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Helicobacter pylori Isolated from Patients with Tonsillar Cancer or Tonsillitis Chronica Could Be of Different Genotype Compared to Isolates from Gastrointestinal Tract

E. PAVLÍK[a], P. LUKEŠ[b], B. POTUŽNÍKOVÁ[a], J. ASTL[b], P. HRDÁ[a,c], A. SOUČEK[a], P. MATUCHA[c], J. DOSEDĚL[d], I. ŠTERZL[a,c]

[a]*Department of Immunology and Microbiology, 1st Faculty of Medicine, Charles University, Prague, Czechia*

e-mail pavlik.emil@seznam.cz

[b]*Department of Otorhinolaryngology and Head and Neck Surgery, 1st Faculty of Medicine and Teaching Hospital Motol, Charles University, Prague, Czechia*

[c]*Institute of Endocrinology and* [d]*Hospital of the Sisters of Order of St. Charles Boromeo, Prague, Czechia*

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ABSTRACT. *Helicobacter pylori* from patients with different diseases, including so-called autoimmune thyroiditis, chronic tonsillitis and tonsillar cancer, was isolated and cultured. It was identified according to the genotype using labeled hybridization probes complementary to six sequences of *cagA* and *vacA* genes. Different types of strains were found in isolates from gastrointestinal tract and patients suffering from thyroiditis. Six out of seven genotyped isolates from patients in our *Department of Otorhinolaryngology and Head and Neck Surgery* exhibited the same genotype, differing from isolates obtained from other patients; the 7th isolate originated from a patient who had undergone surgery for deviatio septi nasi, at the same time suffering from autoimmune thyroiditis, having confirmed gastric infection by *H. pylori* from biopsy. This data made it possible to formulate the hypothesis on probable association of specific *H. pylori* genotype with chronic tonsillitis and tonsillar cancer. We assessed commercial transport media and improved nucleic acid isolation techniques and the RT-PCR-based tests, which allowed us to skip a culture step and to test directly the patients' samples; however, for full confirmation of our hypothesis and explanation of possible mechanisms of the contribution of *Helicobacter* sp. to the pathogenesis of the disease further data are to be collected and evaluated.

Abbreviations

AT	autoimmune thyroiditis
GIT	gastrointestinal tract
MWP	microwell plate
NA	nucleic acid(s)
ORL	otorhinolaryngology
OSAS	obstructive sleep apnoe syndrome
PAI	pathogenicity island(s)
PCR	polymerase chain reaction
RT-PCR	real time PCR
RUT	rapid urea test
UBT	urea breath test

Helicobacter pylori is considered to play an important role in the pathogenesis of type B gastritis and gastric ulcer. On-going studies on functional map of its genome lead to identification of genes encoding several virulence factors (*e.g.*, *cagA*, *vacA*), and some others grouped in PAI. *H. pylori* has also been isolated from other tissues such as lymphatic tissue, atherosclerotic plates in vessels and from carcinomas. These findings generate the question whether these isolations are due to spread of bacteria *via* lymph and blood from GIT infection, or the bacteria play a more important role in the pathogenesis of different diseases by generating inflammations, immunity disorders and harming cell division regulation.

MATERIALS AND METHODS

Autoimmune thyroiditis patients were selected in an out-patient department of the *Institute of Endocrinology*. They underwent specific anti-*H. pylori* antibody detection (IgM, IgG and IgA). For patients with positive antibody tests the urea breath test followed. Patients positive in the *Helicobacter* breath test were indicated for gastroscopy in the gastroenterology department of the *Sisters of Order of St. Charles Boromeo Hospital*; during gastroscopic investigation specimens of gastric mucosa were taken and immediately transported to the laboratory for culture of *H. pylori* and further investigation.

Otorhinolaryngology patients. During operation on indicated individuals at the department of *ORL and Head and Neck Surgery*, specimens of tonsillar tissue and tonsillar cancer tissue were taken and transported to the laboratory immediately after excision. Blood specimens for specific anti-*H. pylori* antibody test were taken on the same day.

Specimens were inoculated on agar medium enriched with equine blood and *Merck* supplement and cultured for 5 d at 35 ± 2 °C. Successful cultures were prolonged through 7 additional passages in order to obtain enough bacterial mass for further analysis.

For *genotyping*, 3 RT-PCR TaqMan® assays had been developed in cooperation with *TIB-Molbiol* (Germany), one for the *cagA* gene, the second for the *vacA*-gene middle region and the last one for the *vacA*-gene signal region. TaqMan® hybridization probes were developed for *cagA*, *vacA* s1a, *vacA* s1b, *vacA* s2, *vacA* m1 a *vacA* m2 specific sequence detection. In order to guarantee possible comparison with previously genotyped isolates from GIT patients made on end-point PCR hybridization probe MWP-format (Potužníková *et al.* 2000) we have not extended the number of genotyping probes and designed the same sequences for primers and probes, which were optimized for RT-PCR.

