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False Positive and False Negative Results in Diagnosis of *Helicobacter Pylori* Infection Can be Avoided by A Panel of Serum Biomarkers (GastroPanel®)

Kari Syrjänen[1, 2*]

[1]Department of Clinical Research, Biohit Oyj, Helsinki, Finland.

[2]Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, SP, Brazil.

Corresponding Author: Syrjänen Kari. Department of Clinical Research, Biohit Oyj, Helsinki, Finland. Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, SP, Brazil, **Tel:** 358-40-5566810; **Email:** kari.syrjanen@biohit.fi

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ABSTRACT

Helicobacter pylori (HP) management (including diagnosis and therapy) has been exhaustively reviewed in several reports. These reports are unanimous in that several clinical conditions seriously hamper the diagnostic value of the two most commonly used HP tests: ^{13}C-Urea Breath Test (UBT) and Stool Antigen test (SAT), both false-negative and false-positive results being not uncommon. Basically, these false-negative results are due to decreased bacterial loads in the stomach mucosa, and include the following clinical conditions: 1) use of PPI medication; 2) use of antibiotics; 3) bleeding peptic ulcer; 4) atrophic gastritis (AG; with or without intestinal metaplasia); 5) gastric cancer; 6) MALT lymphoma, and 7) partial gastrectomy. Since the late 1990's, it has been well established that UBT also gives false-positive results in cases where urease-producing bacterial species are colonizing an acid-free stomach due to AG or a long term use of proton pump inhibitors (PPI). It is to be emphasized that neither UBT nor SAT (or HP serology) is capable of diagnosing AG, caused by HP infection or autoimmune disease, thus missing the patients at high risk for important clinical sequels of AG: I) gastric cancer (GC), ii) esophageal cancer, iii) vitamin-B12 deficiency (due to malabsorption), and iv) malabsorption of calcium, iron, magnesium and certain medicines.

Conclusion: It is mandatory that these serious limitations (i.e., false-negative results in true disease, false-positives with no HP infection, and failure to diagnose AG) in use of UBT and SAT are properly acknowledged by all laboratories offering these two tests for diagnosis of HP infections. Given that this bacteria is the single most important risk factor of GC, it is time to move a step forward also in the diagnosis of *Helicobacter pylori* infections, and start using the test (GastroPanel®, Biohit Oyj, Finland) that is i) free from the shortcoming of the conventional HP tests, and ii) provides an added value by detecting also the other key risk factor of GC, i.e., atrophic gastritis.

KEYWORDS

Helicobacter Pylori; Diagnosis; Urea Breath Test; Stool Antigen Test; Diagnostic Errors; False-Negative; False-Positive; Limitations; Serum Biomarkers; Marker Panel; Gastropanel Test; Gastric Cancer; Risk; Atrophic Gastritis.

BACKGROUND

The understanding on the important role played by *Helicobacter pylori* (HP) infection in pathogenesis of gastric cancer (GC) and peptic ulcer disease has increased progressively since the discovery of the bacteria in 1984 by Marshall and Warren [1]. According to the current concepts, GC develops from HP-infection through precursor lesions of progressively increasing severity: mild, moderate and severe atrophic gastritis (AG), accompanied by intestinal metaplasia (IM) and dysplasia. This sequence of events is generally known as the Correa cascade, and estimated to be involved in around 50% of GC cases, particularly the intestinal type of GC [2- 4].

In parallel with the increased understanding of the pathogenetic mechanisms, also the management of HP- infection has undergone substantial development during the past decade.

In this context, management also covers the complex topics related to the diagnosis of HP-infections. Much of this favorable development can be attributed to the European *Helicobacter* Study Group that took its first initiative in 1996 in Maastricht to gather dedicated experts in the field to review and discuss all relevant clinical data to arrive at recommendations for the clinical management of HP infection [5]. Since then, these Maastricht conferences have been repeated every 4-5 years. Each of these conferences has yielded a Consensus Report, the latest being the 4th in order, published in 2012 [6]. Attempts to standardize HP management (diagnosis and treatment) within countries have led to several national guidelines [7, 8]. In all these reports, considerable attention has been paid to different diagnostic methods available for HP detection, also including comprehensive review of the advantages and limitations of each technique and their utility in different settings, all based on updated literature [6-10].

