

¹³C-Urea breath test for the diagnosis of *Helicobacter pylori* infection

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Abstract. *Helicobacter pylori* (*H. pylori*) is a Gram-negative bacterium able to colonize the gastric mucosa as well as gastric metaplastic areas of the duodenum, producing inflammation. The clinical outcome depends on sophisticated interactions between bacterial factors, such as the expression of determinants of virulence and pathogenicity, and host characteristics. The severity of inflammation, may then vary among different subjects, leading to the occurrence of different gastroduodenal diseases, ranging from chronic gastritis to gastric cancer and MALT-lymphoma, to some defined extragastric manifestations. Many diagnostic tests are available for the detection of *H. pylori* infection including noninvasive methods, such as serology, ¹³C-urea breath test (UBT), and fecal antigen tests and invasive techniques, including a combined use of endoscopic biopsy-based methods, such as rapid urease testing, histology, culture, and molecular methods.

UBT is a highly sensitive and specific and allows to diagnose the presence or absence of infection of *H. pylori*, through the oral administration of a solution containing urea labelled with the non-radioactive natural carbon 13. This review article analyzes microbiological and clinical features of *H. pylori* as well as the different diagnostic tests able to detect this bacterium with a special focus on UBT.

Key Words:

Helicobacter pylori, Virulence factors, Gastric disease, Urea breath test.

Introduction

H. pylori is a Gram-negative bacterium able to colonize the gastric mucosa as well as gastric metaplastic areas of the duodenum, producing inflammation. It may be considered as one of the most common infectious agents of the stomach where is able to determine different diseases,

ranging from chronic gastritis and peptic ulcer to gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma¹.

The prevalence of *H. pylori* infection shows substantial geographical differences¹. Generally, *H. pylori* infection is a feature of about 50% of the world population but with a significantly higher prevalence in less industrialized countries, where is more common even in children². On the other hand, the prevalence of the infection has fallen greatly in many countries nowadays due to the improvement of standards of life and to the eradicating strategies applied by many governments.

The exact mechanism whereby *H. pylori* is acquired is not well defined: it has been hypothesized a human-to-human transmission, by oral-oral or fecal-oral contact or both. *H. pylori* has been detected in saliva, vomitus, gastric refluxate, and feces of infected patients; however, there are no conclusive evidences that one of those fluids is responsible for a major risk of transmissibility than the others³. Some authors have proposed that geographic differences may affect the modality of transmission of *H. pylori*: direct human-to-human contacts have been suggested as the primary route in developed countries, while the fecal-oral route, including contaminated water, could be more frequent in developing countries⁴.

Pathogenic pathways

Colonization of the gastric mucosa with *H. pylori* does not represent a disease itself, as consistent pathological processes develop only in a certain percentage of infected subjects⁵. Moreover, not all *H. pylori* strains are able to cause the same manifestations, as the clinical outcome depends on sophisticated interactions between bacterial factors and host signal transduction, which are strictly required to mediate the induc-

tion of pathogenic downstream processes and disease development⁶.

H. pylori-related pathology consists in a typical cascade of events⁷, finally determining of a specific gastroduodenal disease: colonization of the gastric mucosa first triggers a predominant Th1 inflammatory response, involving the activation of NF- κ B, pro-inflammatory cytokines, and an overexpression of different growth factors, including the vascular endothelial growth factor (VEGF)⁸. The intragastric distribution and severity of this chronic inflammatory process depends on a variety of factors, such as type of strains, host genetic and immune characteristics, diet, and the level of acid production⁹.

Determinants of virulence and of pathogenicity

One of the main characteristics of *H. pylori* is that is not able to determine the same clinical manifestations in all infected patients¹⁰. This is due to the fact that while all *H. pylori* strains possess determinants of virulence, such as the ability to synthesize different enzymes, including urease and the presence of flagella^{11,12}, which represent fundamental prerequisites for colonization of the gastric mucosa¹³⁻¹⁵, only a fraction of them show specific determinants of pathogenicity. The Cag (cytotoxin-associated gene) pathogenicity island (cag PAI) is an important determinant of pathogenicity expressed by approximately 60-70% of *H. pylori* strains present in Western countries and virtually by 100% of East-Asian countries¹⁶⁻¹⁸. It is known that *H. pylori* strains expressing the cag PAI (cag +) significantly increase the risk of severe gastritis, atrophy, dysplasia, and gastric adenocarcinoma compared to strains lacking the cag PAI (cag -)¹⁹. Interestingly, Cag genotype influences also the topography of *H. pylori* colonization in the stomach: Cag + strains are found near gastric epithelial cells, while *H. pylori* Cag-strains are found predominantly in the mucus gel layer²⁰. The CagA (cytotoxin-associated gene A) protein is a highly immunogenic protein encoded by the Cag PAI, present in approximately 50 to 70% of *H. pylori* strains²¹⁻²³, able to induce morphological changes of gastric epithelial cells²⁴. From the clinical point of view, CagA-positive strains are usually associated with a higher inflammatory response, a more severe gastritis, a higher risk for peptic ulcer, atrophic gastritis, and gastric cancer, due to an epithelial-mesenchymal transition in polarized epithelial cells²⁵.