Helicobacter pylori DNA was isolated on MagNA Pure Compact system using protocol "Total_NA 400_100" and MagNA pure compact nucleic acid isolation kit I (*Roche Diagnostics*) with pretreatment in MagNA pure bacteria lysis buffer (*Roche Diagnostics*). Isolated NA specimens were stored at –80 °C until genotyping assays were performed.

TaqMan® RT-PCR assays were run on LightCycler® version 2.0 (six-channel detection at 530, 570, 610, 640, 670 and 705 nm). Commercial LC TaqMan® MasterMix (*Roche Applied Science*) was used – 15 μL of MasterMix including primers and probes added and 5 μL of sample DNA isolate per 20 μL capillary.

RESULTS

After recognition during thyreoiditis trial, culture failure for different reasons could significantly decrease the number of strains for genotyping, so it was attempted to exclude the culture step and to isolate *Helicobacter* DNA directly from patients' specimens. By using the advantages – high sensitivity and specificity – of our RT-PCR assay arrangement, and the possibility to concentrate the eluted sample up to 8× on the MagNA Pure system (protocol "400_50"), the problem of sample preservation during transport was solved. A solution has been found in implementing the Microtest® M4RT transport media (*Remel*, USA) recommended for sexually transmitted infection samples. This medium protects both DNA and RNA in specimens for 1 d at ≤30 °C and for ≈1 week at 2–8 °C. As a result of improved techniques, samples from ORL patients can now be taken in parallel for culture and, in this sophisticated medium, for PCR detection and genotyping.

All results concerning the recently successfully genotyped *H. pylori* isolates are summarized in Table I. The two groups of patients (divided according to the diagnosis) differed markedly in *H. pylori* detection by culturing. One patient (no. *7*) operated for tonsillitis turned negative in all tests while one patient (no. *8*) occurred occasionally in both groups.

DISCUSSION

For comparison of the above genotyped isolates, we present here genotypes of *H. pylori* strains isolated during one of our previous studies (Potužníková *et al*. 2000) (Table II).

Most data on the association of *H. pylori* and carcinoma in the orofacial area or on bacterial colonization of adenoid tissues are based only on serological and/or RUT results. Only a few used the PCR detection of *H. pylori*, mostly in the end-point format with electrophoretic detection of the PCR product. Target sequences were in the urease-A gene (Bitar *et al*. 2005), the urease-C gene (Bulut *et al.* 2006) or the *cagA* gene (Cirak *et al.* 2003; Bitar *et al*. 2005; Bulut *et al.* 2006). None of the authors used RT-PCR or *Helicobacter* genotyping.

In contrast to our findings, Cirak *et al*. (2003) reported detection of *H. pylori* in tonsils and adenoid tissues out of which 71 % were *cagA*+ (positive). The authors analyzed 23 patients; *H. pylori* was detected in 7 patients only, out of which 5 isolates were *cagA*+. Similarly, Bulut *et al.* (2006) found 59 % of *cagA*+ isolates (17 out of 29). Since the oropharynx represents an important crossroads of GIT and respiratory tract and identical strains of *H. pylori* were repeatedly isolated from the stomach, saliva, dental plaque and gingiva, there is no evidence for exclusion of transient colonization of adenoid tissues and tonsils by these bacterial strains. This is most likely the case of our patient no. *8*, who was *Helicobacter*-positive in our AT study.

Table I. *H. pylori* detection[a] in patients who underwent surgery at the *Department of Otorhinolaryngology and Head and Neck Surgery* and patients with autoimmune thyroiditis

Patient			*H. pylori* detection		Genotyping			Serology[b]
no.	sex	diagnosis	culture	PCR	*cagA*	*vacAs*	*vacAm*	
Patients with oropharyngeal pathology								
1	female	tonsillitis chronica	neg	pos	neg	s1b	m1	Ig neg
2	female	carcinoma tonsillae	neg	pos	neg	s1b	m1	IgA+
3	male	carcinoma tonsillae	neg	pos	neg	s1b	m1	IgA+
4	male	carcinoma tonsillae	pos	pos	neg	s1b	m1	IgA+, IgG+
5	female	tonsillitis chronica	neg	pos	neg	s1b	m1	IgA+
6	male	OSAS	neg	pos	neg	s1b	m1	IgA+, IgM+
7[c]	male	tonsillitis	neg	neg	neg	neg	neg	Ig neg
8[d]	male	deviatio septi nasi, AT	pos	pos	pos	s2	m2	IgA+, IgG+
Patients with autoimmune thyroiditis								
8[d]	male	deviatio septi nasi, AT	pos	pos	pos	s2	m2	IgA+, IgG+
9	female	AT	pos	pos	pos	s2	m1	IgA±, IgG+
10	female	AT	pos	pos	neg	s2	m2	IgG±
11	female	AT	pos	pos	pos	s1a	m1	IgA±, IgG+
12	female	AT	pos	pos	neg	s2	m1	IgA+, IgG+
13	female	AT	pos	pos	neg	s2	m2	IgG+
14	female	AT	pos	pos	neg	s1b	m2	IgG±
15	female	AT	pos	pos	neg	s1a/s1b	m2	–

[a]Test results: pos – positive, neg – negative; statistic evaluation of *H. pylori*-gene combination detected in tumor and/or tonsillitis group of patients compared with the AT patient group was based on Fisher's double-side test ($p < 0.001$).

