# Comparison of *Helicobacter Pylori* Genotypes Obtained from the Oropharynx and Stomach of the Same Individuals – A Pilot Study

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**Abstract:** *Helicobacter pylori* has been recently detected in the oral cavity and oropharynx. However, the role it plays in oral and oropharyngeal pathogenesis remains unclear. The virulence of *H. pylori* strains can be distinguished according to the virulence factors genes carried. Our research has been focused on real-time PCR analysis of *cagA* and *vacA* genes of *H. pylori* strains in tonsils and tonsillar squamous cell cancer and their comparison with *H. pylori* strains obtained from

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the gastric mucosa of the same patients. Urea breath test (UBT) test was used to detect a gastric *H. pylori* infection in 20 patients with previously proven *H. pylori* in the oropharynx. Genotyping of *H. pylori* in gastric biopsies was performed in patients with positive gastric infection. Out of 20 patients positive for oropharyngeal *H. pylori*, 8 were positive for concurrent gastric *H. pylori* infection. In 6 of them gastric biopsies were obtained. Comparison of oropharyngeal and stomach *H. pylori* genotypes showed important differences. Four of 6 patients had different *H. pylori* strains in the oropharynx and stomach. The differences were found in *cagA* gene as well as in *vacA* gene. The finding of oral presence of *H. pylori* without concurrent stomach infection was confirmed using UBT. The results show that more than one *H. pylori* strain can be present in oropharynx and stomach in the same patient. The oropharyngeal infection seems to be independent to the gastric infection.

## Introduction

*Helicobacter pylori* is a well known gastric pathogen (Peterson, 1991; Israel and Peek, 2001). *H. pylori* is a declared type I carcinogen by IARC (1994). Since it was detected in the oral cavity (Nguyen et al., 1993; Riggio and Lennon, 1999; Karczewska et al., 2002) and oropharynx (Bulut et al., 2006; Pavlik et al., 2007; Abdel-Monem et al., 2011) a hypothesis about its possible influence in oropharyngeal pathogenesis is being considered (Rubin et al. 2003; Lukes et al., 2008). *H. pylori* is spread from person to person by oral-oral or faecal-oral route (Brown, 2000). Assuming the oral cavity and oropharynx as a gateway of infection, we can expect that in the oral cavity and oropharynx of the same individual we should find *H. pylori* strains of the same genotype. The virulence of *H. pylori* strains differs according to the production of several virulence factors. The most important virulence factors, which were described as associated with gastric pathogenesis, are CagA (cytotoxic associated gene A) and VacA (vacuolizating cytotoxin A). Genome sequence analysis of *H. pylori* DNA led to the identification of genes encoding these virulence factors. The immunodominant CagA protein is encoded by the group of genes called the cagA-PAI (pathogenicity island), which is present in 60–70% of strains (Mobley, 1996). *H. pylori cagA*+ strains are associated with a significantly increased risk for severe gastritis, atrophic gastritis, peptic ulcer disease and distal gastric cancer compared with *cagA*– strains (Portal-Celhay and Perez-Perez, 2006). All *H. pylori* strains carry the *vacA* gene. Significant polymorphism of this gene exists. *VacA* alleles possess one of two types of signal region, s1 or s2, and one of two mid-regions, m1 or m2, occurring in all possible combinations. *H. pylori* strains with different forms of *vacA* have different pathogenetic potential (Portal-Celhay and Perez-Perez, 2006). The question whether the finding of oropharyngeal *H. pylori* indicates permanent colonization or it is only a sign of contamination of this area by gastric juices still remains unclear.

The presence of different genotypes of *H. pylori* in the oral cavity and stomach is now being considered (Wang et al., 2002; Loster et al., 2006). The comparison of oropharyngeal and stomach genotypes has not previously been studied. The aim of this study was to find out whether one person could host different *H. pylori* strains in different locations. The other aim was to determine whether the oropharyngeal presence could be caused by contamination by gastric contents or it is a permanent colonization which could be independent from gastric infection. Previous research in Department of Otorhinolaryngology, Head and Neck Surgery, First Faculty of Medicine, Charles University in Prague, has been focused on real-time PCR analyzing *cagA* and *vacA* genotypes of *H. pylori* strains in tonsils and tonsillar squamous cell cancer (Lukes, 2010). In 6 cases we have compared the DNA sequences of *H. pylori* strains obtained from oropharyngeal area with the strains obtained from gastric mucosa of the same patients.

