Journal of Advanced Research 13 (2018) 51–57

Contents lists available at ScienceDirect

Journal of Advanced Research

journal homepage: www.elsevier.com/locate/jare

Mini Review

Helicobacter pylori urease for diagnosis of *Helicobacter pylori* infection: A mini review

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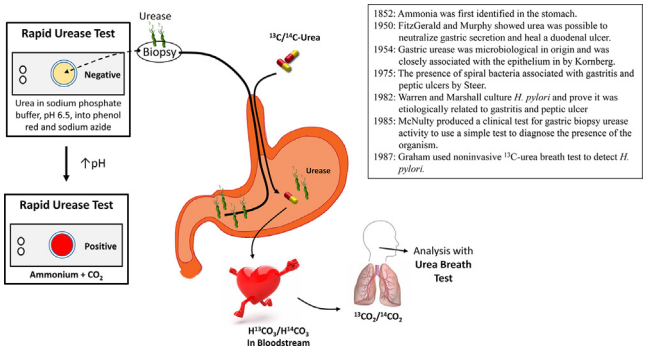
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 19 October 2017
Revised 18 December 2017
Accepted 16 January 2018
Available online 31 January 2018

Keywords:
Helicobacter pylori
Urea breath test
Rapid urea test
Gastric urease
Diagnosis
Confirmation of cure

ABSTRACT

The stomach contents contain of both acid and proteolytic enzymes. How the stomach digests food without damaging itself remained a topic of investigation for decades. One candidate was gastric urease, which neutralized acid by producing ammonia from urea diffusing from the blood and potentially could protect the stomach. Discovery that gastric urease was not mammalian resulted in a research hiatus until discovery that gastric urease was produce by *Helicobacter pylori* which caused gastritis, peptic ulcer and gastric cancer. Gastric urease allows the organism to colonize the acidic stomach and serves as a biomarker for the presence of *H. pylori*. Important clinical tests for *H. pylori*, the rapid urease test and urea breath test, are based on gastric urease. Rapid urease tests use gastric biopsies or mucus placed in a device containing urea and an indicator of pH change, typically phenol red. Urea breath tests measure the change in isotope enrichment of ^{13}C- or $^{14}CO_2$ in breath following oral administration of labeled urea. The urea breath test is non-invasive, convenient and accurate and the most widely used test for non-invasive test for detection of active *H. pylori* infection and for confirmation of cure after eradication therapy.

Introduction

Ammonia was first identified in the stomach in 1852 and since that time has remained a target of investigation [1]. Even today, medical devices designed to detect breath ammonia originally produced in the stomach are in use clinically to detect infection with the Gram negative bacterium, *Helicobacter pylori,* an important human pathogen that despite a decline in prevalence still infects approximately 50% of humans worldwide. *H. pylori* infection is the most common causative agent of gastritis, peptic ulcers and gastric cancer [2]. The presence of urease in the stomach was discovered early in the 20th century (reviewed in [1] and [3]).

Peer review under responsibility of Cairo University.
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https://doi.org/10.1016/j.jare.2018.01.006
2090-1232/

The discovery was followed by widespread interest in gastric urease including the range of animals in which urease could be found as well as its role in health and disease.

In the late 19th century, it was discovered that gastric cancer was somehow related to achlorhydria or loss of the stomach's ability to make acid [4]. This observation prompted research in gastric physiology and which was greatly heightened by the fact that at that time gastric cancer was the most common cause of fatal human cancers [5]. The late 19th century and the early 20th century was a time of great interest and research in gastric physiology and gastric disease [4,6]. That period was also the time many of the great gastrointestinal physiologists were making their discoveries. By 1900, gastric surgery had also begun to emerge as a new field especially devoted to peptic ulcer disease which was then often considered a surgical disease [4]. Duodenal ulcer, previously thought to be rare was found to actually be very common [4]. It was recognized that ulcers were somehow related to acid and that duodenal ulcers were associated with high acidity and gastric cancers with absence of acid.