However, far too often in daily practice, only the merits of the commonly used HP tests are being emphasized while there is a common tendency to neglect the limitations of their use in special clinical settings, although these are clearly discussed in all European Consensus Reports since 1996 [5, 6, 9, 10]. This applies to both of the two most widely used HP tests; the ^{13}C-Urea Breath Test (UBT) and Stool Antigen test (SAT), of which Prof. Marshall who discovered HP (1) made an early warning because of their numerous limitations already 20 years ago [11].

Until now, substantial amount of literature has accumulated on different HP-tests during the past two decades, also emphasizing the limitations of these tests in special clinical settings [12, 13]. Based on these data, there is little doubt that several clinical conditions seriously hamper the diagnostic value of these two HP tests (UBT, SAT): either false-negative (up to 40%) or false-positive results are not uncommon. The present communication makes a short review on the potential limitations of UBT and SAT in special clinical settings.

^{13}C-UREA BREATH TEST (^{13}C-UBT)

The urea breath test is based on the ability of HP to break down urea, into carbon dioxide which then is absorbed from the stomach and eliminated in the breath [5-10]. For the UBT, patients swallow a capsule containing urea made from an isotope of carbon (^{13}C). If HP is present in the stomach, the urea is broken up and turned into carbon dioxide. The carbon dioxide is absorbed from the stomach into the blood, and excreted in the breath. Samples of exhaled breath are collected, and ^{13}C in the exhaled carbon dioxide is measured. If the isotope is detected in the breath, it means that HP is present in the stomach.

Both recent national and European Consensus data are available on the utility of UBT in specific diagnostic settings [5, 6]. These issues were addressed in a recent Italian conference in February 2015 in Bologna, where recommendations were based on the best current evidence to help physicians manage HP infection in Italy. The guidelines have been endorsed by the Italian Society of Gastroenterology and the Italian Society of Digestive Endoscopy [8]. The same topics were also surveyed in the latest Maastricht Consensus Conference [6].

According to the Italian Consensus statement, the report stated that: "several meta-analyses confirmed that UBT is the best test for the non-invasive HP diagnosis, with a 96% sensitivity and a 93% specificity" [8]. With a closer look, however, this statement refers to one meta-analysis only, which included only cross-sectional studies evaluating the diagnostic accuracy of UBT in adult patients with dyspeptic symptoms, making the meta-analysis highly selective [13]. Thus, out of 1380 studies identified in the literature, only 23 met the eligibility criteria [13]. Meta-analysis was associated with a significant statistical heterogeneity that remained unexplained after subgroup analysis. The included studies also had a moderate risk of bias. The authors of the meta-analysis concluded that UBT has high diagnostic accuracy for detecting HP infection in patients with dyspepsia. They admit, however, that the reliability of their meta-analytic estimates is limited by significant between-study heterogeneity [13].

False-negative results

The UBT test has serious limitations when the test results should be interpreted with caution [5-13]. There is firm evidence to implicate that recent use of proton pump inhibitors (PPI) (within 2 weeks) or antimicrobials (within 4 weeks) may lead to a decrease in the gastric bacterial load causing false-negative results [14-16]. Bleeding can also reduce the sensitivity of both UBT and SAT [14, 15]. Data from a systematic review suggests repeating diagnostic tests in patients with bleeding ulcer after at least 4 weeks in case of a negative result [17]. In patients with precancerous conditions (e.g. atrophic gastritis, intestinal metaplasia) or gastric cancer, as well as in patients with partial gastrectomy, diagnostic tests may have lower accuracy [3, 17].