## Material and Methods

*Collection of clinical specimens*

The study was approved by the appropriate ethics committee, and informed written consent was obtained from every participant.

Twenty patients with previously proven *H. pylori* in the oropharynx were selected for routine urea breath testing (UBT) for the presence of *H. pylori* in the stomach. The patients were part of a study on the detection of oropharyngeal *H. pylori* (Lukes, 2010). They underwent surgery and subsequently the tissue samples were analysed for the presence of *H. pylori*. Indications for surgery were tonsillar spinocellular cancer (SCC) (9 patients), obstructive sleep apnea syndrome (OSAS) (5 patients) and chronic tonsillitis (6 patients).

The tonsillar specimens were collected using sterile instruments at the beginning of surgery, immediately after insertion of endotracheal tube, prior to application of local anaesthetics or disinfection substances into the oral cavity. Biopsies were immersed into Microtest$^R$ M4RT transport media (Remel Inc., USA) and transported to the laboratory for real-time PCR analysis. After positive results for *H. pylori* presence in tonsillar tissue, the presence of gastric infection was confirmed using UBT.

Eight patients were found positive for *H. pylori* by UBT. Six of them (3 with cancer, 2 with OSAS and 1 with tonsillitis) gave their consent for gastroscopy (remnant two patients declined any further examination). The gastroscopy was performed subsequently at the Department of Gastroenterology, Hospital of Merciful Sisters of St. Charles Borromeo, Prague. Gastric mucosa samples were taken from the antrum and body of the stomach and immersed in Microtest$^R$ M4RT transport media for real-time PCR analysis. The genotyping of gastric strains was performed. The gastric genotypes were compared to oropharyngeal genotypes in the same individuals.

*Preparation of genomic DNA*
*H. pylori* DNA isolation was performed on MagNA Pure Compact System (Tegimenta AG, Rotkreuz, Switzerland) using Protocol "Total_NA 400_100" and MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Basel, Switzerland) with pre-treatment in MagNA Pure Bacteria Lysis Buffer (Roche Diagnostics, Basel, Switzerland). Isolated nucleic acid specimens were stored in –80 °C until genotyping assays were performed.

*PCR amplification and genotyping*
For genotyping, 3 real-time PCR TaqMan assays had been developed in cooperation with TIB-Molbiol Berlin, Germany; one for the *cagA* gene, the second for the *vacA* gene middle region and the last one for the *vacA* gene signal region. Primers used for PCR detection of *H. pylori* were: cagA F (sense), cagA R (antisense), HPMGF+ (sense), HPMGR– (antisense), VA1F (sense) and VA1R– (antisense) according to Atherton et al. (1995) and van Doorn et al. (1998, 1999) (Table 1).

TaqMan hybridisation probes were developed for *cagA, vacA m1* and *vacA m2* specific sequence detection (Table 2). For the *cagA* assay FAM-BBQ labelled probe was used (detection 530 nm), for *vacA* middle region assay FAM-BBQ labelling was used for M1 TM-probe (530 nm) and HEX-BBQ labelling for M2 TM-probe (560 nm).

TaqMan$^R$ real-time PCR assays were run on a LightCycler$^R$ instrument, version 2.0 (six channel detection: 530, 570, 610, 640, 670 and 705 nm). Commercial LightCycler$^R$ TaqMan$^R$ Master (Roche Applied Science, Basel, Switzerland) was used – 15 µl of MasterMix including primers and probes and 5 µl of sample DNA isolate per 20 µl capillary.

