3. Virulence factors (Cag PAI, Vac A, iceA, babA)

3.01 The detection of tyrosine-phosphorylated CagA and SHP-2 tyrosine phosphatase in the biopsy of Helicobacter pylori positive patients

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Background and Aim: In vitro H. pylori infection as well as cagA gene transfection experiments, we recently discovered that the CagA protein is injected from the bacteria into gastric epithelial cells via the bacterial type IV secretion system and undergoes tyrosine phosphorylation in the host cells, and that the translocated CagA forms a physical complex with the SRC homology 2 domain (SH2) containing tyrosine phosphatase SHP-2 in a phosphorylation-dependent manner. In the present study, we investigated these phenomena in vivo human gastric mucosa.

Methods: Five patients with atrophic gastritis, 5 patients with early gastric cancer, and 5 H. pylori-negative normal controls participated in the present study. The biopsy specimens by endoscopy were obtained from the greater curvature of the gastric antrum and fundic mucosa, and also from gastric cancer tissues. These samples were subjected to immunoprecipitation using anti-CagA antibody, and immunoblot analysis. H. pylori infection was evaluated by histology, culture, and 13C-urea breath test (UBT).

Results: The tyrosine phosphorylated CagA protein and CagA co-immunoprecipitated endogenous SHP-2 were detected in the gastric mucosa from H. pylori-positive atrophic gastritis patients, but not in H. pylori-negative controls. Interestingly, although tyrosine phosphorylated CagA and CagA co-immunoprecipitated endogenous SHP-2 were detected in the non-cancer tissues from H. pylori-positive early gastric cancer patients, CagA protein was not detected in the gastric mucosa of those with intestinal metaplasia or cancer.

Conclusions: The present data provided the first and compelling evidence that CagA is actively translocated from the bacteria to gastric epithelial cells, receives tyrosine phosphorylation, and binds SHP-2 in vivo human gastric mucosa. Degradation of SHP-2 by CagA may play a role in the acquisition of a cellular transformed phenotype at a relatively early stage of multistep carcinogenesis in gastric cancer.

3.02 Detection of tyrosine-phosphorylated CagA protein in biopsy specimens from H. pylori-infected patients and the role of tyrosine-phosphorylated CagA protein associated with Syk in H. pylori-infected epithelial cells

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Background: Helicobacter pylori (H. pylori) is one of the most ubiquitous bacterial pathogens to humans and is colonized in the stomachs of as many as half of the humans worldwide. It may cause a variety of gastroduodenal diseases including gastritis, peptic ulcer, MALT lymphoma and gastric cancer. Our and other studies demonstrated that, in H. pylori-infected epithelial cells, CagA protein was injected into the cytoplasm through type IV secretion system, was tyrosine-phosphorylated via tyrosine kinase (PTKs) which is involved with tyrosine-phosphorylation and binds to a variety of phenomena such as activation of JNK and sustenance of Akt kinase activation, etc. in H. pylori-infected epithelial cells. These results suggest that CagA protein and tyrosine-phosphorylated CagA protein, that were injected into the cytoplasm through type IV secretion system and phosphorylated in the host cells, were deeply involved in gastric ulcer, MALT lymphoma and gastric cancer in vivo H. pylori-infected patients.

Methods: To correlate PAI function with genotype of the Y-972 motif and CagA tyrosine kinase (PTK), and (3) Syk PTKs was a multifunctional second messenger for tyrosine-phosphorylated CagA protein or CagA protein, which initiated the secondary phosphorylation signaling cascades leading to a variety of phenomena such as activation of JNK and sustenance of Akt kinase activation, etc. in H. pylori-infected epithelial cells. These results suggest that CagA protein and tyrosine-phosphorylated CagA protein, that were injected into the cytoplasm through type IV secretion system and phosphorylated in the host cells, were deeply involved in gastric ulcer, MALT lymphoma and gastric cancer in vivo H. pylori-infected patients.

Results: The most frequent motif found was a single SVSPEPIY in 18 strains, a single sequence repeat in 9 isolates, and one strain with 5 repeats, followed by 1b strains with a single SASPEPIY, 6 with a single repeat, and one with 3 repeats. Two strains showed a single SANHEPIY, and one 4 repeats. The motifs SASSEPIY, and SASPEEPIY were only found in one strain, respectively. 4 strains had no Y-972 motif. From each motif one strain was randomly chosen to determine the hummingbird phenotype and only the strain Ly27a harbouring 4 repeats of the motif SANHEPIY was found positive.

Conclusions: The mere PCR-based determination of Y-972 does not necessarily correlate with the sequence-predicted hummingbird phenotype and supports the view that additional bacterial factors may contribute to the full virulence phenotype. Work is in progress to test all isolates for the hummingbird phenotype and, in addition, for IL-8 induction and to correlate PAI function with genotype of the Y-972 motif and CagA function.

3.03 Functional analysis of CagA tyrosine phosphorylation motif Y-972 in clinical Helicobacter pylori isolates

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Objective: Helicobacter pylori CagA protein has been shown to be translocated into gastric epithelial cells and after phosphorylation to be essential for the induction of the so-called “hummingbird” phenotype. Phosphorylation has been demonstrated to occur at tyrosine residue 972 (Y-972). Several phosphorylation motifs have been identified so far. In order to correlate these motifs with the induction of the hummingbird phenotype in AGS cells we determined the cagA Y-972 motifs in different Helicobacter pylori isolates.