Gastric urease

How the stomach protected itself from injury by the highly concentrated acid contained within was unclear and the object of considerable research [7,8]. For example, it was known that placing the leg of a live frog into the stomach through a hole in the abdominal wall would result in digestion of its flesh discounting the protective effect of a living principle [8]. Urea hydrolysis produced alkaline ammonia was thought to be a good candidate for the mechanism of protecting the gastric mucosa from the corrosive acid resent in the stomach [1]. FitzGerald and Murphy provided proof of principle that urea could play an important role in protecting the stomach by showing that it was possible to neutralize gastric secretion and heal a duodenal ulcer by giving urea orally and parenterally to humans [9].

Much of the credit for our current understanding gastric urease comes from decades of experiments by Kornberg et al. who studied gastric urease primarily in cats [3,10]. Their comprehensive studies have served as the basis for modern investigations. The breadth of Kornberg's observations included studies on: (*a*) the effect of acid secretion on urea breakdown, (*b*) the effect of the presence of acid in the stomach, variations in gastric blood flow, and the secretion of non-acid juice on urea hydrolysis, (*c*) urea hydrolysis associated with the passage of urea solution from the gastric lumen to the blood, (*d*) the effect of anti-bacterial substances on urea hydrolysis, (*e*) the deposition of urease in the stomach, (*f*) urea and ammonia content of gastric juice, (*g*) quantitative aspects of gastric urease activity, and (*h*) disappearance of urea from the gastric juice. Their conclusions, regarding the physiology of urea in the stomach, included: (*a*) hydrolysis of urea was associated with its passage from blood in both parietal and non-parietal secretions to the lumen of the stomach, (*b*) the amount of urea hydrolyzed paralleled the rate of secretion of acid juice, (*c*) when urea was added to the stomach, the rate of urea hydrolysis was determined by the rate of passage of urea-containing fluid through the mucosa; the majority of the urea was hydrolyzed in the mucosa before entering the blood, and finally and most importantly, (*d*) gastric urease was microbiological in origin and was closely associated with the epithelium [1,3,10]. The evidence suggesting a microbial origin of urease initially rested on the effects of treatment with antimicrobials, for example, cats with gastric urease activity had a mean gastric juice urea and ammonium concentration of 0.6 mM and 4.2 mM respectively. Following antimicrobial therapy, the mean concentrations of urea rose and that of ammonium fell (3 mM and <0.05 mM, respectively) [3,10].

Overall, interest in gastric urease waned after it was shown that urease was not a mammalian enzyme but rather was likely of microbial origin [1,3]. Interest was rekindled by the discovery of *H. pylori* in 1982 and its role in gastritis and peptic ulcer disease [11]. After the discovery of *H. pylori* interest in the role of urease in human disease was rekindled including the role of gastric urease in relation to the production of ammonia which had a role in the neurologic complications of liver disease ranging from hepatic encephalopathy to hepatic coma [12–18].

The mid-20th century was an era characterized by great interest in peptic ulcer disease. For example, during the 1970s there were more than 140,000 ulcer operations/year in the United States [4] and Congress established specific research centers to solve the ulcer problem known as Centers of Ulcer Research and Education (CURE) [19]. It was known that duodenal ulcer was associated with specific abnormalities in the control of gastric secretion and this guided CURE's research. The hypothesis of a microbial cause of peptic ulcer had gone in and out of favor for decades. In the mid-1970s a group in the UK noted the presence of spiral bacteria associated with gastritis and peptic ulcers [20–22]. Attempts to culture that organism failed. Later, Robin Warren from Australia prompted by his observations using silver stained gastric sections, also noted the association of spiral bacteria and gastric inflammation. He was able to convince a clinical research fellow, Berry Marshall, to join him and together they were able to confirm their observations and with the advice of a microbiologist, Adrian Lee, who had special expertise with spiral bacteria, and the laboratory services of Steward Goodwin were able to culture *H. pylori* and prove it was etiologically related to gastritis and peptic ulcer. For this, Warren and Marshall earned a Nobel Prize in 2005 [23].

H. pylori urease

Although initial microbiological studies pointed away from *H. pylori* being urease positive, subsequent studies by McNulty et al. reported copious urease activity [24–26]. McNulty et al. also produced a clinical test for gastric biopsy urease activity to use a simple test to diagnose the presence of the organism [24,26]. Marshall subsequently added an antimicrobial agent to a common laboratory urease test and produced the first patented test to detect *H. pylori* clinically, the CLO test for *Campylobacter*-like organisms as *H. pylori* was then called. *H. pylori* urease also proved to be highly antigenic and is a component of most anti-*H. pylori* serologic tests and candidate vaccines.

