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Diagnosis of *Helicobacter pylori* infection: a look into molecular aspects of urea breath test

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Running title: *H. pylori* and UBT

Abstract

Helicobacter pylori (*H. pylori*) is a bacterium that selectively colonizes gastric epithelium in more than 50% people over the world. The infection is usually acquired in early childhood and rarely resolved spontaneously; transmission is mostly person to person, and occurs by fecal-oral or oral-oral modality. Diagnosis and antibiotic treatment may lead to eradication of *H. pylori*, improving the prevention and the outcome of gastric and extragastric diseases. Many tests are currently available for the diagnosis of *H. pylori* infection and the choice depends on several clinical aspects including symptoms, age, indications for testing, concomitant medications and comorbidities. Invasive tests (i.e. endoscopy with histologic assessment) are considered the gold standard, but they are expensive and should be performed only in an appropriate context. The most common non-invasive tests are urea breath test (UBT), stool antigens test and serology. UBT is non-invasive, quick, safe, accurate and cheap. This test is performed mainly with ^{13}C and is based on the presence of *H. pylori* urease, an enzyme that converts urea (labelled with an isotope) into CO_2. Labelled CO_2 is then exhaled and measured by dedicated spectrophotometers. This review analyses with special emphasis UBT, focusing on its molecular aspects.

Key words: breath test; diagnosis; *Helicobacter*; urease.

Helicobacter pylori (*H. pylori*) is a spiral shape Gram-negative bacterium, discovered in 1982 by Marshall and Warren,[1] that selectively colonizes gastric epithelium (mainly the antrum) of more than 50% people over the world.[2] Phylogenetic analysis pointed out that *H. pylori* global spreading occurred from East Africa to other continents thousands of years ago.[2,3]*H. pylori* infection is usually acquired in early childhood and the risk declines sharply after 5 years of age. The higher prevalence in older age groups is thought to reflect a cohort-effect related to poorer living conditions during childhood.[4,5]

Unless antibiotic therapy for eradication is administered, infection persists and could lead to chronic gastritis and peptic ulcer (PU).[6] Because of the well-known association with gastric cancer (GC) and mucosa-associated lymphoid tissue (MALT) lymphoma, the World Health Organization (WHO) has categorized *H. pylori* as a group I carcinogen.[7-8] *H. pylori* infection has also been related to several extra-gastric diseases, especially those of autoimmune origin, attracting the attention of researchers.[9] Hundreds of diseases have been investigated for a possible relationship, but only for a few the association was precisely demonstrated (i.e. iron deficiency anemia, vitamin B12 deficit and idiopathic thrombocytopenic purpura), although the exact causative mechanisms remain unexplained. In this context, the prevailing idea is that *H. pylori* is not an etiologic agent but it acts as environmental trigger in genetically predisposed subjects. However, these suggestions are supported by small data and evidence is still lacking.[10-15] Nonetheless, *H. pylori* infection has been inversely associated to different rates of asthma and allergies.[16]

Currently, *H. pylori* prevalence is still high, showing different rates worldwide: in Europe it is lower in Northern countries (ranging from 31.7% among blood donors in Netherlands to 84.2% in Portugal). In North America, rates are similar to Europe, while the prevalence is higher in Central and South America, and in Asia, ranges from 54% to 72%.[17] Some studies conducted in Africa reported a prevalence of infection between 65.7% and 75.5%, with a significant increase with age.[5]

The principal risk factors for *H. pylori* infection are represented by low socioeconomic status (risk of contamination), poor health and crowded living conditions, attendance of children at

day care centres. In low income countries, additional factors can be represented by drinking spring water and vegetarian diet.[5] Regarding the route of transmission, it is mostly person to person, and occurred by fecal-oral or oral-oral way.[7]