The same limitations as acknowledged in the Italian Consensus Report are also emphasized as limitations of the UBT and SAT tests in the latest Maastricht Consensus Report [6, 8]. It is clearly stated that significant decrease of the gastric HP load arises from the following conditions: i) use of antimicrobial agents, ii) use of anti-secretory drugs (PPI), and iii) in bleeding ulcers. Importantly, bacterial load may be permanently low in premalignant and malignant lesions, including i) atrophic gastritis, ii) intestinal metaplasia (IM), or iii) MALT (mucosal associated lymphatic tissue) lymphoma [18, 19].

Use of PPI Medication

In brief, several studies have shown that by increasing the gastric pH, PPI use leads to local changes in the stomach [14]. Because PPI drugs have anti-microbial properties, the bacterial load decreases, especially in the antrum, causing false-negative results of the UBT tests, in contrast to serology (HP IgG or IgA antibodies) that remains unaffected. Most of these studies have been carried out with UBT and showed a 10-40% rate of false-negative results [14, 20]. In addition to PPI treatment, also H2-blockers may lead to some false-negative results of UBT, but to lesser extent than PPI medication [14-16, 12, 21].

Antimicrobials

As to the use of antimicrobials, the evidence is notwithstanding that the local bacterial load of HP in the stomach will be reduced, leading to potentially false negative UBT results, as stated in the Consensus reports, based on several well documented studies [5, 8, 14-16, 22, 23]. Thus, Perri et al. [22] performed UBT in 41 HP-infected subjects before and after 1 day of therapy with (among other drugs) amoxycillin (2.5 g). They showed that even a short course of drugs specific for HP may result in a false-negative UBT [22]. The authors concluded that false-negative results are likely even after 1 day of therapy with bactericidal or anti-secretory (PPI) drugs. In another recent study, Leung et al. examined the serial changes of UBT results in 35 hospitalized patients who were given antibacterial therapy for predominantly chest and urinary infections, most (91 %) receiving a single antibiotic of either a penicillin or cephalosporin group [23]. Serial UBTs were performed within 24 hours of initiation of antibiotics, at one-week and at six-week post-therapy. The results showed that one-third of HP-infected individuals had transient false-negative UBT results during treatment with antibacterial agent, albeit a full clearance of HP infection by regular antibiotic consumption was very rare [23].

Bleeding ulcer

As to the accuracy of UBT in cases of bleeding ulcers, this topic was subjected to a comprehensive meta-analysis recently [17]. It has been suggested that prevalence of HP in peptic ulcer bleeding (PUB) is lower than that in non-complicated ulcers. These authors performed a systematic review of studies assessing the prevalence of HP infection in patients with PUB, including 71 articles, with 8,496 tested PUB patients. In meta-regression analysis, the UBT was reliable only when delayed until at least 4 weeks after the PUB episode [17, 24, 25].

In areas of low HP infection prevalence, a test-and-treat strategy should be considered [6, 8]. In the setting of peptic ulcer bleeding, histology and rapid urease test maintain a high specificity, but are affected by a low sensitivity, possibly leading to under-treatment [17, 24]. Importantly, serology seems not to be influenced by upper gastrointestinal (GI) bleeding [5, 9, 10].

Cancer precursors: atrophic gastritis (AG) and intestinal metaplasia (IM)

Atrophic gastritis (AG) is another clinical condition to be associated with substantial proportion of false-negative UBT results. This subject has been studied in detail by Kokkola et al. [19, 26, 27]. In their first study, patients with atrophic corpus gastritis (AGC) and elevated HP antibody titers but HP-negative UBT and histology, were randomized into eradication therapy or follow-up only [19]. HP antibody levels decreased significantly in six out of seven patients in the eradication group, while in the follow-up group, the titers declined in only one out of eight patients. In patients with AGC, positive HP serology results may indicate an ongoing infection in spite of negative UBT and histology results.

In another study, these authors made a direct comparison of UBT, HP serology and histology in 50 male patients with AGC [26]. The results are revealing: HP was detected in 15 (30%) patients by histology and in 14 (28%) by UBT, whereas increased serum HP-antibody levels were found in 41 (82%) patients ($p<0.0001$). HP infection was associated with AGC in 84% of the present patients. The authors concluded that in patients with AG and IM, prevalence of HP infection will be underestimated if only UBT (and biopsy-based diagnostic methods) are used [26].

