living in Estonia.

3. To assess the diagnostic value of the PCR method in the patients with a late post-treatment setting, applying the tissue samples of gastric mucosa.

4. To evaluate the presence of persistent infection or reinfection with *H. pylori* in the tissue samples of gastric mucosa in patients with the perforated peptic ulcer after surgery and antimicrobial treatment in one-year follow-up.

MATERIAL AND METHODS

1. Subjects and material

In the present thesis the biopsy samples of the gastric mucosa were analyzed by molecular methods with various primers. The results were compared with the clinical and histological data obtained by the medical doctors diagnosing and treating the patients with various diagnoses in the course of collaborative research.

The clinical data (results of endoscopic examination, histology of gastric mucosa, diagnosis and treatment) of the patients with chronic gastritis (CG) and the patients with the peptic ulcer disease (PUD) came from the research database of the Department of Polyclinic and Family Medicine and for patients with the perforated peptic ulcer (PPU) disease from the research database of the Surgery Clinic of Tartu University Hospital (Figure 3, Table 1). The medical doctors of aforementioned units selected the patients, performed the endoscopical procedures, assessed their clinical status and evaluated the histological samples of gastric mucosa. Different treatment procedures were performed.

All the studies (Papers I to IV) had an approval from the Ethics Review Committee on Human Research of the University of Tartu and in every case an informed consent was obtained from each participant.

The schematic presentation of the division of the patients of different gastric diseases in Papers I to IV is depicted in Figure 3.

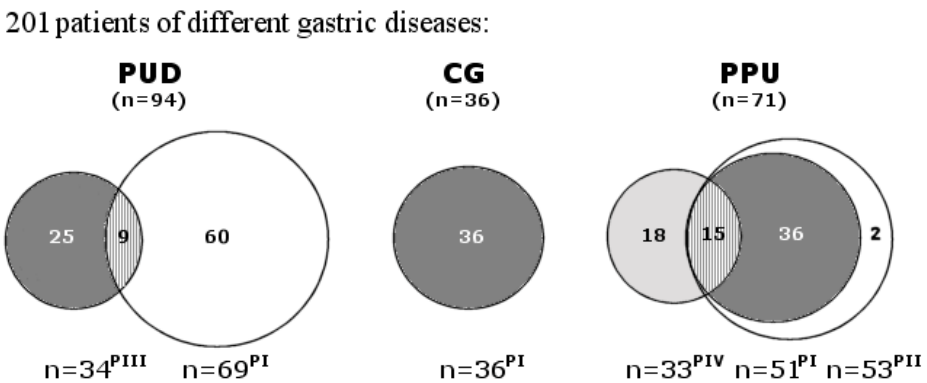

Figure 3. Inclusion of the patients from different investigations. Roman numerals as subscript on patients' numbers indicate the number of original papers (PI-IV).

Table 1. Study subjects.

Aims	No of patients	Diagnosis	Material	Methods	Original papers
Comparison of the presence and the distribution of *H. pylori* virulence markers in different gastric diseases	69* 36 51	PUD CG PPU	*H. pylori* isolates and biopsy samples	PCR of *cagA* and *vacA* genes	I
Detection of the diversity of *H. pylori* among Estonians and Russians living in Estonia	53	PPU	Biopsy samples	Histological grading of *H. pylori* density and PCR of *cagA* and *vacA* genes	II**
Assessment of the diagnostic value of the PCR method in patients with a late post-treatment setting	34	PUD***	Biopsy samples and breath samples	^{13}C- UBT, PCR of *glmM* gene, histological, bacteriological and cytological assessment of *H. pylori.* PCR of *cagA* and *vacA* genes	III Present study
Evaluation of the presence of persistent infection or reinfection with *H. pylori* after surgery and antimicrobial treatment in one-year follow-up	33[#]	PPU	Biopsy samples	Histological grading of *H. pylori* density, *cagA* and *vacA* detection (PCR) and strain typing (PCR-RFLP)	IV

* PUD patients from Paper I includes 9 patients from Paper III
** Paper II includes 51 PPU patients from Paper I and 15 patients of Paper IV
*** After 5-years follow-up
[#] 1-year follow-up study, altogether 99 samples from 3 different time-points.

Paper I: Comparison of the virulence markers of *H. pylori* in patients with different gastric diseases. Biopsy samples from 156 adult Estonian patients with CG, PUD and PPU were collected and investigated between the years 1995 and 2000. The 105 patients suffered from CG and PUD while in 51 patients the PPU was diagnosed.

Paper II: Comparison of the genotypes of *H. pylori* in Estonian PPU patients belonging to different nationalities. Altogether 53 Estonian patients with PPU whose both parents could be identified as being of the same nationality (Estonians or Russians) were included within the cooperative research in 1997–1999.

Paper III: Assessment of the diagnostic value of the PCR method in diagnosis of *H. pylori* infection in a late post-treatment setting. In our study the 34 patients were recruited from the group of PUD patients who had been treated by 7-day triple therapy with metronidazole, amoxicillin and omeprazole in 1996 (Maaroos *et al.*, 2001). The inclusion criteria for this study group were the persistent upper abdominal pain as the predominant complaint and the compliance with all the investigations (clinical symptoms, ^{13}C-UBT, PCR, endoscopy, biopsy, bacteriology and cytology) 5 years after the treatment.

Paper IV: Detection of the presence of either persistent infection or reinfection with *H. pylori* in PPU patients after surgery and eradication therapy. Altogether 33 PPU patients were selected from the consecutive patients operated for PPU and observed clinically during the period 1997–2001. The inclusion criteria were: the agreement to participate in the study and the availability of three consecutive biopsy samples of gastric mucosa during the one-year period (initial, 2–5 months and 1 year later), the first probe being *H. pylori* positive as detected by histology and PCR.

The perforated ulcers were classified in 2 cases as gastric ulcers and in 31 cases as duodenal (including praepyloric, pyloric and bulbar) ulcers. The surgical therapy of 18 patients included definitive (truncal vagotomy combined with drainage procedure) and in 15 patients non-definitive (ulcer excision or suturation) operations. In the latter case, the traditional triple therapy (amoxicillin, metronidazole or clarithromycin, and omeprazole) was applied for 7 days. In the vagotomised patients modified antimicrobial treatment schemes without omeprazole were used for 5 to 7 days. Eradication was defined as a negative histological finding of *H. pylori* during the one-year follow-up.

2. Methods

2.1. ^{13}C-urea breath test

The material for ^{13}C-UBT tests was collected by medical doctors within the cooperative research described in Paper III. The subjects made a baseline breath and after that passed ^{13}C-UBT drinking 100 mg ^{13}C-urea; the test meal was citric acid and the time of specimen collection was 30 min. The test was provided, according to a standard protocol, from the Helsinki Central Laboratory, Finland. The ratio of $^{13}CO_2$ to $^{12}CO_2$ in expired breath was measured by mass spectrometry and expressed in ml/mmol/kg (δ). An automated breath ^{13}C

analyzer (ABCA) with chromatographic purification and a single inlet isotope ratio mass spectrometer (IRMS) were used. A difference from the baseline value of 5‰ in the content ($\delta^{13}C$) was considered positive for *H. pylori* infection.

2.2. Histological examination

Gastrobiopsy specimens from the antrum and corpus mucosa were collected, fixed in neutral buffered formalin solution and embedded in paraffin. The paraffin sections were stained using haematoxylin-eosin and the Giemsa methods. The mucosal specimens were evaluated histologically according to the Sydney classification: the presence of neutrophil infiltration, chronic lymphocytic inflammation, surface epithelial damage, atrophy, intestinal metaplasia, and lymphoid follicles (Misiewicz *et al.*, 1990).

The colonization density of *H. pylori* was evaluated on a three-grade scale as described earlier (Glupczynski, 1996): grade 0, absence of *H. pylori*; grade 1 (low or mild), <20 microbes per field; grade 2 (moderate), 20–60 microbes per field; grade 3 (high or severe), >60 microbes per field (Papers II and IV).

2.3. Bacteriological examination

Specimens from the gastric antrum and corpus mucosa were placed into the Stuart Transport Medium (*Oxoid*) and taken to the laboratory within two hours for bacteriological examination (Papers I and III). The biopsy samples were homogenized with sterile glass powder and under a stream of CO_2 and diluted in the Brucella broth (*Oxoid*). *H. pylori* was isolated on the Columbia Agar Base supplemented with 7% horse blood and 1% Vitox (*Oxoid*) or Isovitalex (*BBL*). The plates were incubated for 3–7 days at 37°C under microaerobic conditions (*CampyBak, BBL or CampyGen, Oxoid*). *H. pylori* was identified by Gram staining, colony morphology and by oxidase, catalase and urease reactions (Chapin, 1995). The sensitivity of the isolated *H. pylori* strains to clarithromycin (Paper III) was estimated by E-test (*Oxoid*). The antibiotic cut-off points employed for the E-test were 1.0 mg/l (NCCLS, 2002).

2.4. Cytological examination

One specimen was used for imprinting the cytology slides from the antrum and corpus mucosa, fixed with 96% ethanol and stained by Acridine Orange (*Difco, BBL*) (Bernhardt and Knoke, 1967). The cytological specimens were studied under a fluorescence microscope (AXI Phot 2) where the morphotypes and the density of bacterial colonization were evaluated. A positive cytological diagnosis was based on the presence of typical helical *H. pylori* cells on the gastric mucosa and in the mucus layer (Paper III).

2.5. Molecular methods

For *H. pylori* isolation most of the biopsy samples from CG and PUD patients (Paper I) were placed into the Stuart Transport Medium and taken to the laboratory within 2h. Biopsy samples from PPU (Papers I, II and IV) and PUD patients (Paper III) were placed directly into the lysis buffer (200mM Tris-HCl [pH 8.0], 25 mM EDTA, 300 mM NaCl, 1.2% sodium dodecyl sulfate) and stored at –20ºC. The further procedures either about *H.* pylori cultivation and DNA extraction or *H. pylori* DNA extraction directly from the frozen gastric biopsy specimen, are described in Paper II.