Methods: The complete cagA gene was amplified by PCR and sequenced from 60 H. pylori strains obtained from different clinical entities (duodenal ulcer, gastric ulcer, gastric carcinoma, and MALToma) and the Y-972 motifs were determined. AGS cells were then infected with H. pylori strains representing each motif and analysed for the induction of the hummingbird phenotype.

Results: The most frequent motif found was a single SVSPEPIY in 18 strains, a single sequence repeat in 9 isolates, and one strain with 5 repeats, followed by 1b strains with a single SASPEPIY, 6 with a single repeat, and one with 3 repeats. Two strains showed a single SANHEPIY, and one 4 repeats. The motifs SASSEPIY, and SASPEEPIY were only found in one strain, respectively. 4 strains had no Y-972 motif. From each motif one strain was randomly chosen to determine the hummingbird phenotype and only the strain Ly27a harbouring 4 repeats of the motif SANHEPIY was found positive.

Conclusions: The mere PCR-based determination of Y-972 does not necessarily correlate with the sequence-predicted hummingbird phenotype and supports the view that additional bacterial factors may contribute to the full virulence phenotype. Work is in progress to test all isolates for the hummingbird phenotype and, in addition, for IL-8 induction and to correlate PAI function with genotype of the Y-972 motif and CagA function.

3.04 Cytolethal distending toxin in Helicobacter cinaedi

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Helicobacter cinaedi is an enterohelobacter helicobacter causing a variety of diseases in humans. While it is an important pathogen, virulence factors have not been reported and little is known about how it causes disease. Recently, other enterohelobacter helicobacters have been reported to produce a cytolethal distending toxin (CDT). These toxins cause cellular distention in tissue cultured cells, and arrest cells in the G2/M phase of division. The toxin is encoded by 3 genes: cdA, cdB, and cdC. The most conserved is cdB. In this study we obtained 7 human isolates and one canine isolate of H. cinaedi from CCUG, 1 isolate from ATCC, one isolate from the mesenteric lymph node of a rhesus macaque with idiopathic colitis and one isolate from an immunocompromised patient. The human isolates were from individuals in Canada, Sweden, Australia, and the US. Isolates were assessed for typical morphological changes in HeLa cells, cell cycle arrest, and by PCR using primers designed for the H. cinaedi cdB gene. All but one isolate demonstrated typical changes in HeLa cells, arrested cell division at the G2/M phase (32-80% cells arrested), and had the expected 600 bp PCR product. The ATCC type strain lacked both the tyrosine-phosphorylated CagA and Cytolethal distending toxin (CDT).
D. Y. Graham.

Conclusions: The infection by H. pylori (HP) strains with different structural types of the cagA gene, may determine different risk of gastric carcinoma (GC) development. As post-genomic approach to the study of H. pylori is restricted to only a few strains, we carried out a comparative proteome analysis of HP with different cagA structural types, isolated from patients with different pathologies.

Methods: The clinical strains of the six strains studied and the patients’ pathologies are reported in the table. Strains were grown simultaneously and protein extracts were submitted to two-dimensional polyacrylamide gel electrophoresis. Immobiline strips covering a pH range of 3-10 were used for isoelectrofocusing, while a polyacrylamide gel separating proteins in the Mr range of 200-8 kDa was used for the second dimension. The digitalized images of the silver stained gels were qualitatively and quantitatively analyzed by Melanie II 2D-PAGE software (Bio-Rad). Spot intensities were obtained in pixel units and normalized to the total absorbance of the gel. Increasing/decreasing indexes (fold change) were calculated as the ratio of spot intensity (relative volumes). Identification of protein spots was performed by N-terminal microsequencing, gel matching and immunoblotting.

Results: We observed: a) a high variability in the expression of proteins sharing of virulence determinants, such as UreB, Cag26, CagA, catalase, UreA, TagD, 26K antigen, HSPI, NapA, etc.; c) a maximal expression of CagA by GC strains, independently of the structural cagA type; d) a quantitatively different expression of CagA in strains from diffuse and intestinal GC; e) a decreased expression of proteins other than CagA in the cagA‘ isogenic mutant.

Discussion: the increased CagA expression by GC strains may be used for diagnostic/prognostic purposes. The role of proteins expressed by different cagA structural types is uncertain.

3.8 Proteome of H. pylori strains with different cagA structural type isolated from patients with gastric carcinoma and controls

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Background: The infection by H. pylori (HP) strains with different structural types of the cagA gene, may determine different risk of gastric carcinoma (GC) development. As post-genomic approach to the study of H. pylori is restricted to only a few strains, we carried out a comparative proteome analysis of HP with different cagA structural types, isolated from patients with different pathologies.

Methods: The clinical strains of the six strains studied and the patients’ pathologies are reported in the table. Strains were grown simultaneously and protein extracts were submitted to two-dimensional polyacrylamide gel electrophoresis. Immobiline strips covering a pH range of 3-10 were used for isoelectrofocusing, while a polyacrylamide gel separating proteins in the Mr range of 200-8 kDa was used for the second dimension. The digitalized images of the silver stained gels were qualitatively and quantitatively analyzed by Melanie II 2D-PAGE software (Bio-Rad). Spot intensities were obtained in pixel units and normalized to the total absorbance of the gel. Increasing/decreasing indexes (fold change) were calculated as the ratio of spot intensity (relative volumes). Identification of protein spots was performed by N-terminal microsequencing, gel matching and immunoblotting.