Due to the increasing rate of antibiotic resistance, reported by a European multinational study, the optimal regimen to cure *H. pylori* infection should be decided regionally. [18] The Maastricht V/Florence Consensus Report[19] and the American College of Gastroenterology Clinical Management Guideline[20] highlight that in countries with low clarithromycin resistance rates (<15-20%), an empiric clarithromycin-based regimen can be used. In countries with high clarithromycin resistance rates or, in the American Guideline,[20] with a previous exposure to clarithromycin, a bismuth-containing quadruple therapy (with metronidazole and tetracycline) is the first choice. In case of persistent infection, after a previous clarithromycin-containing regimen, this drug should be avoided in second line therapy. Options after initial eradication failure include tailored therapy (choosing antibiotic combinations based on antibiotic susceptibility testing), empiric bismuth-containing quadruple therapy or triple levofloxacin-based therapy. Encouraging data are reported, both for the first-line and for rescue treatments, with the use of a formulation of bismuth subcitrate potassium, metronidazole, and tetracycline contained in a single capsule, together with a PPI.[21,22] Rifabutin- and furazolidone-based regimens should also be considered in rescue regimens.[23]

***Helicobacter pylori*: colonization and survival**

H. pylori has an impressive ability to persist chronically in the human stomach.[3,24,25] An essential step in the colonization of gastric epithelium by the bacterium is mediated by outer membrane proteins (OMPs) that serve as adhesion molecules. Most of *H. pylori* OMPs play a role in adherence, associated with elevated gastric epithelial cell damage risk and, possibly, involved in the pathogenesis of GC. *H. pylori* genome has more than 30 genes which encode for OMPs, that can be divided into *Helicobacter* outer membrane proteins (Hop) subgroups.[26-29] Some of these proteins have been identified. Blood group antigen binding adhesin (BabA), the first discovered, is involved

in binding with ABO group (Lewis antigen, expressed in stomach) and leads to the synthesis of pro-inflammatory cytokines. Sialic acid binding adhesin (SabA) is used for binding to sialyl-dimeric-Lewis, a receptor for *H. pylori* whose expression increases in the early infection and is closely associated with gastric atrophy, intestinal metaplasia (IM) and GC development. Also, adherence associated lipoproteins (AlpA/B) are two homologous proteins involved in bacterium adhesion, though the receptor is still undetermined. HopZ is another bacterial adhesin with an unknown receptor and is regulated by contact with gastric cells and by the pH of gastric environment. The outer inflammatory protein (OipA) is encoded by *HopH* gene and induces interleukin (IL)-8 production promoting inflammation; it correlates with severe outcomes such as duodenal ulcer and GC.[1,28,30]

In addition, several factors play an important role independently of bacterial interactions with epithelium. Indeed, during colonization, only 20% of the bacteria interact with the epithelium. *Cag pathogenicity island* (*Cag PAI*) consists of a cluster of 31 genes, most of them coding for a T4SS, a needle-like structure that penetrates the epithelial cell membrane and allow *H. pylori* translocation into the cells. Cytotoxin-associated gene A (CagA) protein is an effector protein of 125 and 140 kDa, encoded in *CagPAI* with no homologous in other bacteria. It is the most virulent factor and a risk factor for PU and GC. In the epithelial cell, CagA initiates signalling events through tyrosine phosphorylation in the Glu-Pro-Ile-Tyr-Ala (EPIYA) domains (with different motifs in different strains). Moreover, peptidoglycan is recognised by the nucleotide-binding oligomerization domain-containing protein 1 (NOD1), a pathogen-associated molecular pattern (PAMP) that induces nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway and up-regulation of pro-inflammatory response. There are other Cag proteins, such as CagL, CagY, CagI, all important for *H. pylori* colonization and under investigation for their potential use in the development of an effective vaccine. CagA is also known as the first bacterial onco-protein, ranking the *H. pylori*-mediated adenocarcinoma as the second for cancer mortality worldwide.[31] Further studies are needed on CagA fragmentation *in vivo*, in order to understand

CagA translocation into cytosol, its interaction with cellular proteins and neoplastic transformation of the infected cells.[32,33]