Approximately 50% of all *H. pylori* strains secrete another important cytotoxin, the vacuolating VacA, a highly immunogenic 95-kDa protein that induces massive vacuolization in epithelial cells *in vitro*²⁶, membrane channel formation and cellular apoptosis^{9,27,28}.

Finally, another determinant of virulence possessed by *H. pylori* is the ability to produce a lipopolysaccharide (LPS), which is able to induce an inflammatory response and, at the same time, to protect *H. pylori* by the action of phagocytes and neutrophils through the production of superoxide dismutase and catalase. Interestingly, considering that LPS has a strong resemblance to the Lewis antigen present on human cells it is able to induce only a low-grade inflammatory response, so that only a fraction of infected patients will then develop symptoms²⁹.

Clinical features

Gastroduodenal colonization with *H. pylori* may cause different diseases, ranging from chronic gastritis to MALT lymphoma and cancer.

The role of *H. pylori* in gastritis and peptic ulcer disease (PUD) is now well recognized³⁰. Definitely, *H. pylori* may confer a lifetime risk of developing ulcer disease and distal gastric cancer with an estimation of about 10-20% and 1-2% respectively³¹.

Interestingly, there is a correlation between the level of acid secretion and the distribution of gastritis. In subjects with normal acid secretion, *H. pylori* is mostly distributed in the antrum realizing then the so called antrum-predominant gastritis. On the other hand, when acid secretion is altered, *H. pylori* may be also colonize the corpus, causing a corpus-predominant gastritis or even a pangastritis³²; interestingly, local inflammatory factors and cytokines, including interleukin-1 (IL-1), have a strong suppressive effect on parietal cell function, resulting in functional hypochlorhydria.

Gastric ulcers are more frequently distributed along the lesser curvature, especially among transitional zone between corpus and antrum³³, while duodenal ulcers usually occur in the duodenal bulb, mostly due to a direct exposition to gastric acid which in turn may be a cause of gastric metaplasia. Even though *H. pylori* is responsible for approximately 85% of gastric ulcers and 95% of duodenal ulcers, there are other environmental factors, such as smoking, alcohol intake and using of non-steroidal anti-inflammatory

drugs, which may increase the risk of peptic ulcer development^{33,34}.

Non-ulcer dyspepsia (NUD) defines the presence of symptoms of upper gastrointestinal disease, like recurrent pain/discomfort without any organic alteration revealed during diagnostic work-up. Organic causes for dyspeptic symptoms has not been found, but it has been revealed that 30-60% of patients with functional dyspepsia show positivity for *H. pylori*. On this subject, many studies have confirmed that dyspeptic symptoms may improve after *H. pylori* eradication³⁵.

The role of *H. pylori* in gastric cancer is well recognized and this bacterium is now classified as a class I carcinogens by the World Health Organization (WHO)³⁶, as it is able to increase the risk of gastric cancer of approximately 10-fold³⁷. Gastric Cancer (GC) is the fourth most common malignancy in the world; interestingly, 63% of all GC may be attributed to the effect of *H. pylori* infection³⁸. Inflammation is a risk factor for different tumors and this is why chronic gastritis is considered to be as the main known risk factor for adenocarcinoma, by inducing significant changes of the gastric mucosal architecture, possibly leading to the occurrence of precancerous lesions, such as atrophic gastritis, intestinal metaplasia and dysplasia³⁹, depending on the severity of inflammation⁴⁰.

H. pylori eradication is effective in preventing gastric cancer development: even if eradication occurs when the cancer development has already started, progression and proliferation are significantly delayed⁴¹. The neoplastic transformation may also occur after the eradication of *H. pylori* but only in patients with pre-existing precancerous lesions and this is why the preventive effect of eradication is reached mainly in subjects without those lesions⁹.