Results: We observed: a) a high variability in the expression of proteins sharing of virulence determinants, such as UreB, Cag26, CagA, catalase, UreA, TagD, 26K antigen, HSPI, NapA, etc.; c) a maximal expression of CagA by GC strains, independently of the structural cagA type; d) a quantitatively different expression of CagA in strains from diffuse and intestinal GC; e) a decreased expression of proteins other than CagA in the cagA‘ isogenic mutant.

Discussion: the increased CagA expression by GC strains may be used for diagnostic/prognostic purposes. The role of proteins expressed by different cagA structural types is uncertain.

3.06 Helicobacter pylori virulence factors and the pattern of gastritis: oipA, vacA, and cagA tyrosine phosphorylation motifs EPIYA

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Background & Aims: The pattern of gastritis determines potential outcomes of H. pylori infection. We studied the pattern of gastritis with uncomplicated H. pylori gastritis in relation to H. pylori virulence factors.

Methods: Gastric mapping was done with jumbo forceps including 3 antral and 3 biopsy specimens. Gastritis was divided into pattern and scored as to severity as 1) superficial gastritis, 2) antral-predominant gastritis, 3) antral and 3 biopsy specimens. Gastritis was divided into pattern and number of Glu-Pro-Ile-Tyr-Ala
This loss of motility was due to the absence of flagella in the flaA1 mutant as judged by electron microscopy. Immuno-detection experiments will reveal if the absence of flagella results from lack of flagellin synthesis or export. The non motile wbpB mutant still produced flagella, indicating that these flagella were not functional. Finally, 2D-gel electrophoresis analysis of total proteins suggested that the flaA1 and wbpB genes were both necessary for the glycosylation of two proteins yet to be identified.

The similar phenotypes observed for the flaA1 and wbpB mutants suggest that both genes participate in the same biological function although they are found remote from one another in the H. pylori genome. The multitude of phenotypes observed suggests that flaA1 and wbpB might be at the interface between several pathways that govern expression of different virulence factors.

3.09 Beyond the vacuole. Trafficking of VacA from Helicobacter pylori within epithelial cells

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VacA, a major virulence factor of H. pylori, forms anion-selective pores within membranes, causes profound vacuolation of epithelial cells, reduces trans-epithelial resistance, and plays a role in apoptosis. Although it is clear that VacA is localised to Rab7-positive vacuoles, little is known about the delivery of VacA to other compartments within epithelial cells.

Methods/Results: To visualise the intracellular location of VacA, we performed direct immunofluorescence using anti-VacA antibodies on RK13 cells treated with culture supernatant from H. pylori strain 60190 (vacA s1-allele). As expected, most VacA localised to the vacuolar membrane, but a small proportion appeared to be free within the cytosol. To provide further evidence for the existence of cytosolic VacA, we treated RK13 cells, co-cultured for 16-24 hours with H. pylori strain 60190, with cytochalasin D (2 μg/ml), which causes disruption of the Golgi apparatus. Cytosolic VacA appears to be biologically important, as VacA has been reported to interact with the cytosolic proteins VIP54 and RACK1, and to cause apoptosis through mitochondrial damage.

Conclusions: Most VacA delivered to epithelial cells becomes associated with vacuolar membranes but a fraction appears to be translocated into the cytosol and may be transported through the Golgi apparatus. Cytosolic VacA appears to be biologically important, as VacA has been reported to interact with the cytosolic proteins VIP54 and RACK1, and to cause apoptosis through mitochondrial damage.

3.10 Nucleotide sequence variation in the pldA gene of Helicobacter pylori isolates from a clinical material

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Objective: Helicobacter pylori phospholipase A (PLA) activity results in degradation of bacterial membrane phospholipids (PL) to lysophospholipids (lysoPL). High levels of lysoPL compared to PL are associated with higher hemolytic activity, increased release of urease and VacA and better adherence to epithelial cells in vitro. The gene coding for PL (pldA) displays phase variation resulting in ON/OFF switching of PL activity. The aim of this study was to determine the pldA status of H. pylori strains in a clinical material.

Methods: Bacterial strains from 42 patients colonized with H. pylori were examined for pldA status by sequencing. The lysoPE/PE ratios were determined by preparative thin-layer chromatography (TLC).

Results: All isolates contained homopolymeric tracts in pldA. Four isolates had repeats in these variable regions in resulting in shifts in the open reading frame leading to OFF switching of PL activity. In addition one isolate had lost 1413 base pairs from pldA and the up-stream gene. The remaining isolates showed an intact open reading frame for pldA. The lysoPE/PE ratio varied from 0.005 to 1.92.

Discussion: Although we found a high degree of intragenic diversity of pldA, 88% of the isolates yielded pldA sequences with the potential of an active PL. Even though isolates yielded pldA sequences with the potential of an active PL, there was a high degree of variation in the observed PL activity.