For real-time PCR for *vacA* gene signal region hybridisation probes S1a LC (LC610), S1b LC (LC640) and S2 LC (LC705) were used together with commercial LightCycler$^R$ FastStart DNA Master$^{PLUS}$ HybProbe (Roche Diagnostics, Basel, Switzerland) – 15 µl of MasterMix including primers and probes and 5 µl of sample DNA isolate per 20 µl capillary.

The real-time PCR assay program consisted of 45 cycles with an initial denaturation step at 95 °C for 10 minutes in first cycle and than for 10 s in remnant cycles. Annealing temperatures were selected 58 and 68 °C for 10, 15 or 20 s according to used probes. An extension step at 72 °C for 45 s was followed by real-time detection at proper wavelength according to used probe.

**Results**
Only 8 of 20 patients with proven oropharyngeal presence of *H. pylori* showed concurrent presence of gastric infection by UBT.

In gastric biopsies the presence of *H. pylori* was proven by real-time PCR in all 6 patients who underwent endoscopy. Endoscopic examination showed mucosal changes typical for gastric inflammation in four patients, the inflammation was

clinically asymptomatic. None of the patients admitted any gastroduodenal complaints.

Comparison of gastric and oropharyngeal genotypes showed important differences. Results are shown in Table 3. Three patients (nos. 1, 2 and 5) had the *cagA* positive *H. pylori* strain in the stomach but negative in the oropharynx. Two of them also showed differences in the *vacA* gene S region (nos. 1 and 5). Patient no. 6 showed a difference in S region alone. In two patients (nos. 3 and 4) the *H. pylori* strains from the oropharynx and stomach were identical.

**Table 1 – PCR primers for amplification of *cagA* and *vacA* sequences**

| Gene | Primer | No. of nucleotides | Sequence |
|---|---|---|---|
| cagA | cagA F | 24 | 5'-TTG ACC AAC AAC CAC AAA CCG AAG-3' |
| | cagA R | 22 | 5'-CTT CCC TTA ATT GCG AGA TTC C-3' |
| vacA s | VA1F | 21 | 5'-ATG GAA ATA CAA CAA ACA CAC-3' |
| | VA1R | 19 | 5'-CTG CTT GAA TGC GCC AAA C-3' |
| vacA m | HPMGF | 21 | 5'-CAG AGC CAC TTT CAA TAA CGA-3' |
| | HPMGR | 21 | 5'-CGT CCA AAT AAT TCC AAG GG-3' |

**Table 2 – PCR probes for detection of *cagA* and *vacA* sequences**

| Gene | Type | No. of nucleotides | Sequence | Detection (nm) |
|---|---|---|---|---|
| cagA | cag_TM | 28 | 6FAM-ATA ACG CTG TCG CTT CAT ACG ATC CTG A-BBQ | 530 |
| vacA s | S1a_LC | 21 | LC Red610-GCR TTR GTC AGC ATC ACA CCG-PH | 610 |
| | S1b_LC | 21 | LC Red640-GCG TTG ATT AGY KCC ATA CCG-PH | 640 |
| | S2_LC | 21 | LC Red705-GCT AAY ACG CCA AAY GAT CCC-PH | 705 |
| vacA m | M1_TM | 30 | 6FAM-ACC ACC ATT ACC CGT ATC AAT ACC TTT AAA-BBQ | 530 |
| | M2_TM | 26 | HEX-CTA GTG TTT AGC CCG TTA TCG CTC TT-BBQ | 560 |

**Table 3 – Results of *Helicobacter pylori* investigation**

| No. | Diagnosis | Oropharyngeal genotype | Gastric genotype | Gastritis |
|---|---|---|---|---|
| 1. | tonsillar cancer | cagA– S1aM1 | cagA+ S1a/bM1 | – |
| 2. | tonsillar cancer | cagA– S1bM2 | cagA+ S1bM2 | – |
| 3. | tonsillar cancer | cagA+ S1bM2 | cagA+ S1bM2 | + |
| 4. | OSAS | cagA– S1aM2 | cagA– S1aM2 | + |
| 5. | OSAS | cagA– S1aM2 | cagA+ S1a/bM2 | + |
| 6. | tonsillitis | cagA+ S1bM1 | cagA+ S1aM1 | + |

OSAS – obstructive sleep apnea syndrome

## Discussion

The results of the present study showed that oropharyngeal lymphatic tissue can harbour *H. pylori* strains independently to the stomach infection. Real-time PCR genotyping of oropharyngeal and gastric strains in patients with present infection in both areas showed that different *H. pylori* strains can be found in oropharynx and stomach of the same individual. Four of 6 investigated patients had different *H. pylori* strains in oropharynx and stomach. The differences were found in *cagA* gene as well as in *vacA* gene.

