**American Journal of Clinical and Experimental Medicine**
2015; 3(6): 364-367
Published online December 28, 2015 (http://www.sciencepublishinggroup.com/j/ajcem)
doi: 10.11648/j.ajcem.20150306.17
ISSN: 2330-8125 (Print); ISSN: 2330-8133 (Online)

# Detection of *Helicobacter pylori* and Human Papillomavirus in Peroperative Tissue Biopsies Collected from Malignancies in Oropharyngeal Area

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## To cite this article:

Emil Pavlik, Eva Nartova, Jaromir Astl, Barbora Drnkova, Petr Lukes, Bela Potuznikova, Rami Katra, Jaroslav Kraus, Ivan Sterzl. Detection of *Helicobacter pylori* and Human Papillomavirus in Peroperative Tissue Biopsies Collected from Malignancies in Oropharyngeal Area. *American Journal of Clinical and Experimental Medicine*. Vol. 3, No. 6, 2015, pp. 364-367. doi: 10.11648/j.ajcem.20150306.17

**Abstract:** *Helicobacter pylori* has been reported as pathogen of human GIT. It is associated with type B gastritis and peptic ulcers. Bacterium´s relationship to cancer has also been declared and H. pylori considered cancer-inductor. A number of studies documented H. pylori residence in oropharynx, generating hypotheses on participation in development of cancer in oropharyngeal area. Human papillomaviruses are DNA viruses colonizing skin and mucoid membranes of the host. Their oncogenic potential, especially in genitourinary system, has been confirmed. High-risk type HPV16 (group A9) is frequently reported as cancer-inductor in oropharyngeal area. The aim of this study is to contribute to discussions on induction of malignancies in oropharyngeal area, providing comparison of incidence of one bacterial and one viral pathogen in the cells and tissues of oropharyngeal neoplasia. Using real-time PCR-based tests, we investigated 70 tissue specimens collected during cancer surgery for detection of bacterial DNA of Helicobacter pylori and viral DNA of High risk HPV (groups A9, A7 and A5/6). Results: Helicobacter pylori DNA was detected in 60 samples (85.7%), while DNA of HPV only in 42 (60%). If focused on HPV-16 as proposed cancer inductor, it was detected in 34 samples (48.5%) only. No DNA of respective agents was detected in 7 samples (10%). There were 21 Helicobacter sole pathogen detections compared with only 3 of HPV. *Conclusions:* There is no doubt, Helicobacter pylori is a long-term resident in oropharynx and tonsils. This residence most likely influences functions of immune system, so that a newly entering contributor could switch-on the process resulting in cancer development. This could support high incidence of common detection of HPV and Helicobacter pylori in 39 samples (55.7%).

**Keywords:** *Helicobacter pylori*, Human Papillomavirus, Real-Time PCR, Cancer, Oropharynx, Genotype

# 1. Introduction

*Helicobacter pylori* had been originally isolated from type B gastritis or peptic ulcer patients [1]. Later on, variety of its virulence factors had been determined to clear pathogenesis of respective diseases [2]. Furthermore, relation of this bacterium to development of gastrointestinal malignancies became confirmed [3].

Human papillomaviruses are reported in relation to carcinoma of cervix uteri. Their oncogenic potential has recently been cleared and described [4]. Therefore, High Risk and Low Risk groups occur in classification. Incidence of malignancies especially in female genital tract led to development of a screening test system and effective vaccine. Despite these achievements, cervical cancer is still one of the most frequent ones in many countries of the world [5].

Detection of *Helicobacter pylori* in saliva [6] supported idea, bacterium could colonize oropharyngeal area and known affinity of Helicobacter to lymphoid tissue in gastrointestinal tract predicted the same in oropharynx [7]. Detection of *H. pylori* in tonsils and Waldeyer´s lymphatic circle is not surprising. In contrary, in our previous trial, out of six patients with parallel gastric and oropharyngeal biopsies, in 3 patients *Helicobacter pylori* isolates from stomach and oropharyngeal tissue showed different genotypes [8].

There is no doubt, that HPV are highly contagious and therefore easily transmitted by direct contact, especially during sexual intercourse. So HPV infection of oropharynx is likely, more in sexually active population.