3.11 Structural examination of Lewis expression and adaptation in lipopolysaccharides of Helicobacter pylori from experimentally infected rhesus monkeys

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Expression of Lewis antigens (Le<sup>a</sup> and Le<sup>b</sup>) in lipopolysaccharides (LPSs) of Helicobacter pylori (Hp) has been hypothesized to be related to host Helicobacter pylori attenuation in humans, connected to protected bacterial populations. Moreover, serological analysis of Hp isolates from an inoculation study of rhesus monkeys showed that monkeys of non-secretor status, Le<sup>a</sup> and Le<sup>b</sup> were the only isolates that showed mono-secretor status, whereas non-secretors, Le<sup>a</sup> and Le<sup>b</sup>, were the only isolates that showed 3-2 secreting isolates. However, to understand the molecular complexity of this adaptation, LPS structural analysis was performed. From the monkey studies, the inoculating strain and three isolates recovered after 40-weeks colonization were studied. Biomass was produced in large quantities, LPS extracted by the hot phenol-water technique and purified by enzymatic treatments and ultra-centrifugation. After hydrolysis, Le<sup>a</sup> and gel chromatography, the saccharide of LPS was isolated and subjected to detailed structural determination using NMR spectroscopy, mass spectrometry, and sugar methylation analysis. The J166 (inoculating) strain, 98-149 and 98-169 (non-secretor monkey isolates) and 98-181 (secretor monkey isolate) produced an O-chain of N-acetylgalactosamine, which causes disruption of the Golgi apparatus. Surprisingly, this result suggested that vacA is localised to Rab7-positive vacuoles, but a fraction appears to be translocated into the cytosol and may be transported through the Golgi apparatus. Cytosolic VacA appears to be biologically important, as VacA has been reported to interact with the cytosolic proteins VIP54 and RACK1, and to cause apoptosis through mitochondrial damage.

3.12 Virulence factors, cagA and vacA, and Lewis antigen expression in Helicobacter pylori isolates from Spanish paediatric patients

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The aim of this study was to determine the occurrence and relationship between the presence of Lewis antigen and other virulence factors (cagA and vacA) in H. pylori strains obtained from paediatric patients.

Methods: 36 H. pylori strains were obtained from paediatric patients attending to the Gastroenterology Unit due to different symptomatology. Upper endoscopy was performed and biopsy cultured following standard methodology. Strains were conserved at -70°C until used. Lipopolysaccharide (LPS) was extracted from a culture of H. pylori with mini-phenol water and detection of Lewis antigen was determined by a standard serodot method using monoclonal anti-Le<sup>x</sup> and anti-Le<sup>y</sup> antibodies. DNA was extracted from a culture of H. pylori with phenol-water technique and PCR performed to detect cagA gene and vacA s1- and s2-alleles.

Results: The frequency of H. pylori strains expressing LewisX was 61.1% (22 strains) and LewisY was 58.3% (21 strains). None of the strains tested expressed LewisX, LewisY, Le<sup>a</sup>, Le<sup>b</sup>, sialyl-LewisX and H type 1. Peroxidase-labelled secondary antibody. DNA was extracted from a culture of H. pylori with phenol-water technique and PCR performed to detect cagA gene and vacA s1- and s2-alleles.

Conclusions: The prevalence of cagA gene and vacA s1-alleles was observed in strains from paediatric patients. Furthermore,
occurrence of \textit{cagA} was associated with \textit{vacA} s1-allele and to LewisX and LewisY in this population.

### 3.13 Search for a pathogenicity marker in the plasticity zone of \textit{Helicobacter pylori} genome

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Comparison of strains isolated from gastric carcinoma and gastritis allowed the identification of genes apparently associated with gastric carcinoma or gastritis. \textit{H. pylori} strains isolated from patients with gastric carcinoma (n=76), gastritis (n=69) and peptic ulcer (n=48) were investigated.

\textit{cagA} status of these strains was known. A PCR was performed with primers designed to detect JHP940, JHP947 as well as a third ORF (JHP966) associated with gastritis. ORF JHP947 was found associated with gastric carcinoma patients (86.8%) and peptic ulcer patients (19.2%) versus a presence of 47.8% in strains from gastritis patients. The difference was significant (p<0.01 versus a presence of 47.8% in strains from gastritis patients). The differential enhancement, proinflammatory cytokines attenuation and leptin and growth hormone increase.

The results of this study suggest that omeprazole micronized could neutralize the vacuolation effect of \textit{H. pylori} cytotoxin on Vero cells probably by targeting v-type ATPase. The bacterial motility was also inhibited by low concentrations of omeprazole. The results of this study concords omeprazole micronized as an effective drug which targets important virulence factors of \textit{H. pylori} including, vacuolating cytotoxin, urease, and motility.

### 3.14 Aspirin-induced gastric damage is attenuated in rats adapted to \textit{H. pylori} lipopolysaccharide (Hp-LPS) and LPS-derived from intestinal bacteria

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\textbf{Background and Aims:} LPS is the major virulence factor in Hp-infected stomach. Whether gastric mucosa can adapt to prolonged treatment with this endotoxin or water extract of Hp (WE-Hp) containing LPS and whether such adaptation can influence aspirin (ASA) induced gastric damage have been little studied.

\textbf{Methods:} We compared the effect of single and 5-day repeated intragastric (i.g.) administration of Hp-LPS or WE-Hp with those induced by LPS isolated from Campylobacter jejuni. Yersinia enterocolitica and Bacteroides fragilis in gastric secretion in rats (series A) and acute gastric lesions induced at day 5 by ASA (series B). Hp-LPS and WE-Hp were prepared from antral biopsy of Hp-infected patients. Gastric blood flows (GBF) and lesions area were measured. Plasma leptin, gastrin, IL-1beta and TNFalpha were determined using RIA or ELISA. Gastric mucosa leptin mRNA was analyzed by RT-PCR.