The vacuole-inducing toxin (VacA), contributes to the binding of the bacterium to the gastric epithelium and plays a key role in cell vacuolization, characterized by the accumulation of large vesicles that possess hallmarks of both late endosomes and early lysosomes. Moreover, VacA leads to mitochondrial disfunction and blocks T-cell activation, modulating immune response to the infection.[34,35]

The high temperature requirement A (HtrA) has a serine-protease enzymatic activity and cleaves, for example, E-caderin, contributing to disruption of cellular junctions. HtrA disrupts epithelial barrier and allows to *H. pylori* to invade the intercellular space. In addition, the E-cadherin ectodomain released is a key prognostic indicator in GC.[32]

Once *H. pylori* is ensconced in the mucus it is able to fight the stomach acid by the production of the enzyme urease. Thus, urease has a key role in the survival of *H. pylori.* Since the translational relevance of the knowledge of urease is the main aim of this review it will be discussed in the appropriate section.

***Helicobacter pylori*: diagnosis**

The methods to diagnose *H. pylori* infection can be classified as invasive or non-invasive, the former being based on biopsy specimens obtained at endoscopy. The choice of the test depends on the clinical context. When a patient needs an upper gastrointestinal (GI) endoscopy, due to alarming symptoms or older age (generally >45 years, but age should be determined locally according to GC risk), *H. pylori* infection can be detected by an invasive test, in the remaining cases a non-invasive test is more appropriate.[36-39]

Non-invasive tests

Non-invasive tests are mostly used and reported. According to Maastricht V/Florence Consensus

Report, urea breath test (UBT) is the recommended non-invasive test in the context of a "test-and-treat" strategy. Monoclonal stool antigens test (SAT) could be used as an alternative method. Proton pump inhibitors (PPI) should be discontinued at least 2 weeks before testing, while bismuth and antibiotics should be discontinued 4 weeks earlier.[19] Furthermore, SAT and UBT cannot be used for patients suffering from ulcer bleeding.[40] Serological tests can be used only after validation; conversely, rapid serology tests using whole blood should be avoided.[19] In Eastern Asia (such as Japan and South Korea), immunoglobulin (Ig)G serological detection is recommended as one of the preferred methods for the initial diagnosis.[41] It is necessary, in every country, to validate *H. pylori* detection kits used for that specific population. The performances of the serological kits may be affected by antibody response (IgG or IgA), age, gender, health status and ethnicity of the host.[42,43] For serologic testing only IgG detection is considered, since *H. pylori* infection is chronic; the preferred method is the enzyme-linked immunosorbent assay (ELISA), with overall sensitivity and specificity of 85% and 79%, respectively. However, there is a marked variability in accuracy according to the kits available and this test cannot be used to confirm *H. pylori* eradication, since after-treatment seronegativity may take months or even years. Then, serology should be considered when other diagnostic tests could be falsely negative, such as in patients with bleeding ulcers, gastric atrophy or recent use of PPI and antibiotics.[19,44,45]

UBT and SAT are very specific (>90%), while *H. pylori* IgG can persist for years and a positive test can indicate both a present or a previous infection. After unsuccessful treatment, culture and antimicrobial sensitivity testing may help to determine an appropriate treatment regimen.[46]

Invasive tests

Conventional endoscopic exam is usually performed to diagnose *H. pylori*-associated diseases. Moreover, it is useful to obtain tissue samples for further tests, including rapid urease test (RUT), histology, culture and molecular biology methods. Gastric biopsy is usually obtained from the

antrum, but greater curve sampling is suggested for patients with antral atrophy or IM to avoid false negative results.[47] In case of clinical indication for endoscopy and no contraindications for biopsy, RUT should be the first-line test for the diagnosis of *H. pylori* infection; to note, this test is not indicated for assessment of the eradication of the bacterium. Several commercial rapid urease tests exist, such as gel-based tests (24-hours CLOtest, HpFast), paper-based tests (1-hour PyloriTek, ProntoDry) and liquid-based tests (5-minutes UFT300, EndoscHp). These tests have specificity above 95%-100% and sensitivity above 85%-95%. Bleeding makes RUT significantly less reliable test than other tests.[28,47,48]