[b]Serology results: (+) – (Ig) positive, (±) – (Ig) moderate; (–) – not determined.

[c]The specimen was taken at the start of tonsillectomia directly to Microtest® M4RT medium as a routine procedure; all *H. pylori* tests turned negative.

[d]Patient no. *8* listed in both groups had been diagnosed in the *Institute of Endocrinology* and included in the *H. pylori* AT trial group. He underwent all indicated investigations including gastroscopy. The specimen taken during gastroscopy gave a positive culture and cultured bacteria mass has been genotyped: *cagA*+, *vacA* s2, m2; this genotype is different from other isolates from ORL patients with oropharyngeal pathology as well as from archived ones from patients with ulcers. Approximately 1.5 year later, this patient underwent surgery for deviatio septi nasi; there were no signs of tonsillitis but still a high anti-*H. pylori* IgA level (5.94 units) was found.

He was culture- and PCR-positive for gastric biopsy; subsequently, he underwent surgery at the ORL clinic for deviatio septi nasi but with no evidence of chronic tonsillitis. His *Helicobacter* genotype (*cagA*+, *vacA* s2 m2) differed from the genotype obtained from all other ORL patients. In contrast, at least seven *H. pylori*-negative patients of Cirak *et al.* (2003) underwent tonsillectomy in local anesthesia. Residua of anesthetics in the specimens could inhibit polymerase in the PCR reaction, thus yielding false-negative results. Yilmaz *et al*. (2006) could meet a similar problem, when they obtained 4 culture-positive/PCR-negative results in gastric lavage, while detection of *H. pylori* in middle ear fluid, middle ear mucosa, adenoid tissue and tonsils by PCR was more sensitive than culturing. We consider the elimination of possible contamination of samples by local anesthetics to be a necessary condition for successful PCR from direct patient's sample.

Table II. Archived strain genotypes of *H. pylori* from patients with gastric and/or duodenal ulcer[a,b]

Strain	Diagnosis	*cagA*	*vacAs*	*vacAm*
99/01	gastric ulcer	neg	s1a	m2
99/02		pos	s1a	m2
99/03		pos	s1b	m2
99/04		pos	s1a	m2
99/05		pos	s1a	m1
99/06		pos	s1a	m2
99/07	duodenal ulcer	neg	s2	m2
99/08		pos	s1b	m1
99/09		pos	s1a	m2
99/10		pos	s1a	m2
99/11		pos	s1b	m1
99/12		pos	s1a	m2
99/13		pos	s1a	m2
99/14		pos	s1a	m2
99/15		pos	s1a	m2
99/16		pos	s1a	m2

[a]*H. pylori* detection by culturing and PCR were positive in all cases; for further details *see* footnotes to Table I.

[b]All patients where archived strains had been taken from, were also serologically tested; in all of them anti-*H. pylori*-specific IgG, and IgA or IgM were found.

A further contribution to the view on *cagA* positivity was brought by immunoblot antibody analysis in an AT study (Šterzl *et al*. 2006); specific anti-CagA immunoglobulins were detected in 13 % of 23 patients with isolated AT, in 7 % of 30 patients with AT and polyglandular activation of autoimmunity and in none of 7 patients with AT and autoimmune polyglandular syndrome of type II. In a control group of 30 healthy individuals, the incidence of anti-CagA immunoglobulins reached 20 %. These data support the view that *cagA*-negative genotypes, commonly considered to be "less pathogenic" (Blaser *et al.* 1996; Dunn *et al*. 1997), may induce changes in immune responses, resulting in serious damage of the host by autoimmune mechanisms or influence the regulatory mechanisms of cell division which can lead to the development of neoplasia.

The results of Akbayir *et al*. (2005) based on histopathological and immunohistochemical evaluation of specimens taken from laryngeal carcinomas and benign laryngeal lesions support the above hypothesis. *H. pylori* was found in 28 of 50 specimens from patients with laryngeal carcinoma and in no one of 50 patients with benign laryngeal lesions.

The detection of *H. pylori* in the tissue specimens from our patients with tonsillitis chronica does not correspond to the results of Di Bonaventura *et al.* (2000, 2001) who considered tonsils not to be an extragastric reservoir of *H. pylori*; however, they used only tonsillar swabs and searched for gastric types of the bacteria. Our detection of specific gene combination, using three different RT-PCR TaqMan® assays at the same time, showed that the design of the assay plays a decisive role for successful detection. Tissue sample collection and immediate immersion into proper transport medium in particular, are essential for reliable test results.

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