\textbf{Results:} Hp-LPS (0.015- mg/kg i.g.) or WE-Hp (1 ml i.g.) inhibited the gastric acid secretion (series A) and produced gastric damage which disappeared after 5 daily exposures. It was accompanied by GBF, gastric gastrin and leptin levels increase (series B). Intestinal LPS (1 mg/kg i.g.) inhibited gastric secretion and produced negligible damage also disappeared at day 5. ASA alone caused gastric lesions, decreased GBF and raised the plasma IL-1 beta and TNFalpha levels 2-3 fold. The ASA damage was attenuated in rats treated 5 times with Hp-LPS and WE-Hp or intestinal LPS. These effects were accompanied by GBF increase, leptin mRNA expression, plasma immuneactive leptin and gastric concentration increase, IL-1 beta and TNFalpha levels decrease.

\textbf{Conclusions:} 1) Gastric mucosa may adapt to intestinal endotoxins similarly to that observed in Hp-LPS and WE-Hp treated mucosa and 2) adaptation to Hp cytotoxins enhances mucosal resistance to subsequent ASA damage probably by inhibition of acid secretion, gastric microcirculation enhancement, proinflammatory cytokines attenuation and leptin and gastrin increase.

### 3.15 Neutralization of Helicobacter pylori cytotoxicity on vero cells by omeprazole micronized

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\textbf{Backgrounds/Aims:} Omeprazole is a gastric parietal cells proton pump inhibitor that is also active against \textit{H. pylori} in vitro. This study was designed to examine the neutralization of \textit{H. pylori} cytotoxicity on Vero cells by omeprazole micronized in strains isolated from gastritis, ulcer, cancer and Barrett’s ulcer. This study could determine whether omeprazole could inhibit vacuolation of the Vero cells induced by cytotoxin of \textit{H. pylori} or by urease. The effect of omeprazole on motility of \textit{H. pylori} was assessed using concentrations lower than MIC.

\textbf{Materials and Methods:} The antimicrobial activity of omeprazole micronized was studied by determining the MICs for 15 cytotoxic and four non-cytotoxic \textit{H. pylori} strains. Water extract of the cytotoxic bacterium (concentrated culture supernant) and different concentrations of omeprazole were added to Vero cells in culture. Also extracted urease from \textit{H. pylori} strains with urea (10 Mm) and omeprazole were added to Vero cells in culture. The inhibitory effect of omeprazole on motility of \textit{H. pylori} was tested in semi-solid medium.

\textbf{Results:} MIC90 of omeprazole micronized was 20 µg/ml. MICs for cytotoxic and non-cytotoxic strains were similar. Omeprazole could inhibit induced vacuolation by the water extract of cytotoxic \textit{H. pylori} strains in Vero cells. It could also inhibit vacuolation induced by urease. Inhibition of vacuolation strains was assessed microscopically and by the neutral red method. It was found that omeprazole inhibits the motility of \textit{H. pylori} strains at concentrations lower than MIC.

\textbf{Conclusions:} The results of this study suggest that omeprazole micronized could neutralize the vacuolation effect of \textit{H. pylori} cytotoxin on Vero cells probably by targeting v-type ATPase. The bacterial motility was also inhibited by low concentrations of omeprazole. The results of this study concords omeprazole micronized as an effective drug which targets important virulence factors of \textit{H. pylori} including, vacuolating cytotoxin, urease, and motility.

### 3.16 Determination of the presence of the whole \textit{Helicobacter pylori} cag pathogenicity island in different clinical isolates by an overlapping PCR-based amplification system

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\textbf{Objective:} The \textit{cag} PAI is considered to be one of the major virulence determining factors of \textit{H. pylori}. Although the \textit{cagA} gene is conventionally used as a surrogate marker for the presence of the \textit{PAI}, several studies have limited the use of \textit{cagA} PCR as a suitable marker. In order to determine the presence of the whole \textit{PAI} we developed a PCR-based system to analyse the \textit{PAI} in different clinical \textit{H. pylori} strains.

\textbf{Methods:} Based on the published sequence of \textit{H. pylori} 26695 a PCR-based amplification system was developed to cover the whole \textit{PAI} with overlapping amplicons of 1000-1200 bp in size. 14 clinical Hp isolates that were all shown to induce IL-8 in AGS cells were chosen to evaluate this assay system. The obtained PCR-product were visualised by gel electrophoresis.

\textbf{Results:} Using 31 primer pairs we could successfully amplify the whole \textit{PAI} in 12 of 14 strains. In two strains there were no amplicons obtained at the N- and C-terminus of HP0547, but the rest of the \textit{PAI} was completely amplified. Comparison of the PCR products revealed that the \textit{PAI} was highly conserved because nearly all amplicons were of equal size. When PCR products were sequenced with the reverse primer it was possible to amplify the missed region with upstream and downstream primers. This was true for HP0527 and HP0544 that could only be amplified by long-distance PCR because of multiple gene rearrangements or sequence variations, respectively.