Similar conclusions were drawn in another recent study by Lahner et al. [28], who examined 27 AGC patients using UBT and SAT, to assess whether the diagnostic yield of HP in AGC could be increased by these two tests, as related to histology alone. Without going into the details, the results implicated that in AGC patients, neither the UBT nor SAT added any useful information regarding HP infection, but a combination of histology and serology are needed to define the HP status among AGC patients [28]. Indeed, when followed-up for long enough, even HP serum antibodies disappear spontaneously within 10 years in almost one fourth of the patients with advanced AGC. This disappearance of HP antibodies is accompanied by no or more than a mild improvement of the gastric mucosa [27]. Thus, as well established, HP test can give a negative result in AGC, either i) due to disappearance of HP during the protracted course of the disease, or ii) because AGC is not caused by HP but an autoimmune disease [2, 4, 27, 28].

MALT lymphoma

MALT (mucosal-associated lymphatic tissue) lymphoma is another specific condition, ascribed to HP infection, but known to be associated with reduced bacterial load and thus susceptible to false-negative UBT results [5, 6, 8-10]. Because gastric

MALT lymphoma is a rare disease, few studies comparing the accuracy of diagnostic tests in this group of patients have been carried out, and only a limited number of tests (essentially histological) were performed. In one of those few studies, a total of 90 patients with low-grade gastric MALT lymphoma were enrolled, comparing histology, serology PCR and culture. Histology (97.5%) and serology (95.0%) were the two most sensitive tests, far superior to the other HP tests [18].

Importantly, HP-negative MALT lymphomas exist, testing repeatedly HP-negative [29]. In the search for HP based on histology and the UBT, there are cases with a series of false-negative results, thus confirming the possibility of a lower detectability of HP in patients with MALT gastric lymphoma and supporting the use of additional tests in diagnosis. Although patients with gastric MALT lymphoma with no HP are less responsive to HP eradication, a portion of the HP-false-negative cases are potentially curable by HP eradication therapy alone. Although the rationale for this finding is not fully elucidated, it is suspected that some HP-negative cases of MALT lymphoma are false-negatives due to patchy distribution of the microorganism in the gastric mucosa and limited tissue sampling during biopsy. In addition, PPI therapy before biopsy reduces the sensitivity of HP detection, and PPI should be discontinued at least 2 weeks before HP testing. The patient's symptoms may disappear and HP become undetectable with a negative UBT, if the therapy is effective, but, importantly, histological response usually lags behind the HP eradication, and a lymphoma infiltrate may persist up to 12 months or even longer. In contrast to serology, the UBT may produce false-negative results if performed after the use of HP- and urease-suppressive therapies, such as PPIs and antibiotics [30].

False-positive results

Apart from false-negative results listed above, UBT also gives false-positive results, which have received more attention during the past 10 years [28, 30-33]. These false-positive results are typical to patients with acid-free stomach (due to AG or a long term use of PPIs), where urease-positive bacterial species or yeast-like organisms colonize [28, 32-33].

In fact, however, this possibility of false-positive UBT results was well known already in the late 1990's, when e.g. the 2005 Nobel Laureate Barry Marshal described (in his Chapter to the Textbook of Lee & Megrau, 1996), that false-positive breath test results have been reported in gastrectomy, generally related to the presence of urease-positive bacteria other than HP [11]. The ^{13}C-UBT was positive and urease-positive bacteria other than H. pylori were recovered in gastric juice in hypochlorhydric children due to PPI use, reported by Michaud et al. in 1998 [34]. We know now, that gastric bacterial overgrowth is a constant phenomenon in acid-free stomach [28]. Already in the late 1990's, it was shown that the number of bacteria in gastric mucosa is comparable to that of gastric juice, and these non-H. Pylori bacteria are also found embedded in the mucus and even in close contact with gastric microvilli, similar as HP per se [35, 36].