During last decade, several studies have been performed on detection of *Helicobacter pylori* in oropharynx [9,10,11] . Other studies supported hypothesis, malignancies in oropharyngeal area are mostly due to cancer potential of Human papillomaviruses [12, 13].

Our recent studies were focused on detection of *Helicobacter pylori* from patients´ operation biopsies, analyzing incidence of this infection in patients with different diagnosis [14, 15]. Profiting of archives of all samples tested, it was possible to screen them on Human papillomavirus. In this study we present data comparing detection of both the pathogens in operation biopsy samples collected from patients with malignancies in oropharyngeal area.

# 2. Material and Methods

### *2.1. Collection and Handling of Samples*

*Clinical selection of samples*: indication for surgery was spinocellular cancer (SCC).

Samples were collected in four departments of Prague University Hospitals and one regional hospital in Central Bohemia. All surgical teams were instructed to handle tissue biopsies according identical protocol.

*Collection of tissue specimens*: Biopsies were taken using sterile instruments at the beginning of surgery, immediately after insertion of endotracheal tube, prior to application of local anesthetics or disinfection substances into the oral cavity. Samples were immersed into Remel Microtest$^{R}$ M4RT Collection and Transport Medium (Remel Inc. USA) and transported into laboratory, where the tubes with samples were vortexed, aliquoted and from one aliquot, nucleic acids were isolated on Roche MagNAPure Compact (Tegimenta AG, Switzerland) automated isolator and investigated by real-time PCR technique for *Helicobacter pylori* detection and genotyping. The tests were developed and optimized in co-operation with MolBiol Berlin, Germany. Primary samples and nucleic acid isolates were then archived frozen at -80°C. Archived samples were later investigated for *H. pylori* flagellar gene, after real-time PCR-based commercial test became available, and, for purpose of this study, investigated by commercial real-time PCR screening test for HPV.

### *2.2. Detection Techniques*

#### *2.2.1. Real-Time PCR Amplification and Genotyping of Helicobacter pylori*

For genotyping, three real-time PCR assays had been developed and optimized in cooperation with TIB-Molbiol Berlin, FRG, one for *cagA* gene, second for *vacA* gene middle region and the last one for *vacA* gene signal region. Primers used for PCR detection of HP were: cagA F (sense), cagA R (antisense), HPMGF+ (sense), HPMGR- (antisense), VAF1F+ (sense) and VAXR- (antisense) according to vanDoorn et al. [16]. Primer sequences are shown in Table 1.

***Table 1***. *Helicobacter pylori assays primer sequences.*

| Primer | Sequence |
|---|---|
| cagAF + | 5-TTGACCAACAACCACAAACCGAAG-3 |
| cagAR - | 5-CTTCCCTTAATTGCGAGATTCC-3 |
| VAF1F+ | 5-ATGGAAATACAACAAACACAC-3 |
| VAXR - | 5-CCTGARACCGTTCCTACAGC-3 |
| HPMGF | 5-CAGAGCCACTTTCAATAACGA-3 |
| HPMGR | 5-CGTCCAAATAATTCCAAGGG-3 |

TaqMan$^{TM}$ hybridization probes were developed for *cagA, vacA* m1and *vacA* m2, and LC hybridization probes for *vacA* s1a, *vacA* s1b and *vacA* s2 specific sequence detection. For *cagA* assay FAM-BBQ labelled probe was used (detection 530 nm), for *vacA* middle region assay FAM-BBQ labelling was used for M1-probe (530 nm) and HEX-BBQ labelling for M2-probe (560 nm). Probe sequences are shown in Table 2.

***Table 2.*** *Helicobacter pylori real time PCR assay probes.*

| Gene | Probe sequence and labeling |
|---|---|
| cagA | 6FAM-ATAACGCTGTCGCTTCATACGATC CTGA-BBQ |
| vacAs1a | red610-GCRTTRGTCAGCATCACA CCG-PH |
| vacAs1b | red640-GCGTTGATTAGYKCCATA CCG-PH |
| vacAs2 | red705-GCTAAYACGCCAAAYGATCCC-PH |
| vacAm1 | 6FAM-ACCACCATTACCCGTATCAATACCTTTAAA-BBQ |
| vacAm2 | HEX-CTAGTGTTTAGCCCGTTATCGCTCTT-BBQ |

TaqMan™ real-time PCR assays were run on LightCycler® instrument, version 2.0 (six channel detection: 530, 570, 610, 640, 670 and 705 nm). Commercial LightCycler® TaqMan™ Master (Roche Applied Science) 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 Light Cycler® FastStart DNA Master PLUS HybProbe (Roche Diagnostics). - 15 µl of MasterMix including primers and probes and 5 µl of sample DNA isolate per 20 µl capillary.

