

# Application of immunoassay for detection of *Helicobacter pylori* antigens in the dental plaque

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## ABSTRACT

**Purpose:** The aim of this study was to determine the viability of the commercial test currently used for detection of *H. pylori* antigens in the stool for detection of *H. pylori* antigens in dental plaque.

**Materials and Methods:** A total of 164 dyspeptic patients entered the study; 95 *H. pylori* infected (positive result of at least 4 of 5 diagnostic tests: *Campylobacter*-like organisms test (CLO test), histology, culture, stool antigens, serology) and 69 non-infected (negative results of 4 diagnostic tests: CLO test, histology, culture, stool antigens). Dental plaque was collected from natural teeth of the patients and incubated in microaerophilic conditions for 72 hours before immunoassay.

**Results:** Experimental findings included that optimal dental plaque weight to perform the examination was over 2 mg and that preliminary incubation increased significantly the number of positive results ( $p < 0.002$ ). It was also found that *H. pylori* antigens in the dental plaque were positive in 81.2% of infected and only 17.7% of non-infected subjects ( $p < 0.001$ ), while the reproducibility of results was 95%.

**Conclusions:** The immunoassay for detection of *H. pylori* antigens in the stool may be used, after minor adaptations (specifically pre-incubation in microaerophilic conditions) for *H. pylori* antigen detection in dental plaque.

**Key words:** dental plaque, *Helicobacter pylori* antigens, immunoassay

## INTRODUCTION

Stomach infection with *H. pylori* occurs following oral ingestion of the bacterium. The strain can reach the stomach either with food or with swallowed saliva; the latter being possible if the strain is present in the oral cavity as a permanent or transient resident. A significant role of oral *H. pylori* residence for stomach infection has been reported [1-5], however difficulty with its detection in the oral cavity as a reservoir still results in controversy regarding the relationship between oral and stomach infection [6].

*H. pylori* requires very specific incubation conditions for optimal growth. A number of factors, such as pH, oxygen supply, temperature, and nutrient availability may affect

the ability of *H. pylori* to grow in the oral cavity. So far, the presence of *H. pylori* has been shown in few oral locations, among them saliva and dental plaque [1,2,5,7-9]. Although detection of *H. pylori* in dental plaque is currently possible using various techniques including molecular [2,3,9], culture [1,7], and microscopy [5], still there is no reliable, simple, medically-practical method assaying for the presence of this digestive system pathogen in the oral cavity.

The aim of the study was to test the immunoassay method successfully applied for detection of *H. pylori* stool antigens [10,11], for detection of *H. pylori* antigens in dental plaque.

**Table 1. Patients' data.**

Number	164
Gender (M / F)	69 / 95
Age (mean, range)	53.5 (19-78)
<i>H. pylori</i> status (+ / -)	95 / 69
Smokers / non-smokers	42 / 122
Drinkers / non-drinkers	41 / 123

## MATERIAL AND METHODS

### Subjects

A total of 164 dyspeptic patients referred for upper endoscopy entered the study: 95 *H. pylori* infected (positive results of at least 4 of 5 diagnostic tests: *Campylobacter*-like organisms test (CLO test), histology, culture, stool antigens, serology) and 69 non-infected (negative results of 4 diagnostic tests: CLO test, histology, culture, stool antigens). Characteristics of the study population are presented in *Tab. 1*. Patients were excluded if they were edentulous or taking antibiotics within last 4 weeks. All subjects gave informed written consent to participation in the study and the local Ethical Committee approved the research.

### Gastroscopic examination and *H. pylori* testing in the gastric mucosa

Endoscopic examination was performed using a gastroscope GIF Q145 (Olympus, Tokyo, Japan) by an experienced endoscopist. During endoscopy, gastric mucosa specimens were taken from both prepyloric and body regions; two specimens for culture, two for CLO test, and four for histologic examination. CLO test was prepared in the Department of Physiology Medical University of Bialystok according to the method described by Marshall *et al.* [12]. Specimens for histological examination were placed in buffered formalin, subjected to standard processing, stained with hematoxylin and eosin, and additionally with Giemsa, followed by microscopic assessment by an experienced pathomorphologist.

Gastric biopsies for culture were inoculated into transport medium (Portagerm pylori, BioMerieux, Marcy l'Etoile, France) and cultured in microaerophilic conditions at 37°C for 3-7 days with Columbia agar supplemented with 5% sheep blood (BioMerieux) and Agar pylori (BioMerieux). *H. pylori* was identified by growth conditions, typical morphology of colony and in Gram-stained preparations, as well as positive oxidase, urease, and catalase reactions.