H. pylori-positive subjects have also a significantly increased risk for the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma⁴². In response to the colonization of *H. pylori*, lymphoid tissue appears on the gastric mucosa, where it is not normally present. Diagnosis is based on microscopy, immunohistochemistry or molecular techniques, including PCR. MALT lymphomas occur in less than 1% of *H. pylori*-positive subjects⁴³. Almost all patients affected by MALT lymphoma are also *H. pylori*-positive⁴⁴ and the eradication may lead to a complete remission in patients with stage IE MALT lymphoma confined to the stomach^{45,46}.

Several studies suggested the presence of an inverse association between *H. pylori* infection and gastro-esophageal reflux disease (GERD). A neuro-immunological mechanism might be responsible for the supposed protective effect⁴⁷: a corpus predominant gastritis induced by *H. pylori* may cause gastric atrophy which in turn has a suppressive effect on acid secretion, while is generally known that GERD is associated with increased exposure to gastric acidity⁴⁸. For the same reasons, there is also evidence that *H. pylori* infection may exert a protective effect against esophageal cancer⁴⁹.

Finally, *H. pylori* infection is reported to be linked to a variety of extra-gastrointestinal disorders, through the occurrence of persistent low-grade inflammation, the impairment of absorption and gastric permeability and the occurrence of molecular mimicry mechanisms between bacterial peptides and antigens of the host. On this subjects, idiopathic sideropenic anemia, idiopathic thrombocytopenic purpura and Vitamin B12 deficiency have already been recognized to be associated with *H. pylori* infection. For other diseases, such as ischemic heart disease, functional vascular disorders and pre-eclampsia further studies are now ongoing in order to clarify the issue⁵⁰⁻⁵⁴.

Diagnosis of *H. pylori* infection

Several test are available for the detection of *H. pylori* infection⁵⁵, including noninvasive methods like serology, urea breath testing, and fecal antigen tests and invasive methods like urease-based tests, histology and culture from biopsy samples⁵⁶⁻⁶⁰. Some non-invasive tests⁶¹, such as the UBT and the stool antigen test are able to detect active infection and this the reason why they are called 'active tests'. On the contrary, other tests, including serological test, urinary test and near-patient tests are good markers of exposure to *H. pylori* but do not necessarily indicate the presence of an active infection and they are called "passive tests"⁶².

One of the most reliable tests for the diagnosis of *H. pylori* infection is ¹³C-urea breath test (UBT)⁶³ which is now marketed for use with a new nondispersive, isotope-selective infrared spectrometers (NDIRS). Those devices have been shown to be as reliable as isotope ratio mass spectrometers (IRMS) but with the advantage of being faster, smaller and cheaper⁶⁴⁻⁶⁶.

Urea breath test is defined as a rapid, simple, innocuous, easy to repeat, reproducible, highly

accurate and economical method that detects the presence of *H. pylori* in the gastric mucosa, by exploiting the urease activity of the germ. It is also particularly suitable in all clinical conditions where endoscopy is not strictly necessary, and to check the success of eradication regimens⁶⁶.

Since the original description by Graham et al⁶⁷, several changes have been proposed. Those changes include the dose of labeled urea used, the type of test meal, the time of breath collection, the cut-off values and the equipment adopted for measure isotope enrichment. Nevertheless, while a multitude of papers regarding UBT methodology have been published a definitive standardization of this test does not exist yet.

The principle of the UBT relies upon the capacity of *H. pylori*, when present in the stomach, to hydrolyze orally administered labeled urea to produce isotopically labeled CO₂ which diffuses into the blood, is excreted by the lungs and can be detected in breath samples by means of a measuring equipment. Since *H. pylori* is the most common urease-containing gastric pathogen, urea hydrolysis may generally be equated with the presence of *H. pylori* infection⁶⁸.

Urea can be labeled with two different isotopes, ¹⁴C (the radioactive isotope) or ¹³C (the non-radioactive stable isotope). Labeling urea with ¹³C has become increasingly popular because the non-radioactive isotope is innocuous, so that the test may be repeated as often as required even in children, pregnant women, and women of child-bearing age. On the contrary, the ¹⁴C UBT has been completely abandoned due to radioactivity.

The main inconvenience of the breath test with ¹³C-urea is that it usually requires a mass spectrometer to obtain the results, with a high initial economical investment. However, since the isotope is not radioactive, just one spectrometer shared by several centers permits the samples to be sent to the reference center, decreasing then the cost of the technique.