For the **PCR analysis** of the s and m regions of *vacA* gene and for the detection of the *cagA*, *ureA* and *glmM* gene, the primers shown in Table 2 were used. The conditions of the reaction mixture and thermal cycling are described in Papers II and IV. The PCR products were identified by electrophoresis on 2% agarose gels.

For the **PCR-RFLP analysis** (Paper IV), a 1,169-bp PCR product of *glmM* gene was ethanol precipitated (Li *et al.*, 1997). The pellets were washed with 70% ethanol, and dissolved in 5 µl of water. The obtained purified PCR products were digested with the restriction enzyme HhaI (*Fermentas AB, Lithuania*) with Tango buffer at 37ºC for 20 hours according to the protocol provided by manufacture. The digested DNA fragments were analyzed by electrophoresis on 3.5% agarose gels (*Agarose MP, Roche Diagnostics, Germany*).

2.6. Statistical analysis

The data analysis was performed using the SigmaStat 2.0 (*Jandel Corporation,* USA) or Statistica 6.0 (*StatSoft Inc., USA*) software programs. According to the data the Fisher's exact test, the Chi-square test, the Student t-test or the Mann-Whitney rank sum test were applied. Differences were considered statistically significant for p values less than 0.05.

Table 2. Primers used in papers I–IV

Amplified region	Primer name	Primer sequence (5'-3')	Product size (bp)	Source of reference	Original paper
glmM	forward	AAGCTTTTAGGGGTGT TAGGGGTTT	294	(Bickley *et al.*, 1993;	III
	reverse	AAGCTTACTTTCTAAC ACTAACGC		Lu *et al.*, 1999)	
glmM	ureC-U	AAGAAGTCAAAAACGC CCCAAAAC	1,169	(Li *et al.*, 1997)	IV
	ureC-L	CTTATCCCCATGCACG ATATTCCC			
ureA	HPU1	GCCAATGGTAAATTAG TT	411	(Clayton *et al.*, 1992)	IV
	HPU2	CTCCTTAATTGTTTTTA C			
cagA	D008	ATAATGCTAAATTAGA CAACTTGAGCGA	297	(Covacci and	I, II, IV
	R008	TTAGAATAATCAACAA ACATCACGCCAT		Rappuoli, 1996)	
vacA				(Atherton *et al.*, 1995)	
s1	VA1-F	ATGGAAATACAACAAA CACAC	259		I, II, IV
	VA1-R	CTGCTTGAATGCGCCA AAC			
s1a	SS1-F[a]	GTCAGCATCACACCGC AAC	190		I, II, IV
s1b	SS3-F[a]	AGCGCCATACCGCAAG AG	187		I, II, IV
s2	SS2-F[a]	GCTAACACGCCAAATG ATCC	199		I, II, IV
m1	VA3-F	GGTCAAAATGCGGTCA TGG	290		I, II, IV
	VA3-R	CCATTGGTACCTGTAG AAAC			
m2	VA4-F	GGAGCCCCAGGAAACA TTG	352		I, II, IV
	VA4-R	CATAACTAGCGCCTTG CAC			

[a] Used in combination with primer VA1-R

RESULTS

1. *H. pylori* genotypes in patients with chronic gastritis, the peptic ulcer disease and the perforated peptic ulcer

The Estonian patients with CG, PUD and PPU were applied for the detection and the comparison of the distribution of *cagA* and *vacA* genes of *H. pylori*. Remarkably, among the 156 patients infected with *H. pylori* strains, no s1b strains were found. Multiple *H. pylori* strains with different *vacA* subtypes were detected in 5 (3%) of the 156 patients studied and therefore they were excluded from further analysis, leaving 151 patients.

The high prevalence (87%) of the *cagA* gene was detected in 132 patients out of the 151 samples (35 from CG, 66 from PUD and 50 from PPU patients). Concerning *vacA* subtypes, the s1a/m1 allelic combination was the most frequent (65%), whereas combinations of s1a/m2 and s2/m2 were found in 24% and 11% of cases, respectively. The presence of the *cagA* gene related well with the *vacA* signal sequence type s1a, whereas the s2 type was predominantly found in *cagA*-negative strains ($p<0.001$).

The relationship between the *cagA* status, the *vacA* subtypes and the patient's disease is shown in Table 3. In CG and PUD patients all *cagA*-negative isolates were associated with s2/m2 genotype. In contrast for the PPU group, four *cagA*-negative isolates exhibited the *vacA* genotype s1a and, conversely, one *cagA*-positive isolate exhibited the *vacA* genotype s2. However, statistically the PPU group did not differ from the CG and PUD groups ($p>0.05$), demonstrating the same tight relation between *cagA*-positivity and *vacA* s1a type.

Table 3. Association between *H. pylori*'s *cagA* and *vacA* status among different patient groups ($p>0.05$).

vacA genotype	No. (%) of isolates [a]					
	CG (n=35)		PUD (n=66)		PPU (n=50)	
	cagA+ (n=29)	*cagA*– (n=6)	*cagA*+ (n=62)	*cagA*– (n=4)	*cagA*+ (n=41)	*cagA*– (n=9)
s1a/m1	23 (79%)	0	43 (69%)	0	29 (71%)	3 (33%)
s1a/m2	6 (21%)	0	19 (31%)	0	11 (27%)	1 (11%)
s2/m2	0	6 (100%)	0	4 (100%)	1 (2%)	5 (56%)
Total	29 (100%)	6 (100%)	62 (100%)	4 (100%)	41 (100%)	9 (100%)

[a] CG, chronic gastritis; PUD, peptic ulcer disease; PPU, perforated peptic ulcer

2. *H. pylori* strains in Estonian and Russian patients with perforated peptic ulcer

The genomic variation of *H. pylori* was studied in the patients with PPU, living in Estonia but belonging to different nationalities. Out of 53 investigated patients, *H. pylori* was detected by PCR in 51 PPU patients (96%). In one Estonian male with a *cagA* positive sample, multiple *vacA* gene subtypes (s1a/m1/m2) were found simultaneously and the patient was excluded from further analysis, leaving 50 patients.

No differences were observed in the distribution of *cagA* positive or negative markers between 32 Estonian and 18 Russian patients with PPU, as the *cagA* gene was revealed in 81% (26 of 32) and 83% (15 of 18) of cases, respectively **(Fig. 1. Paper II)**. In contrast, the distribution of *vacA* subtypes was different in the gastric samples of our Estonian and Russian patients (Figure 4). The s1a/m1 subtype was found in 75% (24 of 32) of Estonians but in only 44% (8 of 18) of Russians. At the same time, the s1a/m2 subtype was more frequent in Russians (44%, eight cases) than in Estonians (13%, four cases). The s2/m2 subtype was detected nearly equally, in four (13%) Estonians and two (11%) Russians.

The comparison of the distribution of the three *vacA* subtypes in the gastric mucosa samples of the PPU patients of different nationalities using the Chi-square test showed significant difference (p=0.037). Mainly, the difference existed in the prevalence of s1a positive patients where the m1 subtype was clearly prevalent in Estonians but the m1 and m2 subtypes were equally distributed in Russians.

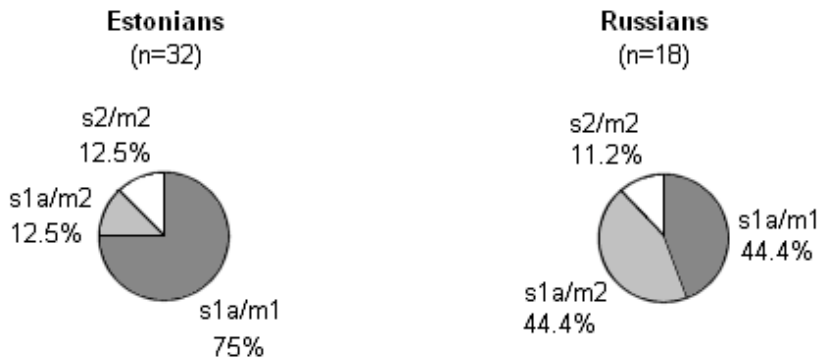

Figure 4. Significant difference in the distribution of *H. pylori vacA* subtypes in Estonian and Russian PPU patients (p=0.037).

3. PCR method for *H. pylori* diagnostics using direct samples of gastric mucosa

The reliability of PCR as a diagnostic tool was evaluated, using direct gastric mucosa samples from the patients of a late post-treatment setting. Five years after the treatment of PUD, 34 patients were investigated with invasive and non-invasive methods (^{13}C-UBT, PCR, endoscopy, histology, bacteriology and cytology) to detect *H. pylori* infection. Half of the investigated patients (17) were *H. pylori* positive by ^{13}C-UBT, PCR and histology. By bacteriological examination there was a non-concordance only in one case that was *H. pylori* positive both by PCR and histological tests, but negative by bacteriological examination (Table 4).

Table 4. Comparison of the test results in *H. pylori* positive and negative cases

Patients n=34	PCR		Histology		Bacteriology		Cytology	
	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)
^{13}C-UBT (+) n=17	17	–	17	–	16	1	4*	–
^{13}C-UBT (–) n=17	–	17	–	17	–	17	Diverse forms of the bacteria	–

(+) *H. pylori* positive samples
(–) *H. pylori* negative samples
* typical morphology of *H. pylori* (the other cases showing diverse forms of bacteria)

Cytological examination failed, as it was possible to detect the typical morphology of *H. pylori* only in 4 patients (Table 4).

By histological examination, statistically significant differences ($p<0.05$) in gastric mucosal findings (activity of neutrophil polymorphs, chronic inflammation, surface epithelial damage, glandular atrophy, intestinal metaplasia both in the antrum and corpus mucosa, and lymphoid follicles only in antrum mucosa) in *H. pylori* positive *vs.* negative patients were detected (Figure 5). No statistically significant differences were seen in glandular atrophy or intestinal metaplasia either in antrum or corpus mucosa samples as well as in lymphoid follicles of corpus mucosa.