The tolerance of *H. pylori* to acid is largely dependent on urease activity, a cytoplasmic enzyme. Access of urea to the enzyme is restricted by the presence of a H^+-gated pore (UreI) such that in acidic conditions urea can enter the cytoplasmic space and be hydrolyzed to CO_2 and ammonia [27–30]. The gene product is directly responsible for urea permeability and is active at acidic pH or it regulates the urea permeability of another cytoplasmic membrane protein. The ammonia produced diffuses into the low pH stomach where it becomes ionized and trapped in the gastric lumen whereas the CO_2 appears in the blood and subsequently is exhaled.

During the Kornberg era ^{14}C and ^{13}C isotope-based methodology was used to identify urease activity non-invasively as quasi-breath tests in experimental animals including the frog [1,3]. Methods using stable and radioactive isotopes were subsequently used to develop diagnostic tests in humans utilizing isotopic enrichment of breath, blood, or urine following oral administration of labeled compounds, most often urea, to detect the presence of *H. pylori* infections [31–37]. Although urea and ammonia can easily be measured in gastric juice [38], the first clinically useful rapid tests for diagnosis of *H. pylori* using gastric contents or biopsies targeted urease and were adaptations of standard laboratory tests for urease activity and named Rapid Urease Tests (RUTs).

Rapid urease test (RUT)

The fact that *H. pylori* is both abundant in the stomach and contains urease has been widely used to assist in clinical diagnosis. As noted above, with a short time after the discovery of *H. pylori*, rapid urease tests had been developed to allow rapid detection of *H. pylori* using gastric specimens (mucus, biopsy, or brushings). Although investigators have exploited essentially every method possible to detect urease activity, methods to detect changes in pH either directly or using color changes following incubation of gastric specimens proved both simple and reliable and were most widely adapted. More sophisticated methods requiring complex devices to accurately measure pH changes have generally not been successful clinically because methods based on change in color of a pH indicator included with the urea substrate are much more convenient and both generally cheap and accurate. Most tests use urea-impregnated agar, liquid, or a dry membrane upon or within which the sample is placed. The fact that *H. pylori* urease has a pH optimum lower (e.g., 5.4) than most other bacteria likely to be encountered in the stomach led to development of potentially more specific tests such as the *hp*-fast® which used two different pH indicators one at a lower and the other at a higher pH (Fig. 1). Choice of RUT test depends on availability, cost, and ease of use. In regions where cost is a significant issue, tests are often made locally from a solution containing 2 g of urea in 100 mL of 0.01 M sodium phosphate buffer, pH 6.5, into which 10 mL of a 0.5% (w/v) phenol red and 20 mg of sodium azide are added. Approximately 0.5 mL of this mixture is then placed into dram vials which are then kept in the endoscopy room. The solution is originally yellow and will turn pink or red typically within 30 min to 3 h after a *H. pylori*-containing specimen is immersed.

The RUT involves an enzymatic reaction and important considerations for test design include substrate concentration, enzyme concentration and activity, time, and temperature. Studies have done to test most of these parameters. The critical variables include sample collection, sample size, the time required before the test can be scored as positive or negative. For gel-based tests warming will reduce the time required and commercial warmers that maintain the sample at 37 °C are available [39]. Some endoscopists place the sample-containing device in their pocket to warm it and check it when convenient. A positive RUT requires approximately 10^5 *H. pylori* in the biopsy sample to produce a positive reaction with an agar-based test [40]. The organisms tend to localize on or near the surface of the specimen such that most of the tissue is "extra" and does not contribute to the reaction. Some investigators have used opened forceps to scrape gastric mucus to ensure that a high concentration of bacteria-rich material is obtained [41].