\textbf{Conclusions:} Although there were differences detectable with respect to sequence variations (primer mismatch) and amplicon size in some regions the genetic variability seems to be limited. Work is in progress to determine now the sequence of the whole \textit{PAI} of all 14 \textit{H. pylori} strains to compare the open reading frames in more detail.
The combined lysophospholipid/phospholipid ratio and cagA status in Helicobacter pylori is identified as a new predictive factor of ulcer disease

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Objective: CagA is regarded as virulence-associated marker of Helicobacter pylori. However, patients colonized with CagA positive strains do not ultimately express virulence. H. pylori phospholipase A (PLA) activity results in degradation of bacterial membrane phospholipids (PE) to lysophospholipids (lysoPE). We have previously shown that variants of H. pylori with high levels of lysoPE compared to PE are selected under acid culture conditions in vitro. High levels of lysoPE compared to PE are associated with higher hemolytic activity, increased release of urease and VacA and better adherence to epithelial cells in vitro. The aim of this study was to determine if there is a correlation between a high lysoPE/PE ratio in the bacterial population from patients with ulcer disease (UD) compared to non-ulcer dyspepsia (NUD).

Methods: 42 patients colonized with H. pylori were examined. Of these 38 had ulcer disease (positive gastroscopy and typical symptoms) and 29 were classified as NUD patients. Bacterial strains from all 42 patients were examined. The lysoPE/PE ratios were determined by preparative thin-layer chromatography (TLC). Genotyping based on cagA, vacA and iceA was also performed.

Results: The lysoPE/PE ratios varied from 0.005 to 1.92. There was a statistical significant association between lysoPE/PE ratio * cagA status and UD when adjusted for a RR for UD of 8.6 (95% CI 1.5 - 49.5, p = 0.016).

Discussion: We demonstrate that infection with strains showing a high lysoPE/PE ratio * cagA status is significantly associated with increased risk of ulcer disease. The cagA status is strain specific while the lysoPE/PE ratio is a result of interaction with the environment in the human ventricle.

3.18 Cag pathogenic island is associated with Helicobacter pylori induced apoptosis in Koreans

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Background & Aims: The increase of apoptotic cells of Helicobacter pylori-infected mucosa suggested that apoptosis could be a major mechanism of cellular damage. However, H. pylori cag pathogenic island(PAI) structure relating to apoptosis research has been little studied. The aim of this study was to analyze the role of cag PAI structure in H. pylori induced apoptosis.

Methods: The H. pylori isolates were classified according to the structure of cag PAI using polymerase chain reaction and then infected in the AGS cell line. Cell cytotoxicity, NF-kB activity, IL-8 production measurements were performed and FACS were able to measure the levels of apoptosis. The expression ratio of the proteins associated with the apoptosis, Bak, Bax, and Bcl-2 were observed and similarly, the expression ratio of the proteins associated with the apoptosis.

Results: The degree of the damage to the cell due to the infection of H. pylori where the cag PAI was completely lost in the H. pylori were remarkably low, and regardless of the structure of cag PAI, in the addition of TNF-α, the cell survival decreased. In the H. pylori strain where the cag PAI was completely lost and also where the cagE was deletion, the production of IL-8 was very low. On the other hand, the activity of NF-kB showed relevant differences according to the structure of cag PAI and the expression of Bcl-2 protein significantly decreased when the H. pylori strain with an intact cag PAI structure was infected. The manifestation of caspase-8 or caspase-3 practically did not show much discrepancy.

Conclusions: Depending on the structure of cag PAI, the secretion of cytokine or apoptosis as an inducing activity revealed dramatic differences, therefore, the structure of cag PAI would be considered very important in regards of H. pylori etiology

3.19 Experimental colonization of mice by Helicobacter pylori is not influenced by the cagA status and the vacA genotype

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Aims: Developing murin models of infection by H. pylori is quite useful but all the strains are not able to colonize the mouse. The aims of this work were to know the percentage of fresh isolated clinical strains spontaneously able to colonize the mouse and to study the influence of the two main virulence factors, CagA and VacA, on the establishment of H. pylori in mice.

Materials and Methods: C57BL/6 mice were orally inoculated with 13 strains randomly chosen among clinical strains freshly isolated from biopsy specimens of infected patients in Russia.

Results: Six (40%) of the clinical strains and 2 (40%) of the reference strains could infect the animals regardless of their cagA status. vacA genotyping showed that 35.3% of the strains that produce an active cytoxin and 66.6% of the strains that do not were established in the mouse stomach. These proportions were not statistically different (p = 0.32).

Discussion/Conclusion: Forty % of the H. pylori strains are able to infect mice and that the capacity of colonization is influenced neither by the cagA status nor by the vacA genotype. These factors cannot be used to predict the success of an experimental infection.

3.20 Helicobacter pylori Cag A, Vac A, Ice A genotype in Korean patients with gastric cancer

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Background: Virtually everyone infected with Helicobacter pylori (H. pylori) develops benign gastroduodenal diseases such as chronic gastritis, gastric ulcer, or duodenal ulcer but only a minority of infected individuals will develop gastric cancer. So, it is important to determine whether strains with specific genotype are associated with the clinical outcome in the patients with H. pylori infection. The present study aimed to define whether any specific genotype was associated with gastric cancer.

Methods: PCR was used to examine cagA, vacA and iceA genotypes of H. pylori isolates obtained from 87 infected patients: 47 cases of nonulcer dyspepsia (NUD) and 40 cases of gastric cancer. Histologic diagnosis of gastric cancer was undertaken according to the Lauren system(29 cases of intestinal type, 11 cases of diffuse type).