In addition, in the present study, the distribution of *H. pylori vacA* alleles and *cagA* gene was examined in parallel in the gastric corpus and the antrum mucosa samples of all the 34 patients (except in one case that lacked the corpus sample). We found no differences in the presence and the distribution of *cagA* and *vacA* genes between those samples. Out of 17 *H. pylori* positive samples, 15 (88%) were of s1a subtype, most in the combination with m1 subtype. With

the exception of one s1a/m2 subtype, 14/15 (93%) were *cagA* positive. Only 2/17 (12%) samples were with *cagA*-negative s2/m2 subtypes.

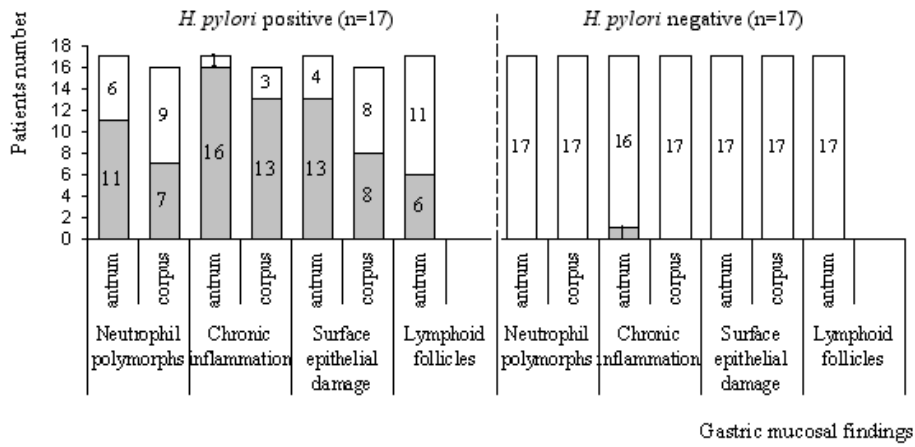

Figure 5. Significant differences in the gastric mucosal findings of *H. pylori* positive and negative cases ($p<0.05$). Grey bars indicate the number of cases with mucosal findings; white bars indicate the number of cases without mucosal findings.

4. Persistence of *H. pylori* infection in perforated peptic ulcer patients

The presence of persistent infection or re-infection with *H. pylori* was evaluated in PPU patients (n=33) in one-year follow-up after surgery and the anti-microbial treatment. By histological investigation the follow-up of *H. pylori* eradication showed that in 21 patients out of 30 (70%) after 2–5 months and in 23 patients (77%) one year after the treatment *H. pylori* was still present in mucosal samples (3 patients with unavailable histology data were excluded from this analysis). The patients of the triple therapy group showed slightly better results of *H. pylori* eradication than the group of the modified therapy (6/15, 40% *vs*. 1/15, 7%).

Similarly, by PCR the *H. pylori* positivity was found in 19 (58%) patients out of 33 after 2–5-month-follow up and in 23 (70%) patients after one year.

At 2–5 months after therapy the histologically assessed colonization density was temporarily reduced in both treatment groups, however, by the time of the final evaluation it had increased in the group that had received modified treatment ($p<0.05$) (Figure 6).

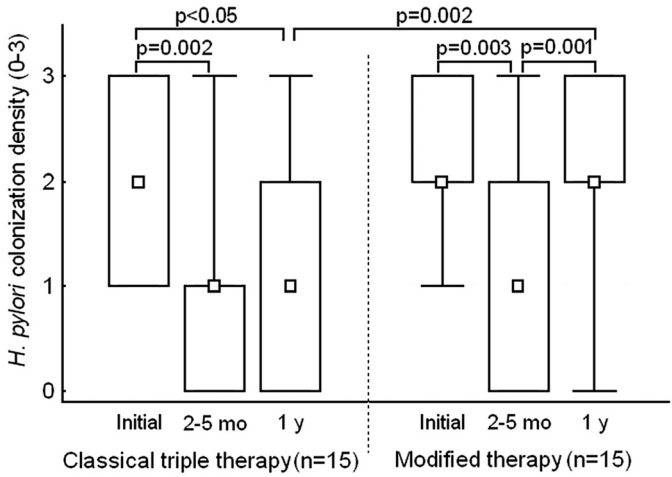

Figure 6. *H. pylori* colonization density in PPU patients during one-year follow-up in the case of classical and modified therapies. Initial biopsy samples before therapy, 2–5 months (mo), and one year (y) after therapy. *H. pylori* density grades are shown as boxes: internal points, medians; tops and bottoms of boxes, 75th and 25th percentiles, respectively; upper and lower bars, 90th and 10th percentiles, respectively.

The recurrent duodenal ulcer was found in 3 patients out of 33 (9%) representing both treatment groups.

Initially, the majority of the patients 31/33 (94%) were infected with *cagA* positive strains of the *vacA* s1a allelic type**.** The distribution of the *vacA* subtypes, comprising 5 different allelic combinations, is shown in Table 5. At the first and at the last follow-up, the infection with the same *vacA* subtypes persisted in most of the patients, 19/33 (58%). Only in 4/33 (12%) patients possible reinfection with the *H. pylori* of virulence markers different from the initial ones was detected.

We have fingertyped the initial and the follow-up samples from 10 patients: 9 belonged to the group of persistent *H. pylori* with similar virulence markers and one patient to the group of reinfection with different markers. The latter patient, initially with the *H. pylori* s1a/m1 strain, showed different patterns both by PCR (s1a allele without the m allele) and PCR-RFLP (Table 5; Figure 7, patient 5). Among the 9 patients with similar virulence markers, the overwhelming majority (8/9) displayed no differences in the restriction pattern between the initial biopsy sample and the follow-up sample (Figure 7, patients 1, 3, 4 and 6–10). Only in one case the PCR-RFLP patterns of the follow-up samples were different from the initial ones due to the variance of one band (Figure 7, patient 2).

Thus, the fingertyping clearly confirmed the persistence of the initial *H. pylori* strains with certain virulence markers detected by PCR in tissue samples.

Table 5. The dynamics of *H. pylori* infection and strains in the follow-up samples of the PPU patients.

The dynamics of the infection	n=33*	*H. pylori vacA* subtypes in follow-up samples		
		1 (operation)	2 (2-5 months)	3 (1 year)
Eradication of *H. pylori*	1	s1a/m1	s1a/m1	neg
(n=10)	4	s1a/m1	neg	neg
	4	s1a/m2	neg	neg
	1	s2/m2	neg	neg
Persistent *H. pylori*	8^5	s1a/m1	s1a/m1	s1a/m1
(n=19)	3^2	s1a/m2	s1a/m2	s1a/m2
	1^1	s2/m2	s2/m2	s2/m2
	1	s1a	s1a	s1a
	2	s1a/m1/m2	s1a/m1/m2	s1a/m1/m2
	4^1	s1a/m1	neg	s1a/m1
Reinfection	1	s1a/m1	s1a/m1	s1a/m2
(n=4)	2^1	s1a/m1	s1a/m1	s1a
	1	s1a/m1	neg	s1a/m2

* The superscript indicates the number of patients enrolled for PCR-RFLP analysis.

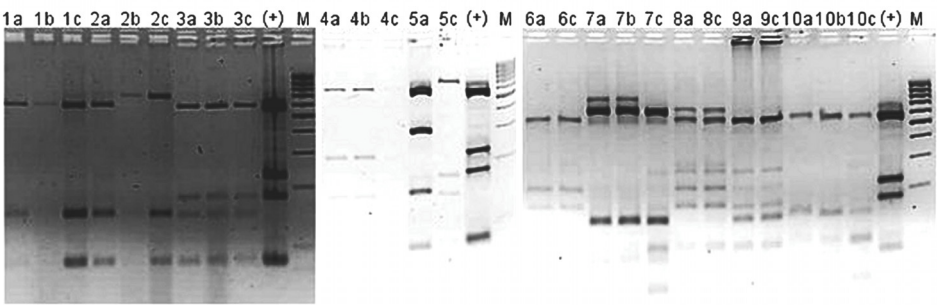

Figure 7. RFLP patterns of the follow-up samples from 10 PPU patients. (a) Before therapy, (b) 2–5 months after therapy and (c) one-year follow-up; the patients are indicated by numerals. Lane M is the 100bp DNA ladder (*Fermentas AB, Lithuania*) and lane (+) is the *H. pylori* strain NCTC 11637 as the positive control.

DISCUSSION

This study summarizes our research on *H. pylori* genotypes in Estonian patients of different gastric diseases in a population with the high prevalence of *H. pylori*. We found the geographic diversity of *H. pylori* genotypes circulating among patients with chronic inflammatory gastric diseases in Estonia. Moreover, the distribution of the *H. pylori* genotypes was characteristic of different ethnic groups like Estonians and Russians, living in the same geographical area. The particular *H. pylori* genotypes were similarly distributed among patients with chronic gastritis, peptic ulcer and perforated peptic ulcer, yet.

1. *H. pylori* genotypes in Estonia

Different roles of the well-accepted molecular virulence markers of *H. pylori*, *cagA* and *vacA* genes, in different gastric diseases have been set for populations with high and low prevalences of *H. pylori* infections (Lee, 1999). We attempted to determine whether the same set of virulence markers could be valid for a population with a high prevalence of *H. pylori*. In Estonia, according to serological studies, *H. pylori* prevalence is 87% in adults and 56% in children (Vorobjova *et al.*, 1994; Vorobjova *et al.*, 2000).

Previously, the high prevalences of the CagA in the populations of Estonian adults (63%) and children (46%) have been demonstrated by the detection of antibodies against CagA protein. (Vorobjova *et al.*, 1998b). In our study, the prevalence of *cagA* gene in CG, PUD and PPU patients was significantly ($p<0.001$) higher than their reported data (81–94% *vs.* 63%) of the prevalence of the CagA antibodies detected in healthy adults (Vorobjova *et al.* 1998). Evidently, even in the populations with a high prevalence of infection, the *cagA* gene of *H. pylori* can be associated with the *H. pylori* derived chronic inflammatory gastric diseases as gastritis and the peptic ulcer disease.