In their study, Gurbuz et al. [31] compared UBT in the detection of HP infection with histology and the rapid urease test (RUT). Histology revealed dense yeast-like micro-organisms in the biopsy specimens in all patients with false-positive results by UBT, making the authors to conclude gastric mucosal colonization by yeast-like micro-organisms with urease activity can account for the high frequency of false-positive results for UBT [31].

Brandi et al [32] evaluated the presence of urease-positive bacteria other than HP in gastric juice and mucosa in 25 hypochlorhydric and 10 control subjects. Altogether, 6 hypochlorhydric patients had 10 strains of urease-positive non-HP bacteria, among which Staphylococcus capitis urealiticum showed the strongest urease activity. The authors concluded that patients with hypochlorhydric or acid-free stomach present with many urease-positive bacteria other than HP. The strong urease activity may be responsible for false-positive results at UBT test in patients with suspected HP-infection [32].

In another study, the UBT gave false-positive results in 4/102 subjects, shown to be caused by the presence of urease-positive bacteria in the oral cavity and stomach [33]. Altogether, 5 bacterial species with urease activity (Proteus mirabilis, Citrobacter freundii, Klebsiella pneumoniae, Enterobacter cloacae and Staphylococcus aureus) were isolated from the oral cavity and/or stomach. Because all of the patients with a false-positive UBT result were suffering from AG, it is obvious that the false-positive results in the UBT are a result from colonization of urease-positive bacteria in hypochlorhydric/acid-free stomach [33].

STOOL ANTIGEN TEST (SAT)

Stool antigen tests (SATs) are non-invasive diagnostic modules for HP-infection. Two types of SATs exist; one based on enzyme immunoassay (EIA) and another on immunochromatography (ICA) [9, 10]. SATs do not require expensive chemical agents or specified equipment; hence, they are less expensive compared with the UBT. Many guidelines have shown that EIA-based SATs using monoclonal antibodies are useful for primary diagnosis as well as for the assessment of eradication therapy. ICA-based tests do not require particular equipment and are therefore useful in developing countries [5, 6]. The accuracy of SATs is lower when the stool samples are unformed or watery, because HP-specific antigens in the stool samples are diluted. Temperature and the interval between stool sample collection and measurement also affect the results of SATs [5-10].

In most occasions listed above for the UBT, the same limitations and potential sources of error apply to the SAT as well [5, 6, 9, 10]. Because addressed in more detail above, a shorted discussion should be enough here. Due to the same inherent reason, i.e. low bacterial load in the stomach, a recent use of PPIs (within 2 weeks) or antimicrobials (within 4 weeks) also contributes to false-negative results in the SAT [14-16]. Similarly, bleeding can also reduce the sensitivity of the SAT [14, 15]. Data from a systematic review suggests repeating the SAT in patients with bleeding ulcer after at least 4 weeks in case of a negative result [17]. In patients with precancerous conditions (e.g. atrophic gastritis) or gastric cancer, as well as in patients with partial gastrectomy, diagnostic tests may have lower accuracy [17].

As discussed for the UBT, several studies have shown that by increasing the gastric pH, PPI use leads to reduced bacterial load in the stomach, especially in the antrum, causing false-negative results of the diagnostic tests, with the exception of HP serology [6,8]. Albeit most of these studies have been carried out with UBT, showing 10-40% rate of false-negative results, similar results have been obtained with the SAT as well [14,15,20,37]. Interestingly, the same bias seems to affect also the biopsy-based tests (culture, rapid urease test and histology) [38].

THE IMPECCABLE DIAGNOSIS OF HP-INFECTION IS POSSIBLE BY A PANEL OF BIOMARKERS

For the general public, it is less well known, however, that there is one test on the market that i) is free from these listed shortcoming of UBT and SAT, and in addition, ii) is capable of diagnosing both HP and AG, with all their potential clinically important sequels. This test is known as GastroPanel®, developed by a Finnish biotechnology company Biohit Oyj (Helsinki) as the first non-invasive diagnostic test for dyspeptic patients and for screening of the risks (HP, AG) of GC [39].