Results: There was no significant difference between NUD and gastric cancer in the positive rates of cagA, vacA s1b, s1c, m1, m2, iceA1, and iceA2. But the positive rates of vacA s1a, s2 were 29.8% (14/47) and 0.0% (0/47) in NUD, and 10.0% (4/40) and 22.5% (9/40) in gastric cancer, showing the significant difference (p=0.023, p=0.001, respectively). The most common genotype in both groups was cagA\+ vacA s1c1 m1 iceA1 (51.1% in NUD, 50.0% in gastric cancer). Histologic type in gastric cancer showed no significant difference in the positive rates of all genotypes.

Conclusion: cagA\+ vacA s1c1 m1 iceA1 genotype is the most common in NUD and gastric cancer in Korean. The higher positive rate of the vacA s2 genotype in gastric cancer is thought to be due to regional difference in part. But, further study is needed to evaluate the relationship of the vacA s2 genotype to gastric cancer.

3.21 Genotyping of H. pylori obtained from patients with duodenal ulcer and gastritis in Moscow region, Russia

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Distribution of H. pylori genotypes was studied in 87 patients (25-55 y.o) from Moscow and Moscow region with endoscopically confirmed DU (n=55) and gastritis (n=25).

Bacterial DNA was extracted from antral biopsies and genotyping of cagA, vacA s1a2s1m1m2, iceA 1/2 &#1080; babA2 was performed with specific primers. Products of amplification were separated in 2% agarose gel which contained 0.5 mcg of ethidium bromide in 1xTAE buffer. Results were documented with videosystem and analyzed by Molecular Analytis Software (BioRad). Distribution of gene types are shown in the table.

Expression of cagA was found in 86% of DU and in 94% with gastritis (p=0.6). There was also no differences found in the rate of expression of vacA s1, vacA m1, iceA1 and babA2 between DU and gastritis patients.

Analysis of combination of different H. pylori genotypes (vacA s1/s2, m1/m2, cagA, iceA1, babA2) demonstrated that from 18 possible combinations the most common was the genotype with simultaneous expression of vacA s1, cagA, iceA1 and babA2 that was found in 31% of cases. vacA s2m1 genotype was not found in this group of patients.
The relationship of the Helicobacter pylori virulence factor gene subtype in gastric adenocarcinoma

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Purpose: The H. pylori cagA gene, vacA gene and iceA gene are considered to be important virulence factors that have been implicated in the development of gastric adenocarcinoma. It was reported that the presence of IS605 elements may be responsible for rearrangements and lead to partial or total deletions of the cag pathogenicity island (PAI) and the virulence of cag PAI may be changed. However, different results regarding the association between these virulence factors and clinical disease have been reported from different geographic regions. This study evaluated the relationship between H. pylori virulence factors such as cagA, vacA, iceA, IS605 and gastric adenocarcinoma.

Materials and Methods: H. pylori isolates were obtained from 54 infected patients (24 cases of gastric adenocarcinoma, 30 cases of control). H. pylori isolates were identified by PCR with ureC gene and 16S rRNA. PCR was performed to examine cagA, vacA, iceA and IS605 genotypes.

Results: Significant difference was found in the negative rates of cagA between gastric adenocarcinoma group and control (62.5% vs. 33.3%; p<0.033). No significant difference was found in the prevalence of iceA, vacA between gastric adenocarcinoma and control. The genotype of cagA positive/vacA s1-m1 iceAI/iceA1+ in H. pylori isolates irrespective of the clinical outcome. IS605 in PAI was not found in gastric adenocarcinoma group and control. The positive rates of IS605 in genome were 33.3% in gastric adenocarcinoma group and 36.7% in control (p=0.05). In gastric carcinoma, the positive rate of cagA+/IS605+ was lower than in control (12.5% vs. 40.0%; p=0.025) and the positive rate of cagA+/IS605+ was higher than in control (54.2% vs. 23.3%, p<0.02).

Conclusion: H. pylori virulence factors had not related significantly with gastric adenocarcinoma. Further study is needed to examine the specificity of H. pylori strains.

Helicobacter pylori genotypes in the Greek population

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Purpose: The prevalence of H. pylori-associated gastric and duodenal lesions is higher in the Greek population than in other European countries. To evaluate the genotypic and type distribution of the isolates, a material of 86 patients (20 with gastric adenocarcinoma, 20 with gastric mucosa-associated lymphoid tissue (MALT) lymphoma, 20 with duodenal ulcer, and 26 with asymptomatic patients) was subjected to genotypic analysis of the following virulence markers: vacA, cagA, iceA, and IS605. To further investigate the occurrence of vacAs2 gene subtype of H. pylori, we carried out analyses in 79 isolates and compared them with other vacA subtypes.