In the countries with a low prevalence of *cagA* positivity (Atherton *et al.*, 1995; Atherton *et al.*, 1997; Rudi *et al.*, 1999), a significant correlation between *vacA* subtypes and *cagA* status has been demonstrated. However, in Asian patients with the high prevalence of the infection over 90% of *H. pylori* strains are *cagA*-positive with *vacA* s1 subtype (Ito *et al.*, 1997; Pan *et al.*, 1997; Maeda *et al.*, 1998). In our studies, a strong association between *vacA* subtypes and the *cagA* status was still found, in spite of the high prevalence of infection. In particular, 68–79% of *cagA*-positive strains had the *vacA* s1a/m1 subtype that has been previously associated with the increased virulence of *H. pylori* strains (Atherton *et al.*, 1995; Atherton *et al.*, 1997). Unfortunately, we have no data of *H. pylori* genotypes in the colonized people without clinical complaints to compare with.

The fact that we have not found any gastric mucosa sample containing the s1b subtype of the *vacA* gene among all the patients investigated by us, confirms the presence of previously postulated geographical differences between *H.*

pylori genotypes. Our findings are similar to those for a population in Asia (Atherton *et al.*, 1996; Ito *et al.*, 1997) and in Poland (Gościniak *et al.*, 1999) for which only one strain with the s1b type out of 72 strains was found. The converse results have been established in several studies conducted, for example, in The Netherlands, Portugal, Brazil, Mexico, and the United States (Atherton *et al.*, 1995; van Doorn *et al.*, 1998a; Morales-Espinosa *et al.*, 1999; De Gusmão *et al.*, 2000). Our finding relates to the widely disparate evolution of circulating *H. pylori* strains in a particular geographical area.

We found that in s1a positive patients the m1 subtype was clearly prevalent among Estonians whereas the m1 and m2 subtypes were equally distributed in Russians. Therefore it is possible that in the countries with a high prevalence of *H. pylori* the distribution of s1a/m1 and s1a/m2 subtypes merely reflects the ethnic affiliation, rather than the association with different gastric diseases. The data about the ethnic tropism of *H. pylori*, have been suggested also earlier by various authors (Campbell *et al.*, 1997; Vilaichone *et al.*, 2004; Ramelah *et al.*, 2005; Tan *et al.*, 2005). Yet, these authors associated differences between the *H. pylori* strains colonising Polynesians and Europeans in New Zealand, with the race-specific specialization of *H. pylori* separate strains. However, this cannot be the reason for *H. pylori* related ethnic differences between Estonians and Russians, as far as both nationalities belong to the same race. Moreover, our Estonian and the Russian PPU patients showed similarity in the distribution of age, sex and smoking habits as well as ulcer localization. Regrettably, we could not follow the socio-economic conditions in the case of the patients and hence it is impossible to conclude if their habits (difference in food or health status etc.) have influenced the distribution of *H. pylori* subtypes. Therefore, the possibility that different predominant *H. pylori* strains were circulating in a particular area among closely related social groups can not be excluded either. In previous epidemiological studies these conditions were strongly associated with the transmission of *H. pylori* infection. In Germany, *H. pylori* infection was established in 6% of native German children versus 45% of Turkish children (Bode *et al.*, 1998). However, the different incidence of *H. pylori* infection in populations from the same country but having a different economic background does not explain the spread of genetically distinct *H. pylori* strains among these populations.

Whether the other determinants, e.g. region/country of birth and childhood, or microbial adherence and host receptors, play a role in a particular distribution of different genotypes of *H. pylori* should be established in further studies with a larger number of subjects. Still, it is important to consider the necessity for the area-and ethnic-specific diagnostics for *H. pylori* virulence markers.

2. *H. pylori* in different gastric diseases

We have investigated the distribution of *H. pylori* genotypes in Estonian patients with the confirmed diagnoses of CG, PUD and PPU. Using the PCR method, the *cagA* gene and *vacA* gene subtypes of *H. pylori* were estimated in

the direct tissue specimens of gastric antral mucosa and in isolated *H. pylori* strains.

In a large number of studies (Cover *et al.*, 1994; Atherton *et al.*, 1995; Atherton *et al.*, 1997; Evans *et al.*, 1998; van Doorn *et al.*, 1998a), the *vacA* s1a/m1 subtype is considered more cytotoxic than s1a/m2 and s2/m2 *vacA* genotypes and is therefore related to the development of PUD. The s2 strains are rarely associated with PUD and are more common in cases of CG or nonulcer dyspepsia (Evans *et al.*, 1998; Strobel *et al.*, 1998; van Doorn *et al.*, 1998c; Rudi *et al.*, 1999; De Gusmão *et al.*, 2000). The virulence markers differentiating *H. pylori* strains in the patients with the complicated and uncomplicated peptic ulcer have not been assessed up to now. In our study, there was no difference in colonization by *H. pylori* cytotoxic strains between the patients with the PUD and those with PPU. Furthermore, a few *H. pylori* strains were of the s2 subtype (6% in PUD and 10% in PPU patients). It could be speculated that, in these patients, the patchy distribution of gastric mucosa obscures the s1a strain, whereas the s2 strains are co-colonizers of the mucosa. Such colonization of the gastric mucosa with several different *H. pylori* strains has been described previously in numerous studies (Taylor *et al.*, 1995; Go *et al.*, 1996; Jorgensen *et al.*, 1996; Morales-Espinosa *et al.*, 1999). However, in the present study we compared the distribution of *vacA* allelic combination and *cagA* gene in the gastric antrum and corpus mucosa samples and found the 100% identity. Therefore the patchy distribution could not be the substantial case.

There has been described a geographical difference in *H. pylori cagA* genotypes caused by highly variable repetitive regions in the 3′ end (Yamaoka *et al.*, 1998a). CagA proteins in Eastern Asian countries are significantly more potent in binding SHP-2 and in inducing cellular morphological changes than the CagA proteins of Western isolates (Higashi *et al.*, 2002a). In addition, there is a correlation between certain *cagA* genotypes and the more severe disease (Yamaoka *et al.*, 1998a; Yamaoka *et al.*, 1999a; Azuma *et al.*, 2002; Azuma, 2004). In our study, from 151 investigated samples in Paper I, the *cagA* gene was detected in 87% of cases. Among PPU patients, the *cagA* status was similar in the samples from Estonian and Russian patients, while the *vacA* s1a subtype prevailed in both nationalities. Similarly, the *cagA*-positive strains from the CG patient samples exclusively carried the s1a subtype though the proportion of *cagA*-negative and *vacA* s2 alleles was the highest (20%) of all the disease groups. Therefore, the unusual result may depend on the exclusive severity of gastritis in these patients. The major effect of VacA is the ability to target the mitochondrial membrane, promoting the release of the cytochrome *c* leading to cell death by apoptosis (Galmiche *et al.*, 2000). The apoptosis-inducing ability of *H. pylori* could be the key to understanding the gastric atrophy in infected persons. In addition, it is likely that the presence of *cagA* and *vacA* s1a genes of *H. pylori* strains is not exclusively specific for the complicated peptic ulcer disease but may merely reflect the circulation of predominant strains in a particular geographic region of Estonia.

One important finding noted in PPU patients in Papers I, II and in the present study was that a small number of patients were apparently colonized by a *cagA*-negative strain that simultaneously expressed the *vacA* s1a subtype. In a recent study (Baglan *et al.*, 2006), conducted in Turkey, where the determination of the several *H. pylori* genotypes failed, the authors suggested that mutations occurred at the primer-binding sites of the genes investigated. The same could be the case in our study where we were seemingly unable to detect *cagA* gene as well as the alleles of *vacA* mid-region in four cases. Consequently, the possibility cannot be excluded that the additional virulence markers of *H. pylori* among other putative factors and microbial colonizers (Bik *et al.*, 2006) may contribute to the development of PPU in some patients with PUD, indicating a role for microbial as well as host genetic diversity.

3. Diagnostic value of PCR method in late post-treatment setting

Several studies (Leodolter *et al.*, 1999; Senturk *et al.*, 2001) have demonstrated the reliability of the *H. pylori* tests used before treatment, while their value in post-treatment testing and in the estimation of persistent *H. pylori* infection are not yet adequately studied. However, in the case of long-lasting recurrent dyspepsia after the *H. pylori* eradication therapy, the endoscopic examination of gastric mucosa for malignancy has been strongly recommended (Malfertheiner *et al.*, 2002). At the same time it is providing the mucosal samples, which could be easily applied for the detection of *H. pylori* putative virulence by molecular methods.

Our aim was to evaluate the PCR method using direct samples of gastric antrum and the corpus mucosa of the PUD patients of late post-treatment settings.

We have found that both in *H. pylori* positive and negative cases by ^{13}C-UBT the results of PCR completely correlated with the findings of histological examination. Although the virulence markers of *H. pylori* were not the scope of the Paper III, we have documented the results in the present study. The most prevalent *vacA* subtype in these PCR-positive samples was s1a. Interestingly, in one of our recent studies (Maaroos *et al.*, 2005), it was found that *cagA* positive *H. pylori* strains with *vacA* s1/m1 subtype were closely associated with the polymorphonuclear infiltration in the antrum mucosa of the patients with PUD and CG. Therefore, the detection of the polymorphonuclear infiltration of the gastric mucosa could help to diagnose the *H. pylori* infection with highly virulent strains and *vice versa,* even after previous treatment. This confirms the validity of the histological evaluation of mucosal specimens in the case of the recurrent peptic ulcer or erosions. Moreover, in the countries with a high rate of *H. pylori* infection and gastric cancer, it is especially important to follow up patients' samples histologically for detecting dysplasia and malignancies (Matysiak-Budnik and Megraud, 1994; Maaroos, 1995; Bray *et al.*, 2002; Kolk *et al.*, 2002).

Therefore, regarding PCR, its main value for obtaining the fast results of *H. pylori* positivity in the patients with dyspeptic complains, is evidently not so important in post-treatment settings. However, this study helped to associate the virulence markers as *cagA* gene and *vacA* s1a of *H. pylori* with more intensive inflammatory defense reaction by the infiltration of gastric mucosa with the phagocytic cells of host.