### Serologic tests

Serum samples were examined for *H. pylori* IgG and IgA antibodies using enzyme-linked immunosorbent assay kit (recomWell *Helicobacter* IgG and recomWell *Helicobacter* IgA, Microgen, Neuried, Germany).

### Collection and examination of fecal specimens

Fresh fecal samples collected pre-endoscopy were stored at – 20° C until examination. An amplified immunoassay was used for detection of *H. pylori* antigens in stool specimens according to manufacturers instructions (Amplified IDEIA™ Hp StAR™, Oxoid, Ely, UK).

### Dental plaque collection

Supragingival dental plaque was removed from the surfaces of natural teeth by scraping. Plaque specimens were taken in the morning after overnight fasting prior to routine oral hygiene practices and gastroscopy. After scraping, plaque samples were placed in Eppendorf tubes filled with 50 µL of 0.15 mol/L NaCl and weighed to determine plaque yield. In several patients, plaque was taken on the next day by different investigators to determine reproducibility of the results.

### Detection of *H. pylori* antigens in the dental plaque

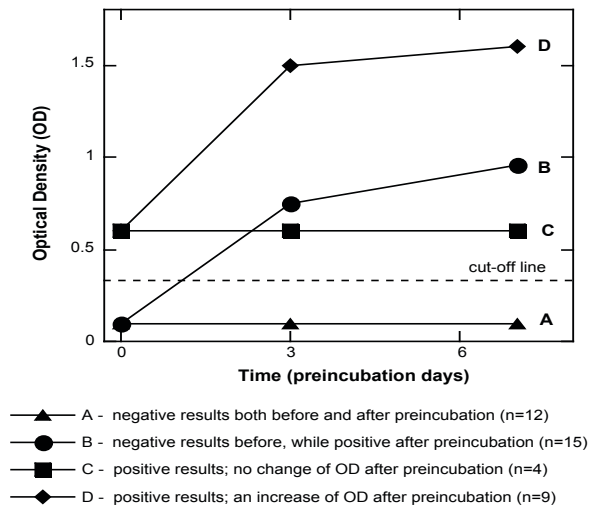
Dental plaque samples were enriched with 50 µL of cultivation medium (Brucella broth, Becton Dickinson and Company, Sparks, MD, USA) and pre-incubated in microaerophilic conditions at 37°C for 72 hours. For some patients whose total dental plaque weight was >7 mg, an additional immunoassay was performed without pre-incubation. Detection of *H. pylori* antigens in the dental plaque was based on the monoclonal antibody test used for *H. pylori* antigens detection in the stool (Amplified IDEIA™ Hp StAR™, Oxoid, Ely, UK). Briefly, after centrifugation of the samples at 5000 rpm for 5 min, 100 µL of the sample diluent (supplied by the manufacturer) was added to the pellet. Then this mixture, along with 50 µL of horseradish peroxidase labeled monoclonal antibodies, was added to the monoclonal antibody-coated microwells of the microtitration plate forming a sandwich complex. After incubation at room temperature for 60 minutes, the microwells were washed with phosphate buffer (pH 7.4) to remove the unbound antibodies. To quantify the amount of bound protein, a colorless enzyme substrate (tetramethylbenzidine) which turns blue after oxidation by horseradish peroxidase was used, followed by a stop solution (sulphuric acid) which changed the color from blue to yellow. The intensity of the color (optical density, OD) was determined spectrophotometrically at 450 nm.

Positive and negative controls established the cut-off point of OD at 0.250. As a control *H. pylori* strain ATCC 43504 (BactiBug™, REMEL, Lenexa, KS, USA) was used.

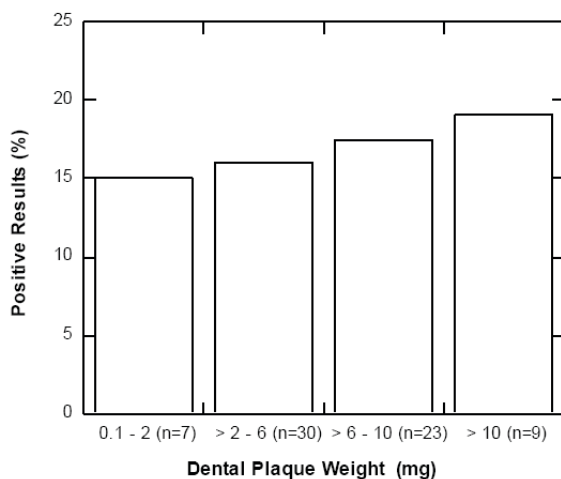
### Culture of *H. pylori* from dental plaque