Conceptually quite simple, UBT is based on the administration of urea labeled with an isotope of carbon. Once ingested, urease hydrolyzes urea into ammonia and carbon dioxide, which is absorbed by gastric wall, then carried by the blood and rapidly excreted in the breath. In the presence of *H. pylori*, urease activity is increased leading then to an enhancement of the exhalation of the labeled carbon dioxide after a few minutes from the beginning of the test. By analyzing the ratio of the isotope of carbon chosen to mark the

urea and the ¹²C, the most frequent isotope present in nature, we may make an estimate of the urease activity inside the stomach, starting from the assumption that in healthy individuals, there is no urease activity in the stomach.

While specificity and sensitivity of UBT are close to 100%, we have to take into account that the close or concomitant use of antisecretory drugs or antibiotics may affect the results of the test.

UBT is preferably performed in the morning after a 6 hours fasting. We proceed with exhaled air sampling baseline (before urea administration): the patient is asked to take a deep breath and blow through a straw in a vial, exhaling all the air contained in the lungs. At this point, the patient is asked to drink a solution of ¹³C labeled urea, and wait thirty minutes sitting in the waiting room without eating, drinking or smoking. At the end, the entire procedure is repeated in a second tube. The exhaled air is then analyzed by a mass spectrometer, which allows to measure the amount of CO₂ ¹³C with respect to the total of expired CO₂ ¹²C.

Several studies have shown that some drugs may alter the gastric *H. pylori* bacterial load leading to false negative results. Those drugs are mainly represented by proton pump inhibitors (PPI), antibiotics and bismuth. This is why we may allow to perform UBT⁶⁹:

- four weeks after the use of antibiotics and associated PPI (the use of a single antibiotic does not seem interfere examination);
- two weeks after the use of PPI

Concerning the use of anti-H₂ drugs, there is a general agreement that their effect on the results of UBT is much less important than that observed for PPI; therefore, it would only be recommended to use citric acid before doing UBT in patients taking this class of drugs.

UBT may be performed with relatively low doses of urea: 100 mg, 75 mg (mean sensitivity and specificity: 97%) or even 50 mg (mean sensitivity and specificity: 98%)^{70,71}. Since endogenous CO₂ excretion in children is less than in adults, less urea is required for children. Accordingly, it has been demonstrated that a dose of 50 mg of urea is sufficient to achieve excellent results in this population^{72,73}. With the most widely used protocols (100 mg without citric acid and 75 mg with citric acid), excellent accuracy is obtained when breath samples are collected as early as 10-15 min after urea ingestion⁷⁴, with a mean sensitivity and specificity of almost 100%. AN

Interesting study⁷⁵ showed that accuracy of capsule UBT (n = 100) with 100 mg of ¹³C urea was higher than that of CLO test, histology and culture (100% vs 92%, 91% and 89%, respectively; *p* = 0.035, 0.018 and 0.005, respectively). While some studies suggest that fasting before UBT should be mandatory, others have not found any significant differences between tests performed under fasting and non-fasting conditions. However, it would be more prudent to perform UBT in fasting conditions until new data definitively clarify this issue⁷⁶⁻⁷⁹.

Citric acid⁸⁰ solution is currently one of the most widely used, and it has been stated that it may increase the maximum concentrations of ¹³CO₂ in comparison with other semiliquid test meals previously used. In the absence of further studies, test meals (and especially citric acid) should probably continue to be used in UBT protocols.

The precise choice of the cut-off point to define whether the UBT is positive or negative still represents a controversial issue. Overall, at the beginning the cut off value was 5‰ but was then lowered to 3.5‰. Anyway, it depends on the protocol we apply (dosage of Urea, timing of the test, etc.). At present, there is general agreement on the use of two breath samples, one collected before and another collected, in most cases, 20-30 min after urea ingestion. On the other hand, it has been shown that sampling too early (at 5 or 10 min) may produce false-positive results because of urease activity of oral bacteria.

Concerning the confirmation of *H. pylori* eradication, UBT may be considered as a gold standard test but it should be performed at least four weeks after treatment, even though is our opinion that 6 weeks would be the preferable choice in order to avoid false negative cases^{69,81}.

Conclusions

H. pylori infection is the leading cause of different gastroduodenal diseases and the major risk factor for gastric cancer. Determinants of pathogenicity together with host characteristics may play an important role in discriminating patients experiencing more severe diseases. Different methods may be used to detect *H. pylori* infection, either non-invasive or invasive. In patients in whom endoscopy is not recommended UBT represents one of the best method to detect *H. pylori* either before or after the eradicating treatment. The

procedure is very simple and easily reproducible; we believe that the administration of 100 mg without citric acid or 75 mg of ¹³C-labeled urea together with citric acid and with a time of breathing of 30 minutes, represents the most reliable procedure to detect *H. pylori* infection.

Conflict of interest

The Authors declare that they have no conflict of interests.

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