4. Persistence of *H. pylori* infection in patients with peptic ulcer perforation

In PPU patients we explored the direct samples of the gastric mucosa for molecular detection and the histological evaluation of persistent colonization by *H. pylori.* It has been under debate whether the recurrence of ulcers following *H. pylori* eradication is due to persistent infection (recrudescence) or as a result of reinfection by another strain. Moreover, many authors have used the word "reinfection" inappropriately for the recurrence of *H. pylori* infection. Therefore, according to the annual seroconversion rate, reinfection after a true eradication in adults is probably less than 1% (Xia *et al.*, 1997). Distinguishing between these should help to improve our understanding of the epidemiology of the infection and suggest optimal strategies for treating and preventing the infection.

We found that one year after treatment the eradication of *H. pylori* in PPU patients was rarely (23%) achieved and the same strains continued to persist. This result is quite different from another study where the eradication rate of the *H. pylori* infection was 98% registered at 2-month-follow-up (Ng *et al.*, 2000).

It would be beyond the aim of our study to discuss the success rate in the case of different *H. pylori* treatment methods as the traditional means for the therapy of PPU patients were applied. Yet, we noted that the density of *H. pylori* was significantly lowered 2–5 months after therapy even when different antimicrobial treatment schemes were applied. Therefore, it could be speculated that unlike previous suggestions, the time span of 4 to 8 weeks is not sufficient to confirm eradication (Gisbert, 2005). In our study, the density of colonization clearly returned to the values of the original sample after the one-year period in several patients from either treatment group but more in the group that received modified therapy.

The use of the PCR method supported the data of histological evaluation, showing that in most (58%) of the PPU patients *H. pylori* infection persisted for one year after the operation and eradication therapy. Previously, the ability of *H. pylori* of increased virulence to survive inside epithelial cells has been described (Björkholm *et al.*, 2000). This can also be the case with the PPU patients, studied by us, in whom mostly the highly virulent *cagA* positive strains (94%) with the *vacA* s1a/m1 alleles prevailed (68%). It is possible that in this way the bacterium can partly evade antimicrobial therapy, at the same time showing decreased colonization in follow-up diagnostics shortly after therapy. This possibility was recently suggested also by Gisbert (Gisbert, 2005).

One of the most important observations is that the successful eradication of *H. pylori* infection in the patients with the peptic ulcer disease is associated with a significant decrease in the rate of ulcer relapses (Hopkins *et al.*, 1996). In our PPU patients, who displayed quite a low eradication rate of *H. pylori* infection, the rate of ulcer relapse (9%) was not significantly different from the corresponding data (5%) of a study where a markedly higher eradication rate was reported (Ng *et al.*, 2000). The rate of possible reinfection according to the molecular analysis turned out to be quite low (12%), showing that in the communities with a high prevalence of *H. pylori* but yet a good hygiene standard, the development of the infection usually occurs with one strain. Hence our study, which revealed a low rate of ulcer relapse, did not confirm the assumption (Figura *et al.*, 1989; Gisbert, 2005) that new non-ulcerogenic strains (*vacA* s2 alleles) may colonize the gastric mucosa after therapy. Apparently, the lowered density after eradicative therapy postpones the development of recurrent ulceration in the one-year follow-up period irrespective of the recrudescence of the presence of ulcerogenic strains. Moreover, our study showed a mixed colonization pattern in only 6% of the patients. These results are in good accordance with those of the studies comparing the pre- and post-treatment strains of *H. pylori*, where approximately 80% of the strains turned out to be identical (Gisbert, 2005).

The application of direct samples of gastric biopsy for the detection of the RFLP pattern has been shown to be effective for differentiating between reinfection and the persistent strains of *H. pylori* after using different therapy schemes in PUD patients or in the patients with upper gastrointestinal complaints (Li *et al.*, 1997; Stone *et al.*, 1997; Adamsson *et al.*, 2000; Jeen *et al.*, 2001). The results of these studies show that mostly identical pre- and post-treatment strains can be detected, whereas different strains are found in only a minority of the cases (Peitz *et al.*, 1999). In the present study we compared the restriction pattern of the *glmM* gene in 9 patients with persistent *vacA* alleles before and after therapy. In most patients (89%) the RFLP profiles of the subsequent samples were indistinguishable. Only in one patient there was a minor one-band difference between the initial sample and the two follow-up samples despite the fact that they were of the same *vacA* genotype.

Thus, we found that in patients with PPU, one year after surgical and antimicrobial treatment, not the reinfection but the persistent infection with *H. pylori cagA* positive and *vacA* s1a subtype strains is common.

CONCLUSIONS

1. In Estonia, the carriage of s1a subtype and the complete absence of the s1b subtype of *vacA* gene is characteristic of *H. pylori* strains circulating among patients with chronic inflammatory gastric diseases. This geographical diversity is similar to Asian but in opposite to most of the Southern-European and American isolates.

2. The distribution of *vacA* s1a/m1 and s1a/m2 subtypes of *H. pylori* strains is different in Estonian and Russian patients with perforated peptic ulcer yet living in the same geographical area. It may indicate the spread of particular strains among different ethnic groups.

3. In Estonia, the high prevalence (81–94%) of the *cagA* gene of *H. pylori* is common in patients with chronic gastritis, the peptic ulcer disease and the perforated peptic ulcer. Thus, the *cagA* gene as an important virulence marker of *H. pylori* is well associated with the chronic inflammatory gastric diseases even in a country with the high prevalence of infection and CagA antibodies.

4. The closely associated *cagA* gene and *vacA* subtypes (s1a/m1, s1a/m2 and s2/m2) of *H. pylori* are similarly distributed among the patients with different diagnoses like chronic gastritis, peptic ulcer and perforated peptic ulcer. Thus, the studied *H. pylori* genotypes do not differentiate between various chronic inflammatory gastric diseases in Estonia.

5. The PCR-based diagnostic method, applying the tissue samples of gastric mucosa, shows a tight concordance with ^{13}C-UBT and histology in the follow-up of peptic ulcer patients 5 years after the treatment. Thus, this method provides fast and reliable results of the presence of *H. pylori* in the late evaluation for the success of the treatment.

6. In patients with the perforated peptic ulcer, one year after surgical and antimicrobial treatment, not the reinfection but the persistent infection with *H. pylori cagA* positive and *vacA* s1a subtype strains is common.

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SUMMARY IN ESTONIAN

Helicobacter pylori genotüübid Eesti krooniliste põletikuliste maohaigustega patsientidel

Alates *Helicobacter pylori* avastamisest 1982. aastal, on *H. pylori* infektsiooni seostatud erinevate maohaiguste laialdase esinemisega kogu maailmas.

Eestis on *H. pylori* levikut populatsioonis ja seoseid selliste maohaigustega nagu krooniline gastriit, mao- ja kaksteistsõrmiksoolehaavand, maovähk ning perforeerunud peptiline haavand, uurinud mitmed töögrupid, kasutades peamiselt histoloogilisi, bakterioloogilisi või seroloogilisi uurimismeetodeid. *H. pylori* vastaseid antikehi on leitud üle 80% Eesti elanikkonnast. *H. pylori* tüvede täpsemaks kirjeldamiseks epidemioloogilistes või kliinilistes uuringutes oleksid vajalikud aga kaasaegsemad molekulaarsed meetodid.

Maailma erinevates populatsioonides on täheldatud geneetiliselt erinevate *H. pylori* tüvede levikut. *H. pylori* mitmeid geene (*vacA*, *cagA*, *iceA*, jm.) seostatakse mao erinevate patoloogiliste seisunditega. *H. pylori* genotüüpide iseloomustamisel Lõuna-Eesti gastriidi ja peptilise haavandiga patsientidel oleme oma varasemas uurimuses määranud *cagA* geeni ja erinevate *vacA* geeni subtüüpide esinemist kui olulisi virulentsusmarkereid. Ei ole aga selge, kas raskemate maohaiguste, nagu näiteks perforeerunud peptilise haavandi korral on patsiendid koloniseeritud virulentsusgeenide erilisemaid kombinatsioone omavate *H. pylori* tüvedega kui peptilise haavandiga patsiendid. Sellisel juhul võiks selliste molekulaarsete markeritega tüvede püsimine peptilise haavandiga patsientidel raskendada haiguse kulgu.

Mitmed uurimused viitavad erinevustele *H. pylori* leviku ja genotüüpide osas erinevatel etnilistel gruppidel ja erinevates geograafilistes piirkondades. Siiski pole seos *H. pylori* genotüüpide ja inimeste etnilise kuuluvuse vahel veel laiemalt kinnitust leidnud.

Uurimistöö eesmärgid ja ülesanded

Töö peamiseks eesmärgiks oli uurida, kas Eestis ringlevad mingid spetsiifilised *H. pylori* tüved, mis vastutavad erinevate krooniliste põletikuliste maohaiguste eest.

Sellest tulenevalt, olid käesoleva töö ülesanded järgmised:

1. Võrrelda geneetiliste virulentsusmarkerite (*cagA*, *vacA*) esinemist ja jaotumust *H. pylori* tüvedel Eesti kroonilise gastriidi, peptilise haavandi ja selle komplikatsiooni — perforeerunud peptilise haavandiga patsientidel.
2. Uurida *H. pylori* tüvede mitmekesisust, võrreldes *H. pylori* genoomset erinevust mao limaskesta proovides, mis on saadud Eestis elavatelt eesti ja vene rahvusest perforeerunud peptilise haavandi patsientidelt.

3. Hinnata PCR meetodi diagnostilist väärtust ravi hilise järelkontrolli patsientidel, kasutades mao limaskesta koeproove.
4. Hinnata mao limaskesta koeproovides püsiva *H. pylori* infektsiooni või taasnakatumise esinemist perforeerunud peptilise haavandiga patsientidel üheaastase järelkontrolli jooksul peale operatsiooni ja antimikroobset ravi.