Histologic assessment is considered the gold standard for the direct detection of *H. pylori*, but can be influenced by site, size, number of biopsies, staining methods, use of PPI or antibiotics and pathologist experience. Immunohistochemical stain is the most sensitive and specific approach, but in most cases hematoxylin-eosin stain is sufficient for the diagnosis. Other stains and/or immunohistochemical testing of *H. pylori* can be used as ancillary tests, only if histology is not normal.[47]

Fluorescent in-situ hybridization using peptide nucleic acid probes (PNA-FISH) method is useful for the diagnosis of infection and to assess clarithromycin resistance of *H. pylori*.[49] New tests have been developed, such as polymerase chain reaction (PCR)-based Amplidiag *H. pylori* test, for the detection of clarythromicin resistance, since adapted therapy increases eradication rates.[50]

From urease to UBT: An example of translational medicine

Since the human stomach is an unfriendly place, to overcome the barrier represented by its acidic environment (pH < 2), *H. pylori* produces a large amount of the nickel (Ni)-containing enzyme urease, which provokes the breakdown of urea (of which there is an abundant supply in the stomach originated from saliva and gastric juices) with production of the cell-toxic ammonia in the gastric epithelium. After exit from the bacterial cell, ammonium neutralizes the acidity and creates a nearly neutral microenvironment for *H. pylori* survival. This phenomenon allows the bacterium to safely

cross the mucus layer to the epithelium surface.[3,51] Urease represents 10% of total proteins among those expressed by *H. pylori*. Urease is found both inside and outside the bacterium. The relative contributions of these ureases are determined both by their respective amounts and the pH of the medium. At acidic pH of 4.5 or less, the outer urease is inactive; therefore, it does not contribute to survival from acid. The inner urease, however, shows 10-20 folds increase in activity when external urease is present but it rises much more if the latter is inactivated. At least seven gene products of this pathogen are required for the catalytic urease activity. *UreA* is a species-specific gene present in all strains of *H. pylori.* While *ureA* and *ureB* encode for the two structural subunits that constitute the apoenzyme,[52-54] the other five genes (*ureE, ureF, ureG, ureH* and *ureI*) encode for accessory proteins involved in the incorporation of Ni ions, crucial for the activation of the urease system, into the apoenzyme.[3] One active urease molecule requires 24 Ni ions for full enzymatic action. After entering the outer membrane, the Ni molecules are transported to the cytoplasm through the protein channel NixA, a monomeric high-affinity Ni-uptake protein localized in the cytoplasmic (inner) membrane of the bacteria. [3] *NixA* deletion mutants still retained urease activity to some extent (up to 50% in some strains), indicating the existence of an alternative Ni transporter.[55] A reported Ni transport system, required for Ni-dependent urease activation and acid survival, is the gastric *Helicobacter* species-specific NiuBDE.[56] Another type of Ni-uptake strategy requires the multiple-component ATP-binding cassette (ABC)-transporter operon consisting of four genes (*abcABCD*), and which is potentially involved in NixA-independent Ni uptake.[57] Insertional mutagenesis in *nixA*, *abcC* or *abcD* genes significantly reduce urease activity (42-72%), whereas abolition of urease activity is achieved by double mutations in both *nixA* and *abcC* genes.[58]

The intra-bacterial concentration of Ni ions is well regulated. Too low amount of Ni entry impairs urease activity and acid survival, while too much amount of Ni entry generates reactive oxygen species leading to cell damage. A family of DNA binding Ni-responsive regulatory protein (NikR) regulates the Ni-responsive genes including *ureA, ureB, nixA, frpB4*, and *fecA3*.[3]