Fresh dental plaque was pre-incubated in microaerophilic conditions at 37.0° C in 100 µL of Brucella broth cultivation medium enriched with antibiotics (vancomycin 10 µg/mL, bacitracin 20 µg/mL, amphotericin B 5 µg/mL, and trimethoprim 5 µg/mL; SIGMA-ALDRICH Corp., St. Louis, MO, USA). After 24 and 96 hours of pre-incubation dental plaque samples were inoculated on Pylori agar and Columbia agar supplemented with 5% sheep blood. The culture was

**Figure 1.** The influence of dental plaque pre-incubation on *Helicobacter pylori* antigens detection.



**Figure 3.** The relationship between dental plaque weight and detection of *Helicobacter pylori* antigens in non-infected subjects.

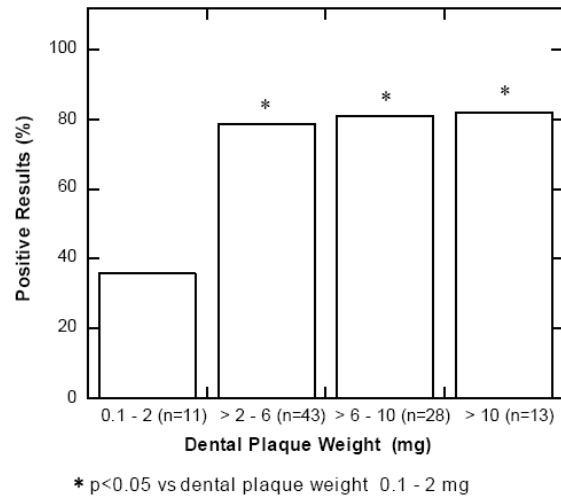


continued to 7 days in microaerophilic conditions at 37.0° C. As a control *H. pylori* strain ATCC 43504 was used. *H. pylori* was identified by growth conditions, typical morphology of colony and in Gram-stained preparations, as well as positive urease, oxidase, and catalase reactions.

**Statistics**

The results were evaluated statistically using Mann-Whitney U test and Fisher exact test as appropriate. A p value < 0.05 was accepted as significant.

**Figure 2.** The relationship between dental plaque weight and detection of *Helicobacter pylori* antigens in infected subjects.



**RESULTS**

**The effect of preliminary incubation of dental plaque**

In 40 dental plaques weighing above 7 mg, *H. pylori* antigens were examined with and without preliminary incubation lasting 72 hours and 7 days. *H. pylori* antigens were found in 13 of 40 (32.5%) plaque samples which had not been preliminary incubated, while pre-incubation for 72 hours increased this incidence to 28 of 40 (70.0%) (p<0.002); pre-incubation prolonged to 7 days did not increase the number of percentage of positive results. As many as 55.6% of negative results for the presence of *H. pylori* plaque antigens tested without pre-incubation became positive when pre-incubation was used (p<0.0001).

Moreover, pre-incubation increased significantly the OD in 69.2% of samples being already positive for *H. pylori* plaque antigens without pre-incubation (p<0.0005) (Fig. 1).

**The influence of dental plaque weight**

Dental plaque taken from natural teeth ranged in weight from 0.1 mg to 34.0 mg, however most of the samples ranged between 2 and 10 mg (Fig. 2, Fig. 3). In the *H. pylori* infected population, *H. pylori* antigens from plaques weighing less than 2 mg (with 72-hour pre-incubation) were positive at a rate less than half of those weighing over 2 mg (p<0.05), while in *H. pylori* non-infected population plaques of different weights showed very similar results.

**Culture from the dental plaque**

Only 3 of 8 subjects with documented *H. pylori* antigens in the plaque were positive in culture, while none of 9 subjects with negative plaque antigens (Tab. 2).

**Table 2. *Helicobacter pylori* culture from the dental plaque of subjects with positive and negative results of *H. pylori* plaque antigens.**

		Plaque antigens	
		+	-
Culture	+	3	0
Culture	-	5	9

### Reproducibility of results

In 40 subjects, dental plaque was taken two times, before and a day after gastroscopy. The second sampling was performed by different investigator from the first. It was found that with 72-hour preliminary incubation, the reproducibility of results was 94.1% and 95.7% for infected and non-infected subjects, respectively. In the former, one positive result changed into negative, in the latter, one negative result changed into positive.