Uuritav materjal ja meetodid

Antud uuring hõlmas 201 kroonilise gastriidi, peptilise haavandi ja perforeerunud peptilise haavandiga patsienti, kelle andmed olid aastail 1995–2001 sisestatud Tartu Ülikooli Polikliiniku ja peremeditsiini õppetooli ja SA Tartu Ülikooli Kliinikumi Kirurgiakliiniku teaduslikesse andmebaasidesse. Mao limaskesta proovitükid saadi neilt patsientidelt endoskoopilise uuringu ja/või operatsiooni (perforeerunud peptilise haavandi korral) käigus.

Koostöös kaasautoritega kasutati *H. pylori* infektsiooni tuvastamisel ^{13}C-märgistatud uurea hingamistesti, histoloogilist, bakterioloogilist ja tsütoloogilist uuringut. *H. pylori* molekulaarsel määramisel ja mikroobi virulentsusmarkerite (*cagA* geen ja *vacA* geeni alleelid) selgitamisel kasutati PCR (polümeraasi ahelreaktsioon) meetodit. *H. pylori* tüvede püsimist ja reinfektsiooni tuvastati perforeerunud peptilise haavandiga patsientidel PCR-RFLP (restriktsiooni fragmendi pikkuse polümorfism) meetodi abil. Molekulaarseks analüüsiks vajalik *H. pylori* DNA eraldati kas otse mao limaskesta koeproovi tükkidest või isoleeritud mikroobide suspensioonist Brucella puljongist. Viimast kasutati vaid 93 kroonilise gastriidi ja peptilise haavandi patsientide korral (Paper I).

Uurimistööst tulenevad järeldused

1. Eestis on krooniliste põletikuliste maohaigustega patsientidel esinevatele *H. pylori* tüvedele iseloomulik *vacA* geeni s1a subtüübi kandlus ja s1b subtüübi täielik puudumine. Selline genotüüpiline eripära on sarnane Aasia tüvedele, kuid vastupidine enamusele Lõuna-Euroopa ja Ameerika isolaatidele.
2. *H. pylori* tüvede *vacA* s1a/m1 ja s1a/m2 subtüüpide jaotumus on eesti ja vene rahvusest perforeerunud peptilise haavandiga patsientidel erinev, vaatamata elamisele samas geograafilises piirkonnas. See võib viidata kindlate tüvede levikule erinevates etnilistes gruppides.
3. Eestis on kroonilise gastriidi, peptilise haavandi ja perforeerunud peptilise haavandiga patsientidele iseloomulik *H. pylori cagA* geeni kõrge levimus (81–94%). Seega on *cagA* geen *H. pylori* oluliseks virulentsusmarkeriks krooniliste põletikuliste maohaigustega haigetel ka sellises riigis, kus esineb kõrge infektsioonisagedus ja CagA antikehade levimus.
4. Omavahel tihedasti seostatud *H. pylori cagA* geeni ja *vacA* geeni subtüüpide (s1a/m1, s1a/m2 ja s2/m2) jaotumus on erinevate diagnoosidega, nagu kroonilise gastriidi, peptilise haavandi või perforeerunud peptilise haavan-

diga, patsientidel sarnane. Seega ei erista uuritud *H. pylori* genotüübid omavahel erinevaid kroonilisi põletikulisi maohaiguseid Eestis.

5. PCR-põhinev diagnostiline meetod, kasutades mao limaskesta koeproove, on peptilise haavandiga patsientidel 5 aastat peale ravi heas vastavuses ^{13}C-märgistatud uurea hingamistesti ja histoloogiaga. Seega võimaldab antud meetod anda hilises ravitulemuste hindamises kiire ja usaldusväärse vastuse *H. pylori* esinemise kohta.
6. Üks aasta peale kirurgilist ja antimikroobset ravi pole perforeerunud peptilise haavandiga patsientidel tegemist mitte taasnakatumise, vaid püsiva infektsiooniga peamiselt *H. pylori cagA* positiivsete ja *vacA* s1a subtüüpi tüvedega.

ACKNOWLEDGEMENTS

This work was carried out at the Department of Microbiology in collaboration with the Department of Polyclinic and Family Medicine and Surgery Clinic, Faculty of Medicine, University of Tartu.

This study could not have been accomplished without the aid and support of a number of people to whom I would like to express my sincere gratitude. In particular, I would like to thank:

- Professor Marika Mikelsaar, my supervisor, for introducing me the field of microbiology, for sharing her knowledge about the science and the research, for her enthusiasm, advice and support throughout my studies.
- Professor Heidi-Ingrid Maaroos, for fruitful collaboration, for rapid manuscript corrections and for always being ready to help.
- Professor Ants Peetsalu and Dr. Toomas Sillakivi, for excellent collaboration and support, for the help with the manuscript and for explaining the clinical aspects concerning the peptic ulcer perforation.
- All the co-authors of the publications included in this thesis for fruitful partnership.
- All my colleagues from the Department of Microbiology for sharing the joys and frustrations of being a student or a researcher.
- Professor Irja Lutsar, Reet Mändar, Krista Lõivukene and Epp Sepp for critical reading of the manuscript and useful comments.
- Professor Raivo Uibo and Dr. Marje Oona for reviewing the thesis and for their valuable comments.
- Mall Tamm and Ester Jaigma for the linguistic revisions of the manuscripts of the thesis and the publications.
- My parents, sister, grandparents and other family members for their endless love, trust and encouragement.
- My dear husband Reidar and our son Oliver for their love and patience, for believing in me and for bringing happiness to my heart.

The investigations were supported by Estonian Target Funding No. 0418 from the Estonian Ministry of Education, and some finances were obtained from the Estonian Science Foundation (Grants No. 4898 and No.5266).

PUBLICATIONS

Persistence Of *Helicobacter pylori* Infection In Patients With Peptic Ulcer Perforation

Helena Andreson (1), Toomas Sillakivi (2), Margot Peetsalu (2), Ants Peetsalu (2), and Marika Mikelsaar (1)

1) Department of Microbiology, University of Tartu, Tartu, Estonia
2) Department of Surgery, University of Tartu, Tartu, Estonia

Background. In patients with perforated peptic ulcer (PPU) the convergence between the high eradication rate of *Helicobacter pylori* infection and low rates of ulcer relapse after treatment has been associated with reinfection by non-virulent strains. The persistence of infection by virulent *H. pylori* strains and ulcer recurrence were evaluated in 33 patients with PPU one year after surgery and antimicrobial treatment. **Methods.** The histological evaluation and molecular detection of *H. pylori cagA* and *ureA* genes, *vacA* allelic types and the PCR-RFLP analyses of the *glmM* gene products from antral mucosa specimens initially, 2–5 months and 1 year after therapy were performed. **Results.** Density of *H. pylori* colonization was temporarily decreased ($p<0.05$) 2–5 months after therapy. After one year the complete eradication was achieved only in 7 patients (23%) at histological examination and recurrent ulcers were revealed in 3/33 (9%) patients. The *vacA* s1a allelic type of *cagA* positive strains persisted in 19 (83%) non-eradicated 23 PPU patients with identical PCR-RFLP fingerprints in 8/9 (89%) of the patients. **Conclusions.** In PPU patients with low eradication rate of *H. pylori* after surgical and antimicrobial treatment the frequent recrudescence of the infection is mostly caused by the persisting virulent strains of the *cagA* and *vacA* s1a subtypes. In the one-year follow-up period the recurrent ulceration may be postponed just by lowered colonization density of *H. pylori* after eradicative therapy.

Key words: *cagA*; *Helicobacter pylori*; perforated peptic ulcer; persistent infection; *vacA*; virulence

Introduction

Helicobacter pylori infection is closely associated with chronic gastritis, non-complicated peptic ulcer disease (PUD) and gastric cancer. Similarly, in the case of perforated peptic ulcer (PPU), which is a potentially fatal complication of PUD (both duodenal and gastric ulcer), *H. pylori* prevalence is high, varying between 50 and 96% (1–4). Using classical quadruple therapy, high *H. pylori* eradication rate, 80–98%, has been achieved 8 weeks after treatment. One year later, however, the data of outcome, e.g. ulcer relapse rate, are quite different: 33% (5) versus 5% (6).

The contradictory results reported on the incidence of recurrent ulcer after surgical intervention and eradication treatment in PPU patients (3, 5–7) may be attributed to different infection rates in population where the high prevalence could facilitate the reinfection.. Besides, the precise clinical relevance of persistent infections or reinfections is unknown. Reappearance of the microbe without associated ulcer recurrence has been explained by reinfection with some non-virulent strains (8, 9).

In peptic ulcer disease *H. pylori* strains possess the cytotoxin-associated gene (*cagA*) with the vacuolating cytotoxin gene (*vacA*) of the s1/m1 allelic combination (10), while the *vacA* type s1a strains appear to be more cytotoxic than the s1b or s2 strains (11). In our previous study, no significant differences were found in the *cagA* and *vacA* markers between the strains from the perforated and non-complicated peptic ulcer groups (12). There are no such investigations where molecular virulence markers were applied for differentiation between true reinfection with a new strain and recrudescence when eradication of the permanent strain of *H. pylori* in PPU patients failed. Populations with high *H. pylori* incidence and hence at risk for reinfection, like Estonia (13), provide a good opportunity for elucidation of the problem in the course of follow-up studies after treatment.

Our study aimed at evaluating the putative eradication, persistent infection or reinfection by *H. pylori* and ulcer recurrence in patients with PPU in a one-year follow-up study after surgery and antimicrobial treatment. The PCR-based molecular virulence markers, the RFLP pattern and the colonization density of *H. pylori* were established directly from gastric antrum mucosa samples.

Methods

Patients

For this study 33 persons (mean age 43 ± 14, 29 men, 4 women) were retrospectively selected from among the patients operated for perforated peptic ulcer and observed clinically during the period 1997–2001 in the Clinic of Surgery of Tartu University Hospital. The inclusion criteria were agreement to

participate in the study and availability of three consecutive gastric mucosa biopsy samples during the one-year period (initial, 2–5 months and 1 year later), the first probe being *H. pylori* positive as detected by histology and PCR.