These results are in concordance with the first study that compared cytotoxin genotypes of *H. pylori* between the stomach and oral cavity published by Wang et al. (2002). Authors used saliva for genotyping of oral *H. pylori*. They showed that more than one *H. pylori* strain may exist in the saliva and the stomach of the same individual. According to our knowledge, our study is the first to compare *H. pylori* genotypes between the stomach and tonsillar tissue biopsies. In this study the patients were selected according to the oropharyngeal *H. pylori* infection. None of them admitted any gastrointestinal complaints. Gastroscopy showed the presence of gastritis in the majority of patients although all were clinically asymptomatic. This differs from previous studies, which were primarily focused on patients with gastric disease. Only a few other studies focused on comparison of *H. pylori* strains in the oral cavity and stomach. Shames et al. (1989), Khandaker et al. (1993), Zhang and Lu (1997) and Hu et al. (2002) showed evidence, that both sites are infected with the same *H. pylori* strain. These authors used a DNA fingerprinting method, PCR adopted single strand conformation polymorphism (SSCP) or restriction endonuclease analysis respectively. None of these studies compared the different gene sequences of VacA gene. The variance of our results could be explained by different diagnostic methods used. Nevertheless real-time PCR genotyping is considered to be highly specific with high degree of reliability (Burgers et al., 2008).

Another study that used real-time PCR to detect *H. pylori* in the oral cavity (Martinez-Gomis et al., 2006) did not find any individual positive for oral *H. pylori*.

The authors did not mention in their paper the use of any transport medium for preserving *H. pylori* DNA, which seems to be very important for successful PCR detection (Pavlik et al., 2007).

The presence of oropharyngeal *H. pylori* without concurrent stomach infection was confirmed using UBT and is in accordance with previously published data by Burgers et al. (2008), who showed that *H. pylori* can be present in the oral cavity independently of stomach colonization. UBT has proved to be one of the most accurate methods for assessing gastric *H. pylori* status (Gisbert and Pajares, 2004). Loster et al. (2006) used a modified UBT for detecting oral *H. pylori*. The solution containing of $^{13}$C-urea was kept in the oral cavity for 5 min to detect oral urease producing bacterium. When using a standard UBT procedure, the solution of $^{13}$C-urea is swallowed immediately, so the time of contact with oropharyngeal mucosa is too short detect oropharyngeal *H. pylori*.

The finding of presence of *H. pylori* in the oropharyngeal tissue independently from the gastric infection confirms that *H. pylori* can permanently colonize oropharyngeal tissue and the oropharyngeal presence is not caused only by contamination by gastric contents. This is further confirmed by the finding of different strains in the stomach and oropharynx in the same individuals.

On the other hand the results re-open the question of *H. pylori* transmission. When we consider the oral cavity and oropharynx as a gateway for infection of stomach, we would expect to find identical strains in both areas. This inconsistency must be resolved by further analysis.

Although *H. pylori* is proved to be highly pathogenic bacterium with carcinogenic potential, the data about oropharyngeal pathogenesis are insufficient. Culture of *H. pylori* from oral and oropharyngeal samples is extremely difficult (reviewed by Lukes et al., 2008). PCR techniques are not able to solve the question of viability of bacterial cells. The lower number of *cagA* positive oropharyngeal strains compared to gastric strains could shed some light on the question of oropharyngeal pathogenesis, nevertheless the number of investigated patients is very small. Further improvement of investigation methods such as culture is needed for obtaining more information about possible involvement of *H. pylori* in oropharyngeal diseases.

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