Generally, the concentration of *H. pylori* is highest in the antrum, however, if the patient has recently taken proton pump inhibitors (PPIs) the concentration can be markedly reduced resulting in a false negative test [42]. Another common cause of false negative tests, especially in areas where atrophic gastritis is common, is the presence of intestinal metaplasia which is often devoid of *H. pylori*. We recommend that at least two large-cup biopsies be taken from normal appearing mucosa, one from the antrum and one from the corpus, avoiding obvious areas of intestinal metaplasia. The two biopsies are then combined within the same test well [40,43]. Many take samples for RUT or culture before taking samples for histology with the thought that formalin may interfere with the results of culture or RUT. This caution is unfounded and there is no reason to request a new forcep if the forceps has been previously immersed in formalin [43–47]. The RUT is examined as convenient over 24 h. After 24 h the device should be discarded as color changes after this time are not a reliable indicator of *H. pylori* infection and can usually be attributed to a low level of contaminating mouth bacteria finally beginning to be detected.

The sensitivity of various RUT tests as primary diagnostic tests is high and has been reported to vary between approximately 80% and 100% with a specificity between 97% and 99% [40,48]. The sample imbedded in the gel can also subsequently be used for additional testing. For example, the sample can be used for molecular testing for antimicrobial susceptibility [49,50].

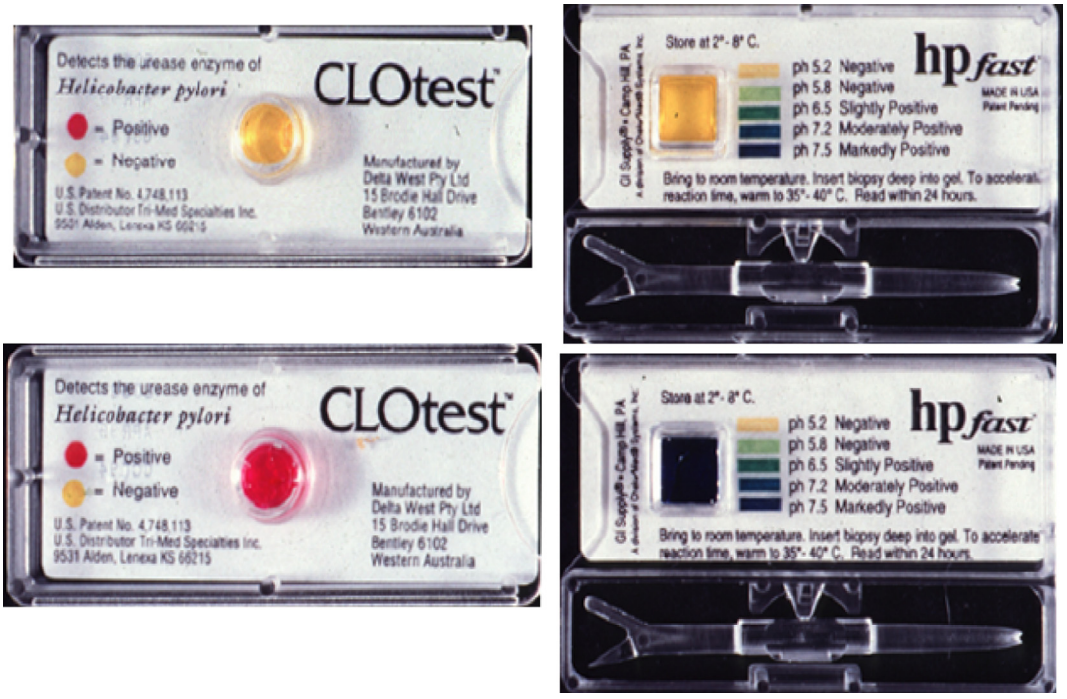

Fig. 1. Examples of commercial rapid urease tests commonly available in the United States. The CLOtest® and a second generation test, the hp_{fast} test are shown. Both use urea impregnated agar and a pH indicator system. The upper one of each pair is the control and the lower shows the color change indicating probable *H. pylori* infection.