A detailed presentation and specifications of the GastroPanel® test are found elsewhere [40]. This ELISA-based biomarker panel includes 3 markers of mucosal atrophy (PGI and PGII for the corpus; G-17 for the antrum), combined with HP IgG antibody assay [40-42]. The results of GastroPanel are interpreted by a special software (GastroSoft®), another innovation of the company. During the past decade, GastroPanel has been tested in both diagnostic and screening settings. In a recent meta-analysis covering all the published literature, GastroPanel proved to be a highly accurate test for diagnosis AG (antrum and/or corpus) [43].

GastroPanel test has been on the market for roughly 10 years by now. This test is the first non-invasive diagnostic tool based on physiology of 3 stomach-specific biomarkers both in health and disease. The test also includes testing for HP-infection, the key etiological factor in pathogenesis of peptic ulcer disease and GC. In its current version, the Unified GastroPanel test is fully automated, and all 4 biomarkers being processed under identical conditions [40]. The test will be soon available in the quick test version as well, particularly suitable for POC (point-of-care) testing in doctors' offices lacking the facilities for blood sample centrifugation.

With the refined diagnostic algorithm of the GastroSoft, the results are classified into 8 diagnostic categories, of which 5 represent functional disturbances (in acid output) and 3 indicate AG (and its topographic location) [42]. In GastroPanel test, the HP antibody measurement is complemented by the other 3 biomarkers (PGI, PGII, and G-17) which are sensitive indicators of mucosal inflammation. This is important because like all bacteria, also HP will induce acute inflammation in the gastric mucosa, with a usual onset in the antrum [39]. Accordingly, in GastroPanel test, three different marker profiles can be encountered in association with HP-infection [40].

First, in an active HP-infection, HP-antibody titers are raised, which can be the only abnormal finding in GastroPanel, with all other markers falling within a normal range. Not infrequently, however, an active ongoing HP-infection causes a severe inflammatory reaction which, due to increased cell permeability, can lead to increased leakage of PGI, PGII and even G-17 from the cells and result in elevated serum levels of any or all of these three biomarkers [6, 40, 42]. **Second**, a successful HP-eradication by active treatment should result in normalized values of all three markers, however, with a delay of some weeks to months. HP-antibody titers can remain elevated for a longer period of time which is unpredictable (usually months) and should be taken into account while interpreting the GastroPanel results after HP-eradication [6, 40]. **Third**, in cases where HP-eradication attempt fails, HP-antibody titers remain elevated (usually slightly), PGI and PGI/PGII ratio usually fall within a normal range, whereas PGII and/or G-17b may be slightly elevated due to ongoing inflammatory reaction [6, 40, 42]. The result can be confirmed after 5-6 months, followed by new treatment attempt if indicated.

With all these sophisticated diagnostic properties, this panel of 4 biomarkers makes GastroPanel test the most comprehensive HP test, devoid of the known shortcomings of the conventional HP tests [11-38, 40]. In 2012, the International *Helicobacter Pylori* Study Group stated in their Maastricht IV Consensus Conference, that the blood biomarker tests are a reliable means to identify and screen for gastric diseases and their risk status [6]. In the same year, 16 experts from 12 countries in the HSI (Healthy Stomach Initiative, http:\www.hsinitiative.org) drafted a set of recommendations implicating that

the biomarker tests are suitable for both screening of asymptomatic patients and for diagnosis of dyspeptic patients [39].

CONCLUSIONS

Because firmly documented and repeatedly emphasized in several international consensus reports, it is mandatory that the serious limitations of the two globally most used HP detection tests (UBT and SAT) are properly recognized [11-38]. It is important that both false-negative and false-positive results are acknowledged as established shortcomings of these diagnostic HP tests. Furthermore, it should be made perfectly clear that these conventional HP tests are not capable of diagnosing atrophic gastritis with all its potentially severe clinical sequels, including the risk of GC. Given that this bacteria is the single most important risk factor of GC, it is time to move a step forward also in the diagnosis of *Helicobacter pylori* infections, and start using the test that is i) free from the shortcoming of the conventional HP tests, and ii) provides an added value by detecting (with high precision) also the other key risk factor of GC, i.e. atrophic gastritis [43].

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