The role of UreI-mediated urea transport is crucial in the control of urease and in handling excess NH_3. It is based on an integral cytoplasmic membrane protein that might form a urea-specific pore, controlled by external pH via a shift in periplasmic pH. As the external pH drops, UreaI opens up allowing urea to reach cytoplasmic urease, that neutralizes the acid; as the pH approaches neutrality, the enzyme is denied access to the substrate to avoid excessive NH_3 production.[59]

The knowledge of urease's activity has been the basis for the development of UBT. This is a semiquantitative accurate test with high sensitivity and specificity. It is a direct method able to detect the active *H. pylori* infection, assessing the whole stomach and avoiding sampling errors. Patient should be fasting (for at least 6-8 hours before the test) and off medications for 4 weeks; no further precautions are required.[59,60] It is important to report demographics, indication for the study (i.e. suspected *H. pylori* infection, follow-up after eradicating therapy), the procedure (i.e. radiopharmaceutical and dosage, number and timing of breath samples collected), result (i.e. disintegrations per minute in each sample), reference ranges, study limitations, confounding factors and interpretation (i.e. positive, negative, indeterminate).[60,61]

Main causes of potential false-negative results are concomitant medications, in particular with antibiotics, bismuth, sucralfate and PPI. Moreover, false-negative results can be due to non-fasting, gastric surgery and difficulty with swallowing (additional breath samples may be helpful).[62] False positive results may be related to oropharyngeal bacteria, if breath samples are taken within 10-15 minutes of swallowing the isotope, gastric surgery with potential resultant bacterial overgrowth (non-*H. pylori* urease) and achlorhydria.[62]

It is possible to perform the test using either ^{13}C or ^{14}C. The former is a nonradioactive isotope of carbon that is measured by isotope-ratio mass spectrometry. ^{14}C is a radioactive isotope of carbon that is measured by a scintillation counter, a pure beta-emitter with a physical half-life of 5730 years and maximum energy of keV.[60]

In the standard procedure, a breath sample is collected at baseline and 30 minutes, respectively, after drinking a 100 mg dose of ^{13}C-labeled urea with 1.2 g of citric acid in 100 ml of water.[62,63] The UBT relies on the ability of *H. pylori* urease to convert into carbon dioxide (CO_2) the urea that has been labelled with isotopes and then ingested by the patient. The converted labeled CO_2 diffuses across the epithelial cells, is conveyed by the bloodstream and ultimately is exhaled by the lungs. A breath sample from the patient is finally collected to measure the amount of labeled CO_2 exhaled and thus the presence or absence of *H. pylori* infection (Figure 1).[62-65] The difference in CO_2 levels between the baseline breath sample (before ingestion of labelled urea) and the post-administration breath sample can be detected by a specific mass spectrometer or infrared spectrophotometer. Balloons with breath samples can be shipped to another laboratory.[62] Since ^{13}C-urea is a non-radioactive isotope, samples can be easily sent for analysis to a central laboratory; thus, ^{13}C-UBT can be performed also in outpatient clinics.[62]Samples are analyzed for the $^{13}C/^{12}C$ ratio with a mass spectrometer. Results are expressed as excess $\delta^{13}CO_2$ excretion per ml, which represents ^{13}C enrichment over and above the baseline sample.[64,66]

Several modified versions have been proposed (Table I). For example, the patient swallows the ^{14}C urea in a capsule form containing 1 mg urea, labelled with 37 kBq, with 20 ml tepid water. At 3 minutes post-dose, the patient drinks another 20 ml tepid water; 7 minutes later, the patient is asked to take a deep breath, hold it for 5-10 seconds and then exhale through a straw into a mylar balloon. Another optional breath sample can be obtained at 15 min post-dose using another balloon.[60] Ferwana *et al*. compared ^{13}C and ^{14}C UBT; both demonstrated high performance against the gold standard test without a significant difference: sensitivity 0.96 (95%Confidence Interval [CI]: 0.95-0.97), specificity 0.93 (95%CI: 0.91-0.94).[67]