In the United States (US) the RUT has fallen out of favor in part because *H. pylori* infection has become increasingly less frequent, there is often no reimbursement, and many of the patients are currently taking or have recently received PPIs resulting in false negative tests. Those with gastric ulcer or a mucosal abnormality would normally also receive biopsies of the ulcer margin and base along with other specimens to examine for *H. pylori*. Whether only taking samples for RUT is sufficient is not settled but in most instances additional biopsies are indicated. While, a positive RUT test in the presence of a high pretest probability (e.g., duodenal ulcer) would be sufficient to confirm the diagnosis of *H. pylori*-related duodenal ulcer, the test results are generally not available during the endoscopy procedure and because the patient with a duodenal ulcer may have a false negative test (e.g., due to PPI or antibiotic use), most would take additional biopsies for histology. In the US, it was suggested that the endoscopists hold the samples for histology until the results of the RUT test were available. If the RUT were positive, the biopsies could be discarded thus saving the patient many hundreds of dollars. It is unclear how often this was actually done. Nonetheless, a negative RUT result is not a reliable indicator of no *H. pylori* infection as many factors can influence the result. The role of RUT testing will therefore depend on the clinical situation and the pretest probability (i.e., the prevalence of *H. pylori* locally). Culture for antimicrobial susceptibility testing is still generally difficult to obtain and expensive. We suspect that molecular testing will soon become widely available and the combination of RUT followed by molecular testing of positive specimens will likely become the most common indication for RUT testing.

Studies of liquid-based RUTs and dry filter-paper test have suggested that some new platforms have shorter times to interpretability than agar tests such as the CLO test [51]. Some RUTs marketed in Europe have reported accurate results within minutes [52,53]. However, clinical experience has not shown rapid or ultrarapid results to be advantageous clinically especially when one considered the overall problem which includes diagnosis, treatment, follow-up, and associated issues (e.g., gastric cancer risk).

Urea breath test (UBT)

As noted previously, the end products of urease hydrolysis of urea, ammonia and CO_2 can be detected non-invasively and thus form the basis for the innumerable tests that have been developed for the clinical diagnosis of *H. pylori* infection. The most commonly used are breath tests that employ labeled carbon either the stable isotope ^{13}C or the radioactive ^{14}C (Fig. 2) [31,33]. The labeled urea is ingested and the enrichment of blood or breath with the isotope is assessed using a specific detector. The radioactive ^{14}C is usually detected by scintillation, in contrast the stable isotope ^{13}C measured by isotope ratio mass spectrometry [54]. Hundreds of variations of the UBT have been described in which the dose, timing, formulation of the substrate, use of adjuvants, test meals, type of detector from different manufactures, etc. have been varied [55,56]. All have been shown to be effective.

The clinical problem is the possibility of false positive or false negative tests. False positive tests are most often caused by urea hydrolysis by mouth bacteria or by urease-containing bacteria in the stomach [57]. This is particularly likely to occur in the presence of achlorhydria or hypochlorhydria. Another potential problem is substrate exhaustion because of the small quantity of ^{14}C-urea administered or emptying of the labeled substrate before it can react with the bacterial urease [58]. The ideal test would use a formulation that was immediately available for hydrolysis, remained in the stomach during the test period, was distributed throughout stomach, and was exposed to most of the gastric surface. Almost every imaginable variation has been tired such as having the patient recline and roll during the test, prior mouth cleansing, and prior administration of meals to retard gastric emptying [55,56]. The ^{14}C-urea containing tests typically contain no non-radioactive (i.e., "cold") urea and the tiny amount of substrate can be rapidly exhausted by hydrolysis by mouth, swallowed, or non-*H. pylori* urease-containing organisms in the stomach producing false positive tests [58]. Radioactive carbon has the advantages of being cheap and as there is no natural $^{14}CO_2$ the analysis is simple. The disadvantage is that many patients wish to avoid exposure to radioactive material despite the very low doses being administered. The test should not be used in children or pregnant women. Despite these concerns, it has proven to be clinically useful.

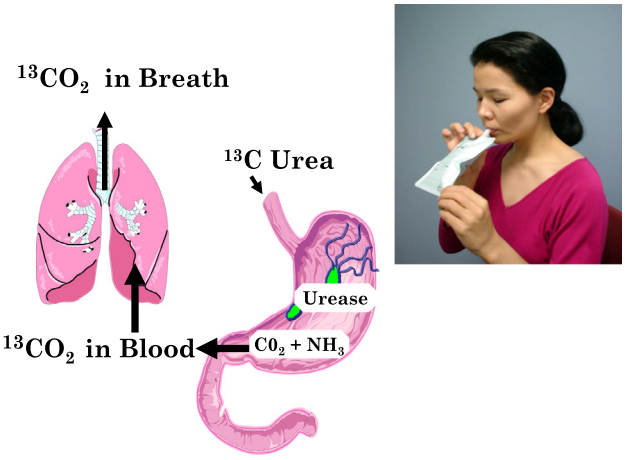

Fig. 2. This illustrate the details of the urea breath test using ^{13}C-urea which when ingested is hydrolyzed into $^{13}CO2$ and ammonia. The labeled CO_2 then is captured in a bag for subsequent analysis of the relative enrichment of $^{13}CO_2$:$^{12}CO_2$.