The perforated ulcers were classified into gastric (2 cases) and duodenal ulcers (31 cases) according to the location. The surgical therapy of 18 patients included definitive (truncal vagotomy combined with drainage procedure) and in 15 patients non-definitive (ulcer excision or suturation) operations. In the latter case, traditional triple therapy (amoxicillin 1000 mg b.i.d., metronidazole 500 mg q.i.d. or clarithromycin 500 mg b.i.d. and omeprazole 20 mg b.i.d.) was applied for 7 days. In vagotomised patients different antimicrobial treatment schemes without omeprazole were used for 5 to 7 days (14). Eradication was defined as a negative histological finding of *H. pylori* during one-year follow-up.

Material

Two gastric biopsy samples of the antral mucosa were taken intra-operatively through the perforation, or postoperatively on panendoscopy, before eradicative treatment, for histological evaluation and molecular analysis. Similarly, the second and the third biopsy samples were taken on endoscopy 2–5 months and 1 year after the first intervention.

The biopsy samples for molecular analysis were placed into a lysis buffer (200 mM Tris-HCl [pH 8.0], 25 mM EDTA, 300 mM NaCl, 1.2% sodium dodecyl sulfate) and stored at -20^0C.

Histological evaluation

For histological evaluation, 95 samples from the antrum were used. Due to technical failure, altogether 4 histology preparations from 3 patients (1 from initial, 3 from 2–5 month samples) were unavailable. The biopsy specimens were fixed overnight in neutral buffered formalin, dehydrated and embedded in paraffin. Tissue sections were stained by the modified Giemsa method for further semiquantitative assessment of *H. pylori* colonization as described earlier (15). The colonization of the antral mucosa by *H. pylori* was graded as follows: grade 0, absence of *H. pylori*; grade 1 (low or mild), <20 microbes per field; grade 2 (moderate), 20–60 microbes per field; grade 3 (high or severe), >60 microbes per field.

Extraction of DNA

Altogether 99 antral mucosa biopsy samples were collected for molecular analysis. For DNA extraction from the frozen gastric biopsy specimens the previously described procedure was used (16).

Primers

The presence of *cagA* in each strain was determined by PCR using D008 and R008 primers (17). The *vacA* signal- and midregion were typed by PCR (10).

The primers VA1-F, VA1-R and SS2-F were used to categorize the *vacA* s region as type s1 or s2. For subclassification of the s1 allele, the reverse primer VA1-R was used together with the allelic subtype-specific forward primers (SS1-F primer for the subtype s1a and SS3-F primer for the subtype s1b). The *vacA* midregion was typed by PCR as m1 (primers VA3-F and VA3-R) or m2 (primers VA4-F and VA4-R). The presence of *H. pylori* was confirmed with the primers HPU1 and HPU2 for detecting the urease A gene (*ureA*) (18, 19). For RFLP, the 1,169-bp fragment of the *glmM* (formerly *ureC*) gene was amplified with the primers ureC-U (5'- AAG AAG TCA AAA ACG CCC CAA AAC-3') and ureC-L (5'-CTT ATC CCC ATG CAC GAT ATT CCC-3') (20).

PCR conditions and amplification

The reaction mixtures were prepared and the PCR reactions were performed for the *ureA, cagA*, s1a and s1b primer pairs (16). Thermal cycling for the *vacA* primer pairs (except s1a and s1b) comprised 4 min of preincubation at 94^0C, followed by 30 cycles of 94^0 C for 30 sec, 58^0 C for 30 sec, 72^0C for 1 min 30 sec, and a final extension of 72^0C for 4 min. For amplification of the 1,169-bp fragment of the *glmM* gene, initial preincubation at 94^0C for 5 min was followed by 35 cycles of 94^0 C for 45 sec, 59^0 C for 30 sec, 72^0C for 1 min 30 sec, and final extension of 72^0C for 10 min. The PCR products were identified by electrophoresis on 1.5% agarose gels in the case of the 1.169-bp PCR fragment and 2% agarose gels were used for all other PCR products.

PCR-RFLP analysis

From 10 patients initial and follow-up samples with a sufficient concentration of DNA were selected for further PCR-RFLP analyses (20). The PCR-amplified 1,169-bp products of the *glmM* gene were ethanol precipitated, the pellets were washed with 70% ethanol, and dissolved in 5 µl of water. The obtained purified PCR products were digested with the restriction enzyme Hha1 (Fermentas AB, Lithuania) with the recommended Tango buffer at 37^0C for 20 hours. The digested DNA fragments were analysed by electrophoresis on 3.5% agarose gels (*Agarose MP, Roche Diagnostics, Germany*).

Statistical analysis

Differences in the treatment schemes and the association between the *cagA* and *vacA* genes were analyzed by the Fisher exact test. The Chi-square test was used for comparison of the PCR and histology methods. The data of *H. pylori* colonization density for different treatment groups were analyzed by the Mann-Whitney Rank Sum Test. Significance was set at a P value less than 0.05. The Statistica 6.0" (StatSoft Inc., USA) software program was used for all analyses.

Ethics

The study was approved by the Tartu University Research Ethics Committee and informed consent was obtained from each participant in all cases.

Results

Clinical and histological data

The follow-up of *H. pylori* eradication showed that in 21 patients out of 30 (70%) after 2–5 months and in 23 patients (77%) one year after treatment *H. pylori* was still present in mucosal samples (3 patients with unavailable histology data were excluded from this analysis). The patients of the triple therapy group showed somewhat better results of *H. pylori* eradication (6/15, 40% *vs*. 1/15, 7%). At 2–5 months after therapy the colonization density was temporarily reduced ($p<0.05$) in both treatment groups, however, by the time of final evaluation it had increased in the group that had received modified treatment ($p<0.05$) (Fig. 1).

Recurrent duodenal ulcer was found only in 3 patients out of 33 (9%) of both treatment groups. In two *H. pylori* non-eradicated cases recurrent ulcer was detected after one year and in one patient at both follow-up endoscopic examinations after surgical and eradicative triple therapy.

H. pylori ureA, vacA and cagA markers

Judging by the detection of *ureA* and *vacA* genes all samples were initially *H. pylori* positive. *H. pylori* positivity was found in 19 (58%) patients out of 33 at 2–5-month follow up and in 23 (70%) patients at one year. None of these findings differed ($p>0.05$) from those of histological evaluation.

Initially, the majority of the patients (31/33) were infected with *cagA* positive strains of the *vacA* s1a allelic type**.** All signal- and midregion *vacA* alleles were found except for s1b. The dynamics of *H. pylori* infection by PCR and the distribution of the *vacA* subtypes, comprising 5 different allelic combinations, are shown in Table 1. At the first and at the last follow-up, infection with the same *vacA* virulence markers persisted in most of the patients, 19/33 (58%). Only in 4/33 (12%) patients there was detected possible reinfection with the *H. pylori* virulence markers different from the initial ones.

PCR-RFLP typing

We fingertyped the initial and the follow-up samples from 10 patients: 9 belonged to the group of persistent *H. pylori* with similar virulence markers and one with different ones. The latter patient, initially with the *H. pylori* s1a/m1 strain, showed different patterns both by PCR (s1a allele without the m allele) and PCR-RFLP (Table 1; Fig. 2, patient 5). Among the 9 patients with similar virulence markers, the overwhelming majority (8/9) displayed no differences in the restriction pattern between the initial biopsy sample and the follow-up sample (Fig. 2, patients 1, 3, 4 and 6–10). Only in one case the PCR-RFLP patterns of the follow-up samples were different from the initial ones due to the variance of one band (Fig. 2, patient 2).

Discussion

Our study aimed to evaluate the change or persistence of *H. pylori* virulent strains in patients with perforated peptic ulcer one year after surgery and antimicrobial treatment and the recurrence of peptic ulcer. Direct gastric mucosa samples were investigated by histological and molecular methods. We found that in our patients one year after treatment the eradication of *H. pylori* in PPU patients was rarely (23%) achieved and the same strains continued to persist. This result is quite different from the above mentioned high eradication rate (84%) registered at 2-month follow-up (6).

It would be beyond the aim of our study to discuss the success rate in the case of different *H. pylori* treatment methods as the traditional means and duration for the therapy of PPU patients were applied. Yet we noted that the density of *H. pylori* was significantly lowered 2–5 months after therapy even when different antimicrobial treatment schemes were applied. Therefore, it could be speculated that unlike previous suggestions (8), the time span of 4 to 8 weeks is not sufficient to confirm eradication. In our study, the density of colonization started to return to the values of the original sample after the one-year period from either treatment group but more in the group that received modified therapy. At the same time, these results indicate that the lowered susceptibility of *H. pylori* strains could not be the reason for the treatment failure, which remains to be elucidated.

In addition to histological evaluation, we explored the direct samples of the gastric mucosa for molecular detection of persistent colonization by *H. pylori.* The use of the PCR method supported the data of histological evaluation, showing that in most (58%) of the PPU patients *H. pylori* infection persisted for one year after the operation and eradication therapy. Previously, Björkholm et al. (21) described the ability of *H. pylori* of increased virulence to survive inside epithelial cells. This can also be the case with the PPU patients studied by us in whom mostly the highly virulent *cagA* positive strains (94%) with the *vacA* s1a/m1 alleles prevailed (68%). It is possible that intracellularly the bacterium can partly evade antimicrobial therapy, at the same time showing decreased colonization in follow-up diagnostics shortly after therapy. This possibility was recently suggested also by Gisbert (8).

In our PPU patients, who displayed a quite low eradication rate of *H. pylori* infection, the rate of ulcer relapse (9%) was still not significantly different from the corresponding data (5%) of a study where markedly higher eradication rate was reported (6). The rate of possible reinfection according to molecular analysis turned out to be very low (12%), showing that in communities with a high prevalence of *H. pylori* but yet a good hygiene standard, development of the infection usually occurs with one strain. Hence our study, which revealed a low rate of ulcer relapse, did not confirm the assumption (8, 9) that new non-ulcerogenic strains (*vacA* s2 alleles) may colonize the gastric mucosa after therapy. Apparently, the lowered density after eradicative therapy postpones

development of recurrent ulceration in the one-year follow-up period irrespective of recrudescence of presence of ulcerogenic strains. Moreover, our study showed a mixed colonization pattern in only 6% of the patients (Table 1). These results are in good accordance with those of studies comparing the pre- and post-treatment strains of *H. pylori*, where approximately 80% of the strains turned out to be identical (8).