Low dose UBT has been proposed since 2002, in this case 50 mg ^{13}C-urea using a simple test meal and a 15 min sampling interval appeared cost-effective and convenient. Using a low cut-off (2.5‰), no loss of diagnostic accuracy was observed, with high sensitivity (99.1%) and specificity (97.3%).[68] Low dose UBT with 50 mg tablet (instead of 75 mg) was proposed by Mattar

et al. The relative sensitivity of ^{13}C-urea with capsule was 100% at 20 minutes and 88% at 10 and at 30 minutes. The relative specificity was 100% at all-time intervals. Among 83 patients that underwent capsule breath test and endoscopy the capsule breath test presented 100% of sensitivity and specificity.[69] Low dose UBT with 25 mg tablet does not reach sufficient accuracy and, although the data are controversial,[70] at the moment, is not recommended.[71] Nawacki *et al.* compared UBT with RUT collected during endoscopy in a group of 50 patients: consistency of the results of both tests was 96%. The only discrepancy concerned two women with grade 1 changes in gastroscopy.[72] Tepeš *et al.* investigated a modified UBT in patients taking PPI using ^{13}C test meal Refex. Using a cut off 2.5‰, sensitivity was 92.45% by per-protocol (PP) analysis and 78.13% (95%CI: 66.03%-87.49%) by intention-to-treat (ITT) analysis. Specificity was 96.00% in the ITT population and 97.96% in the PP population (95%CI: 89.15%-99.95%). In conclusion, this new test meal based ^{13}C-UBT is highly accurate in patients on PPIs and can be used in those unable to stop their PPI treatment.[73] Gilardi *et al*. analysed the accuracy of BreathID®, a device with continuous breath test sample collection and analysis after labelled urea intake. This approach was compared with the classical method. Correlation between the two methods was excellent with a Cohen's k=1.00. Furthermore, using a visual analogue scale, the authors showed that BreathID® had a significant greater acceptance.[74]

In preschool children, cut off value may be adjusted to reduce false positive results, that are 10 times higher in children aged 6 years old or less. The reasons could be ascribed for instance to urease-producing microorganisms in the oral cavity of young children or increase production of CO_2. Optimal cut-off value could be 4‰ for children older than 6 years old and 7‰ for children younger than 6 years old.[4]

The use of an indeterminate zone of result values (for example 2.5-3.5‰) may help to improve the diagnostic accuracy of the test, in order to minimize false-positive and false-negative results.[64]

Conclusions

Breath tests are commonly used in several clinical settings.[75,76] For the diagnosis of *H. pylori* infection, UBT is quick, safe, accurate and requires very few precautions when the isotope ^{13}C is used. Adjustment of the cut-off value or modified versions of UBT may be helpful in special settings, such as young patients or those who cannot stop PPI treatment.

In conclusion, the use of UBT in clinical practice represents a model of translational medicine, beginning from the knowledge of *H. pylori* urease and ending in the easy and cheap diagnostic application.

Conflict of interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Table I.-Main studies assessing sensitivity and specificity of urea breath test (UBT)

Name	Year	Se (%)	Sp (%)	Isotope	Cut-off	Note
Liao *et al.*	2002	99.1	97.3	^{13}C	2.5‰	50 mg
Coelho *et al.*	2011	83.5	99.4	^{13}C	4‰	25 mg *vs* 75 mg
Mattar *et al*.	2014	100	100	^{13}C	4‰	50 mg *vs* 75 mg
Tepes *et al.*	2017	92.5	96.00	^{13}C	2.5‰	PPI treatment
Nawacki *et al.*	2018	88.9	87.5	^{13}C	5‰	UBT *vs* Biopsy

Se: sensitivity; Sp: specificity; PPI: proton pump inhibitors

Figure 1.- Principle of the urea breath test