^{13}C-urea is relatively expensive and requires special machines to detect the enrichment of $^{13}CO_2$ in the breath. Currently, there are a number of companies that produce gas isotope ratio spectrometers that are portable and accurate which has greatly simplified testing. ^{13}C-urea has been formulated as a powder to be dissolved in water prior to use, in capsules and in tablets. The dosage of urea has ranged widely. Because of the expense, the optimum dose is a tradeoff between cost and adequacy. Most of the tests use between 50 and 100 mg of ^{13}C-urea. Tablets and capsules have been used to try and reduce contact with oral urease-containing organisms; however, solid dose formulation also introduce the problems of emptying before dissolving, dissolving slowly, or not being well distributed within the stomach. That said, each formulation appears about equal clinically in most countries especially if citric acid is included. Testing is typically done at least one hour after the last solid food ingestion. However, small studies have not shown that food significantly interferes and similar results were obtained fasting and after a hamburger and fries and fasting is not an absolute requirement [31,59]. The duration of the test is important and times from 10 min to 30 min after ingestion have proved successful. The initial delay is designed to allow any effects of oral bacteria to dissipate and to obtain a value near the peak of isotope enrichment. Most of the tests use times of 15–30 min which is generally near the plateau levels. The results are expressed as delta over baseline (DOB). A baseline breath sample is collected and the isotope enrichment is compared some specified time after ingestion of the labeled substrate. The cut-off is typically a DOB between 2‰ and 5‰ with 2.4‰ and 2.5‰ being used most often. In most series only about 5% of samples are near the cut-off. However, this proportion can be much greater especially in regions where achlorhydria and hypochlorhydria are common (see below).

Improving the reliability of the UBT

The presence of UreI can be exploited to increase the accuracy of the UBT especially in stomachs were acid secretion is low, suppressed, or the bacterial load is low. In normal adults the fasting gastric pH is approximately 1.8. This may increase to 3 or more in *H. pylori* infected individuals by the ammonium produced by *H. pylori* urease. The intragastric pH will thus be the result of the intrinsic rate of basal acid secretion and the bacterial load. When urea is ingested in a stomach with a relatively high pH the acid gated pore, UreI, may restrict its access to *H. pylori* urease and result in low measure urease activity [27–30]. When urea is administered with citric or malic acid the access of urea to *H. pylori* urease is enhanced and the measured urease activity increases. At the same time, the low gastric pH inhibits non-*H. pylori* bacterial ureases from hydrolyzing the labeled substrate and causing false positive results. Overall, the addition of citric or malic acid in the presence of *H. pylori* results in an increase in urease activity (i.e., a higher signal) and a reduction in urease activity in those without *H. pylori* infections (a lower baseline) thus improving the sensitivity and specificity of the test [60]. This is especially important in populations where atrophic gastritis is present and in those taking acid suppressing medicines such as H_2-receptor antagonists. In those populations the proportion of false positive or false negative tests is often clinically important [48,56,58,61–65]. There is little justification for not using citric or malic acid as adjuvants to the urea breath test. The problem of false positive tests has proven clinically important in Spain and Korea with tablet formulations without citric acid [59] and is likely important in other similar populations although this has not been examined systematically.

UBT in children

The diagnostic accuracy of the standard adult UBT is reduced in young children [66–69]. The problem is with the scoring of the urea breath test which is based on the ratio of $^{13}CO_2$ to $^{12}CO_2$ in the breath associated with the influence of age, gender, and basal metabolic rate to CO_2 production. In small children, the relatively low CO_2 production will result in a DOB that is proportionally higher for a given amount of labeled urea hydrolyzed producing false positive test results [69]. One common attempt to overcome this problem has been to arbitrarily increase the UBT cut-off value [69,70]. This method is arbitrary and a better alternative to assess the outcome based on the urea hydrolysis rate (UHR) in which anthropometric variables are used to determine the rate of CO_2 production in children. The ^{13}C-urea hydrolysis rate can be estimated from the algorithm developed by Klein et al. [69] which is based upon measurement of isotopic enrichment and determination of the carbon dioxide rate derived from estimated resting energy expenditure (joules).