#### *2.2.2. Helicobacter pylori Confirmation by Commercial Real-Time PCR Assay*

After its launching on the market in 2012, commercial real-time PCR assay BIORON Real-Line Helicobacter pylori Fla-Format Assay (Bioron Diagnostics GmbH., Germany) has been used to confirm presence of bacteria in the tissue biopsies. New arrivals to the laboratory were parallely tested, for earlier arrived samples testing frozen archived nucleic acid aliquots were used. The assay was performed according to the manufacturer instruction manual.

#### *2.2.3. Human Papilloma Virus DNA Detection*

For HPV detection in archived tissue samples commercial Sacace HPV High Risk Screen Real-TM Quant Assay had been chosen. This real-time PCR-based test following HPV types from groups A6, A7 and A9: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59. Assays were performed according to manufacturer´s manual handbook on Qiagen Corbett Rotor-Gene 6000 Instrument using a four channel detection.

## 3. Results

In our study we have investigated 70 specimens taken from tumors in oropharyngeal area using techniques for detection of DNA sequences specific for cagA gene, vacA gene and fla gene of Helicobacter pylori and High risk types of Human Papillomavirus groups A9, A7 and A5/6. There were 41 samples of tonsillar cancer tissue, 18 tissue samples from tumors in oropharynx, 7 from larynx and 4 lingual. Test results are summarized in Table 3.

***Table 3.*** *Detection of Helicobacter pylori and High Risk HPV in different cancer specimens.*

| Cancer Location | Number of Samples | Both Infections detected | Only H. pylori detected | Only HPV detected | Infection not detected |
|---|---|---|---|---|---|
| Tonsils | 41 | 22 | 11 | 3 | 5 |
| Oropharynx | 18 | 10 | 7 | 0 | 1 |
| Larynx | 7 | 4 | 3 | 0 | 0 |
| Lingua | 4 | 3 | 0 | 0 | 1 |
| Total | 70 | 39 | 21 | 3 | 7 |

Helicobacter pylori was detected in 60 samples (85.71%), while High Risk HPV in 42 samples (60%). In contrary with recent studies supporting possible dominant role of HPV in induction of cancer in oropharyngeal area [17, 18] sole HPV detection occurred in 3 samples only (4.28%), making total HPV detection rate with added 39 mixed infections with H. pylori to total of 42. Helicobacter was detected and subsequently genotyped as a sole agent in 21 samples (30%). In majority of studies, HPV type 16 has been reported as the main cancer inductor [19]. This type is classified in group A9. In a commercial screening test we used, A9 group has a specific probe with unique fluorescent signal. Surprisingly, we detected this group only in 34 samples out of 42 HPV positive. The lack of A9 was replaced by group A5/6 – (HPV51 and 56).

## 4. Conclusions

Considering result data, full acceptance of theory on HPV-induced oropharyngeal cancer could be revised. In contrary, despite the fact of 21 Helicobacter sole pathogen detections compared with only 3 of HPV, conclusions on Helicobacter contribution in induction of malignant process should be aware. There is no doubt, Helicobacter pylori is a long-term resident in oropharynx and tonsils. This residence most likely influences functions of immune system, so that a newly entering contributor could switch-on the process resulting in cancer development. This could be supported by relatively high rate of common detection of HPV and Helicobacter pylori in 39 samples (55.7%).

## Acknowledgements

The research was supported by the Internal Grant Agency of the Ministry of Health of the Czech Republic No.: NT 11523-6, Project of Ministry of Defense MO 1012 and Project OP Prague Competitiveness “Material and technical base for the research of diagnostics and therapy of civilization and oncological diseases and their significant risk factors in the General University Hospital in Prague”, reg.no. CZ.2.16/3.1.00/24012, co-financed by European Regional Development Fund.

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