During follow-up we found that in two patients the usual *vacA* s1a/m1 allelic combination in the initial PCR sample was replaced by a strain of the mere s1a subtype. PCR-RFLP analysis confirmed this difference in one tested patient, yielding clearly distinguishable RFLP patterns for the two samples. Similarly, one patient had persistent infection with the s1a subtype of *H. pylori* in all three follow-up samples without the mid-region allele of the *vacA* gene. It is possible that with the primers used we could have missed some mid-region variants described previously in addition to the m1 and m2 alleles (22–24).

Application of direct samples of gastric biopsy for detection of the RFLP pattern has been shown to be effective for differentiating between reinfection and the persistent strains of *H. pylori* after using different therapy schemes in PUD patients or in patients with upper gastrointestinal complaints (20, 25, 26). In the present study we compared the restriction pattern of the *glmM* gene in 9 patients with persistent *vacA* alleles before and after therapy. In most patients (89%) the RFLP profiles of the subsequent samples were indistinguishable. Only in one patient there was a minor one-band difference between the initial sample and the two follow-up samples despite the fact that they were of the same *vacA* genotype.

Conclusions

In PPU patients with low eradication rate of *H. pylori* after surgical and antimicrobial treatment the frequent recrudescence of the infection is mostly caused by the persisting virulent strains of the *cagA* and *vacA* s1a subtypes. In the one-year follow-up period the recurrent ulceration may be postponed just by lowered colonization density of *H. pylori* after eradicative therapy.

Acknowledgements

This study was supported by Grants No. 4898 and No. 5266 of the Estonian Science Foundation, Estonian Target Funding No. 0418 from the Estonian Ministry of Education and by the Centre of Molecular and Clinical Medicine, Faculty of Medicine, University of Tartu.

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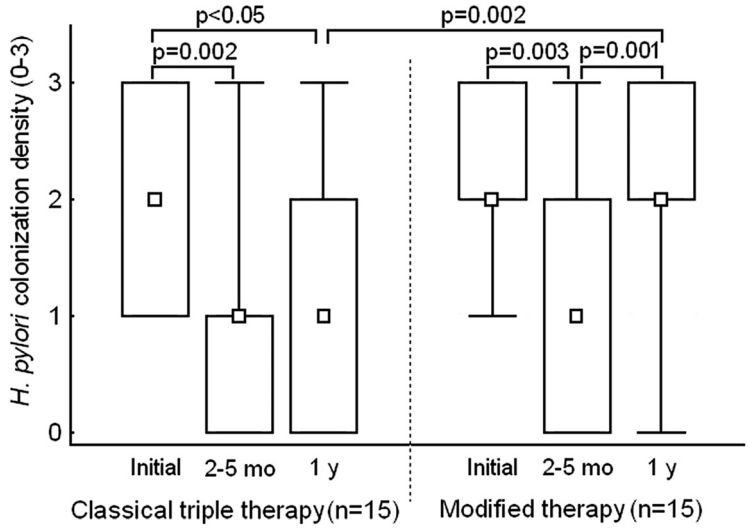

Fig. 1. *H. pylori* colonization density in PPU patients during one-year follow-up in the case of classical and modified therapies. Initial biopsy samples before therapy; 2–5 months (mo), and one year (y) after the therapy. *H. pylori* density grades: (0) absence of *H. pylori*; (1) <20 microbes per field; (2) 20–60 microbes per field; (3) >60 microbes per field. *H. pylori* density data are shown as boxes: internal points, medians; tops and bottoms of boxes, 75^{th} and 25^{th} percentiles, respectively; upper and lower bars, 90^{th} and 10^{th} percentiles, respectively. Significant p values ($p<0.05$) for the sample groups are denoted by arrows.

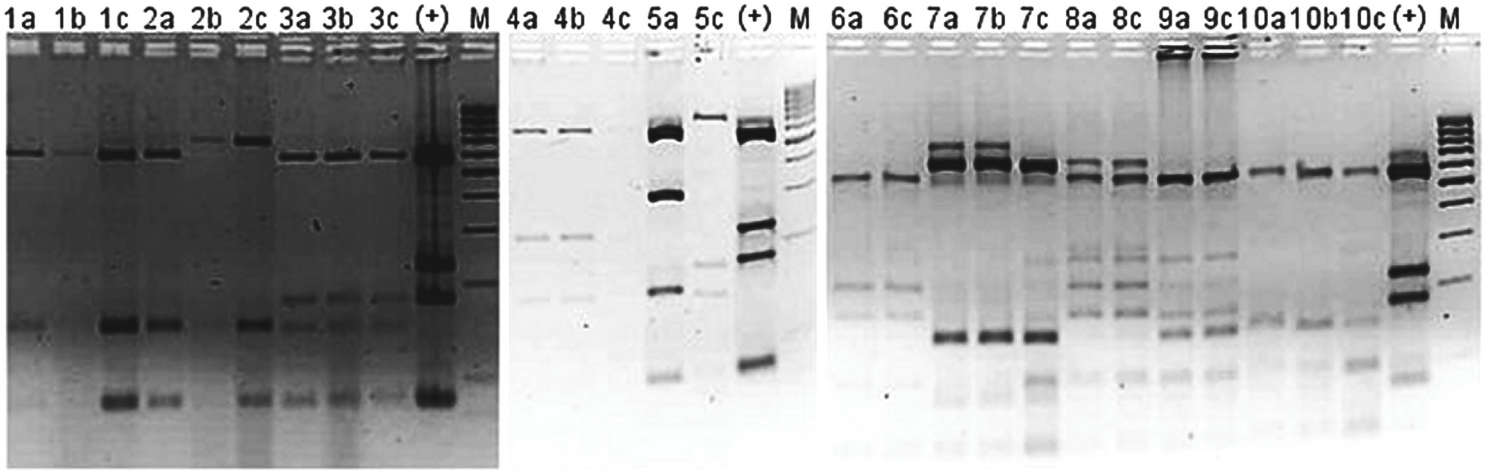

Fig. 2. RFLP patterns of the follow-up samples from 10 PPU patients. (a) Before therapy, (b) 2–5 months after therapy and (c) one-year follow-up; the patients are indicated by numerals. PCR-amplified 1,169 bp fragments of the *glmM* gene of *H. pylori* were digested with the HhaI restriction enzyme. Lane M is the 100bp DNA ladder (*Fermentas AB, Lithuania*) and lane (+) is the *H. pylori* strain NCTC 11637 as the positive control.

Table I.
The dynamics of *H. pylori* infection and strains by PCR in the follow-up samples of the PPU patients.

The dynamics of the infection	n=33*	*H. pylori vacA* subtypes in follow-up samples		
		1 (operation)	2 (2-5 months)	3 (1 year)
Eradication of *H. pylori*	1	s1a/m1	s1a/m1	neg
(n=10)	4	s1a/m1	neg	neg
	4	s1a/m2	neg	neg
	1	s2/m2	neg	neg
Persistent *H. pylori*	8^{5}	s1a/m1	s1a/m1	s1a/m1
(n=19)	3^{2}	s1a/m2	s1a/m2	s1a/m2
	1^{1}	s2/m2	s2/m2	s2/m2
	1	s1a	s1a	s1a
	2	s1a/m1/m2	s1a/m1/m2	s1a/m1/m2
	4^{1}	s1a/m1	neg	s1a/m1
Reinfection	1	s1a/m1	s1a/m1	s1a/m2
(n=4)	2^{1}	s1a/m1	s1a/m1	s1a
	1	s1a/m1	neg	s1a/m2

* The superscript indicates the number of patients enrolled for further PCR-RFLP analysis.

CURRICULUM VITAE

Helena Andreson (born Aro)

Citizenship: Estonian
Born: July 15, 1978, Pärnu
Married, one child
Address: University of Tartu, Department of Microbiology,
Ravila 19, 50411 Tartu, Estonia
Phone: +372 737 4170
Fax: +372 737 4172
E-mail: Helena.Andreson@ut.ee

Education and professional employment

1985–1996	Saaremaa Co-educational Gymnasium
1996–2000	University of Tartu, Faculty of Biology and Geography, Institute of Molecular and Cell Biology, B.Sc
2000–2002	University of Tartu, Faculty of Medicine, Department of Microbiology, M.Sc *cum laude*
2001–	University of Tartu, Faculty of Medicine, Department of Microbiology, technician
2002–	University of Tartu, Faculty of Medicine, Department of Microbiology, postgraduate student

Scientific work

The main subject of the research work has concerned the molecular virulence markers of *Helicobacter pylori* and the development of diagnostic methods of *H. pylori* infection. 4 scientific publications and 7 presentations at the conferences.

ELULOOKIRJELDUS

Helena Andreson (sündinud Aro)

Kodakondsus: Eesti
Sünniaeg: 15. juuli, 1978, Pärnu
Abielus, 1 laps
Aadress: TÜ mikrobioloogia instituut,
Ravila 19, 50411 Tartu, Eesti
Telefon: +372 737 4170
Faks: +372 737 4172
E-mail: Helena.Andreson@ut.ee

Haridus ja erialane teenistuskäik

1985–1996	Saaremaa Ühisgümnaasium
1996–2000	Tartu Ülikool, Bioloogia-Geograafia teaduskond, molekulaar- ja rakubioloogia instituut, B.Sc
2000–2002	Tartu Ülikool, Arstiteaduskond, mikrobioloogia instituut, M.Sc *cum laude*
2001–	Tartu Ülikool, Arstiteaduskond, mikrobioloogia instituut, laborant
2002–	Tartu Ülikool, Arstiteaduskond, mikrobioloogia instituut, doktorant

Teadustöö

Peamiseks uurimisvaldkonnaks on *Helicobacter pylori* virulentsusmarkerite uurimine ning *H. pylori* infektsiooni diagnostiliste molekulaarsete meetodite väljatöötamine. 4 teaduspublikatsiooni ja 7 konverentsiteesi.