In essence, the urea hydrolysis rate (UHR) is expressed as the adjusted product of change in the isotopic enrichment of paired breath samples expressed as DOB times the carbon dioxide production rate (CO2-PR):

$$UHR = DOB \times CO2PR \times 0.346294$$

CO2-PR is calculated from the product adjusted VCO2 value [VCO2 × 0.6944]. The VCO2 is calculated from the resting energy expenditure equation [VCO2 = EE/134.25] from which the EE value is calculated from the adjusted basal metabolic rate [EE = 334.6 × BMR]. This is also available on line (https://puhrca.otsuka-us.com/-files/Pediatric%20UHR-CA_UG%20v2.0.pdf). This method is accurate in both children and adults with a cut-off of 10 μg/min [68,69].

Comparison of RUT, UBT, and other methods

The UBT and RUT both have advantages and disadvantages [71]. The UBT is noninvasive, simple, and accurate and detects active *H. pylori* infections making it the preferred test for both initial diagnosis and confirmation of cure. The weaknesses are cost and relative unavailability in developing countries. The advantages of RUT are its wide availability and low cost. The main weakness of the RUT is the requirement for obtaining gastric specimens (i.e., invasiveness). It is especially useful when endoscopy is being done for another indication. Positive tests provide reliable evidence of infection whereas negative tests cannot be relied on to determine absence of infection. Both should be available.

H. pylori infections can also be identified by histology of gastric biopsies. The characteristic histologic finding is the presence of acute and chronic inflammation called acute-on-chronic inflammation. In addition, the bacteria can often be seen on standard hematoxylin and eosin staining, however, confirmation by Giemsa, a silver stain or immunohistochemical staining is more accurate [72]. Biopsy specimens can also be used for polymerase chain based molecular testing or for culture. Because everything in the stomach must eventually appear in the stool, stool antigen tests have been devised and tests using monoclonal *H. pylori* antibodies have proved similar in sensitivity and specificity to the urea breath test [71]. Finally, antibodies to *H. pylori* appear in the blood and a variety of tests mostly testing for specific IgG anti-*H. pylori* antibodies are widely available. Since the antibody titer often remains elevated serologic testing is best used for epidemiologic studies rather than for the detection of active infection or for test of cure. The choice of the best test is influenced by local *H. pylori* prevalence, availability, cost and clinical setting [72].

Conclusions and future perspectives

What was a medical curiosity, gastric urease, evolved into the mechanism in an important human pathogen, *H. pylori*, was able to thrive in the acidic environment of the stomach which until recently had been thought to be sterile. *H. pylori* urease is now used as a biomarker for the infection and a variety of techniques have been developed to reliably detect its presence and thus diagnose the infection. Attempts to find urease inhibitors to use to cripple the organism and aid in treatment are ongoing. Countrywide *H. pylori* eradication programs are being developed as a strategy to eliminate gastric cancer. Urease inhibitors and/or vaccines which include urease antigens are likely to play an important role in these endeavors.

Disclosures

Dr. Graham is a consultant for RedHill Biopharma regarding novel *H. pylori* therapies and has received research support for culture of *Helicobacter pylori* and is the PI of an international study of the use of antimycobacterial therapy for Crohn's disease. He is also a consultant for BioGaia and Takeda in relation to probiotic therapy for *H. pylori* infection and for Takeda. Dr. Miftahussurur does not have any relevant disclosures.

Conflict of Interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Acknowledgements

Dr. Graham is supported in part by the Office of Research and Development Medical Research Service Department of Veterans Affairs, Public Health Service grant DK56338 which funds the Texas Medical Center Digestive Diseases Center. Dr. Miftahussurur is supported in part by grant from the Ministry of Research, Technology and Higher Education of Indonesia for World Class Professor Program (no. 105/D2.3/KP/2017).

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