### *H. pylori* antigens in dental plaque of infected and non-infected subjects

Using plaque samples weighing over 2 mg and incubated for 72 hours before immunoassay, *H. pylori* antigens were found in the plaque of 82.1% of *H. pylori* - positive subjects and only in 17.7% of *H. pylori* - negative subjects; the difference in incidence of positive oral *H. pylori* antigens between infected and non-infected subjects was highly significant ( $p < 0.001$ ).

## DISCUSSION

A number of methods have been used in the past to detect and identify *H. pylori* in the oral cavity, but all of these have been of low sensitivity and high specificity, or *vice versa* [13]. In the current study, we have found that the test for *H. pylori* stool antigens can be used after small adaptation to detect *H. pylori* antigens in the dental plaque. We have also established that for this method, optimal dental plaque weight to perform examination was over 2 mg, and preliminary incubation of the plaque before examination increased significantly the number of positive results.

Since the culture of *H. pylori* from dental plaque is very often unsuccessful compared to other techniques [8,13], it may be tempting to conclude that *H. pylori* in the dental plaque is not vital. However, the results of the present study indicate that this bacterium in dental plaque is not dead. Pre-incubation in microaerophilic conditions for 72 hours increased OD in the majority of studied samples by more than two times, indicating that during incubation the number of *H. pylori* strains increased significantly, thus improving the sensitivity of immunoassay. Interestingly, in 30% of subjects positive for *H. pylori* plaque antigens tested without pre-incubation, OD remained at the same level following pre-incubation. This finding suggests that some *H. pylori* strains were dead in the plaque at the moment of harvest or died during processing.

Plaque taken from the same subject at two different times, before and a day after gastroscopy, only one individual from infected and one from the non-infected group demonstrated inconsistent results for *H. pylori* plaque antigens detected between samplings. This small variation could be due to oral infection with stomach bacteria present on the withdrawing gastroscopist [14], or that during second harvest, a different set of teeth was chosen by another investigator for plaque collection. The prevalence of *H. pylori* in dental plaque tested by PCR was reported to be different in molars, premolars, and incisors [9]. For this study, to collect more than 2 mg of the plaque from dentate patients, we had to combine dental plaque from different teeth (incisors, canines, premolars, and molars), while in some patients the total amount was too small to perform the examination, or not available at all. Thus, using *H. pylori* antigens as a marker, we cannot answer the question as to whether *H. pylori* is uniformly distributed in the plaque or what the variance of *H. pylori* occurrence is in the dentate as a whole.

By using different diagnostic tests, the presence of *H. pylori* in the oral cavity was confirmed in about half of subjects with non-infected stomachs [1,9]; according to our data that rate was only 17.7% but we used highly selective group. These findings prompt consideration into why so many non-infected subjects are positive for *H. pylori* antigens in the dental plaque. We find three possibilities as the most likely reasons for this seemingly counterintuitive result. First, *H. pylori* in the plaque is more likely to be transient, rather than a permanent resident. Second, the number of *H. pylori* strains in the plaque is small and immunologic conditions in the oral cavity are strong enough to protect against the stomach infection. Third, *H. pylori* antigens may overlap with antigens of other oral bacteria, e.g., *Campylobacter rectus* [15]. If the latter is the case, the sensitivity of this test for *H. pylori* detection in the dental plaque is likely lower than its specificity.

The present study has some limitations. We did not use antibiotics in the medium for plaque pre-incubation before immunoassay. This was related to the fact that in preliminary tests performed before present study began the number of positive results of *H. pylori* plaque antigens pre-incubated with and without antibiotics was the same. However, the use of antibiotics in pre-incubation medium should be recommended, because no difference found then by us could be the consequence of small number of samples studied ( $n=18$ ). Moreover, we were not able to confirm all *H. pylori* positive antigens found in the plaque by culture; unfortunately, the method we have chosen appeared not good enough for this aim.

Because of some discrepancies between the presence of *H. pylori* in the dental plaque and the stomach, the immunoassay for detection of *H. pylori* antigens in the dental plaque will require a support with other tests before potential clinical application. Nevertheless, we suppose the method described here could be used as a simple verification of other tests for determining *H. pylori* presence in the oral cavity. We also

believe that the assay for *H. pylori* antigens in the dental plaque, which simplifies the procedure for finding this microorganism in the oral cavity, may represent a valuable tool for *H. pylori* detection in clinical diagnosis and epidemiology of stomach infection by this prevalent pathogen.

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