Results: Of the isolates, 52% were from patients with gastric adenocarcinoma and 48% from patients with duodenal ulcer. We detected vacA s1 and m1, iceA1, and IS605 in all isolates. The prevalence of vacA s1/m1 was highest in gastric adenocarcinoma patients (66% and 34% respectively). Out of 74 strains analysed, 33 were cagA-positive, and 41 were vacA s1/m1-positive. Out of the 79 strains, 52 belonged to the vacA s1/m1 subtype and 27 to vacA s1/m2 subtype (66% and 34% respectively). Out of 74 strains analysed, 23 (31%) were of the vacA s1/m1 and 48 (65%) vacA s2/m2 subtypes while 3 (4%) remained untypeable. The combinations of vacA subtypes observed are summarized in the following table:

<table>
<thead>
<tr>
<th>Type of gene</th>
<th>Patients n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vacA s1/m1</td>
<td>55 (65.3%)</td>
</tr>
<tr>
<td>vacA s2/m2</td>
<td>33 (39.5%)</td>
</tr>
<tr>
<td>cagA positive</td>
<td>81 (93%)</td>
</tr>
<tr>
<td>vacA m2/m1</td>
<td>35 (45%)</td>
</tr>
<tr>
<td>iceA2</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>babA2 positive</td>
<td>34 (39.3%)</td>
</tr>
<tr>
<td>babA2 negative</td>
<td>33 (39.3%)</td>
</tr>
</tbody>
</table>

Conclusion: A high frequency of cagA positive strains was observed in the Greek population. There was no significant difference in the frequency between the vacA mid- and signal-region subtypes. No statistically significant differences among the subtypes were detected between the adult and the children study groups.

References


3.24 The dynamics of Helicobacter pylori infection in patients with perforated peptic ulcer

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Background: The s1a/m1 Helicobacter pylori strains are known to be highly toxigenic. There are few data of virulence markers of H. pylori strains connected with noneffective vs. effective eradication, particularly in patients with perforated peptic ulcer (PPU).

Aims: To study the dynamics of H. pylori infection using vacA subtypes as molecular virulence markers among patients with PPU.

Materials and Methods: The study was carried out in Tartu University during 1997 - 2001. Thirty-seven PPU patients were operated and treated using 6 different antimicrobial treatment schemes (within 5-7 days). DNA of H. pylori was isolated from gastric antral biopsy specimens, taken before (index) and two months after treatment. From 21 patients the 1-year follow-up specimen was taken. PCR amplification was applied for examination of vacA allelic types of H. pylori.

Results: Two months after the treatment H. pylori was found in 22 cases from 37 (59%). Half of these (50%) were s1a/m1 type of strains. One year after the treatment, 67% of patients were H. pylori-positive with the same vacA subtype as in index-probe, most (64%) of these with s1a/m1 type. The reinfection rate 1 year after the treatment was 14%, where H. pylori strain with s1a/m1 type was replaced with s1a/m2 type of strain. Only four patients remained H. pylori-negative after both two months and 1-year follow-up.

Conclusion: It wasn’t possible to find connection between the H. pylori strains with different markers of cytotoxity and the dynamics (stable, disappearing or changing) of the infection. To lower the high rate (67%) of relapses, the longer H. pylori eradication treatment (>7 days) can be suggested.

3.25 Helicobacter pylori negative patients and gastric cancer: High seroprevalence of Cag A and Vac A antibodies

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Background/Aim: Helicobacter pylori (Hp) has been assigned as a class I carcinogen because of its relation to gastric carcinoma (GC). Infection with different genotypes of virulent Hp strain, CagA and/or Vac A positive, play a role in the development of atrophic gastritis, duodenal ulcer (DU), and GC.

Methods: This study was undertaken to investigate if patients with GC with Hp negative (Hp-) histological staining had a past infection by virulent strains of Hp CagA and/or Vac A positive. 20 GC, 15 males and 5 females (mean age±SD) 68.14±9.8 y old Hp- to histological staining in antrum, corpus and in the lesion take part to the study. Two control group were included: 19 Hp infected patients with DU eradicated 10 years before, 14 males and 5 females, (mean age±SD) 58±18.2 y old. Hp- status was determined every year with histology Giemsa staining and follow-up after therapy was (average±SD) 120±32 months; range 96-44 months. 20 asymptomatic children, 10 males and 10 females, (mean age±SD) 7±4.47 years old, with Hp- faecal test (HPSA-Meridian). The immunoblot assay

<table>
<thead>
<tr>
<th>Groups</th>
<th>VacA-total patients</th>
<th>CagA-total patients</th>
<th>VacA-h/total patients</th>
<th>CagA-h/total patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>19/20</td>
<td>19/20</td>
<td>12/20</td>
<td>2/20</td>
</tr>
<tr>
<td>DU</td>
<td>16/19</td>
<td>16/19</td>
<td>3/19</td>
<td>3/19</td>
</tr>
<tr>
<td>Children</td>
<td>1/20</td>
<td>2/20</td>
<td>19/20</td>
<td>15/20</td>
</tr>
</tbody>
</table>

Prevalence of VacA and CagA in gastric cancer, duodenal ulcer and children
3. Virulence factors (Cag PAI, Vac A, iceA, babA)

(Mikrogen-Germany) was used to detect serum antibodies against CagA and VacA of all patients.

**Results**: The prevalence of CagA and VacA seropositivity was 90% and 95% in GC, 84% and 84% in DU Hp+, 25% and 5% in children Hp- respectively. Serum CagA and VacA antibody positivity was not significantly different between GC and patients with DU eradicated 10 years before. In contrast a true significant positivity was found against children (t-student test; P<0.0001). Statistical difference was found in age between groups P<0.03.

**Conclusions**: Patients with GC, although Hp at present, could be infected by Hp before the appearance of the disease as confirmed by CagA and VacA seropositivity. These data, therefore, may reinforce the idea to consider Hp as a direct carcinogenetic agents of GC.