Occult *H. pylori* infection partially explains ‘false-positive’ results of ¹³C-urea breath test

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Abstract

Background: In a previous study, UBiT-100 mg, (Otsuka, Spain), a commercial ¹³C-urea breath test omitting citric acid pre-treatment, had a high rate of false-positive results; however, it is possible that UBiT detected low-density ‘occult’ infection missed by other routine reference tests. We aimed to validate previous results in a new cohort and to rule out the possibility that false-positive UBiT were due to an ‘occult’ infection missed by reference tests.

Methods: Dyspeptic patients ($n = 272$) were prospectively enrolled and UBiT was performed, according to the manufacturer’s recommendations. *Helicobacter pylori* infection was determined by combining culture, histology and rapid urease test results. We calculated UBiT sensitivity, specificity, positive and negative predictive values (with 95% CI). In addition, we evaluated ‘occult’ *H. pylori* infection using two previously-validated polymerase chain reaction (PCR) methods for urease A (UreA) and 16 S sequences in gastric biopsies. We included 44 patients with a false-positive UBiT, and two control groups of 25 patients each, that were positive and negative for all *H. pylori* tests.

Results: UBiT showed a false-positive rate of 17%, with a specificity of 83%. All the positive controls and 12 of 44 patients (27%) with false-positive UBiT were positive for all two PCR tests; by contrast, none of our negative controls had two positive PCR tests.

Conclusions: UBiT suffers from a high rate of false-positive results and sub-optimal specificity, and the protocol skipping citric acid pre-treatment should be revised; however, low-density ‘occult’ *H. pylori* infection that was undetectable by conventional tests accounted for around 25% of the ‘false-positive’ results.

Keywords

Bacterial infection, diagnostic tests, false positive results, *Helicobacter pylori*, sensitivity, specificity, urea breath tests

Received: 18 November 2014; accepted: 20 January 2015

Introduction

Evaluation of diagnostic technologies is fundamental to ensuring quality of care. Unreliable tests lead to unnecessary treatments and/or underuse of effective therapies. In a previous study, we found a very high rate of false-positive results and low specificity with a commercially available urea breath test (UBT), specifically UBiT-100 mg (Otsuka, Barcelona, Spain), for

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Helicobacter pylori (*H. pylori*) when compared with the combination of histology, stool test, rapid urease test (RUT) and culture.¹ As this finding was totally unexpected, we performed a number of control measurements to confirm its validity. The analysis led us to hypothesize that the false-positive results may be attributable to the omission of the pre-administration of citric acid, within the UBiT protocol. The inclusion of citric acid has been shown to increase the accuracy of UBT.^{2,3}

A subsequent study was devised to confirm our findings, suggesting that a Delta cut-off value of 8.5‰ (instead of the 2.5‰ value recommended by the manufacturer) could partially correct for the high rate of false-positive results; however, there may be alternative explanations for these ‘false-positive’ results. One possibility is that the ¹³C-UBiT may be more sensitive than other tests, and may detect ‘occult’, low-density infection missed by other reference tests. In fact, data from many different settings suggest that subclinical infection exists, and causes false-negative results, in conventional *H. pylori* tests. This may happen, for example, during antisecretory or antibiotic therapy; or in patients with extensive gastric mucosal atrophy, intestinal metaplasia, gastric mucosa-associated lymphoid tissue (MALT) lymphoma^{4,5} or upper gastrointestinal (GI) bleeding.⁶ Even in normal individuals who were *H. pylori*-negative according to conventional tests, molecular methods are able to detect active infection.⁷ Interestingly, in these situations, a polymerase chain reaction (PCR) was able to detect a low-density infection in a significant number of patients.^{8–10}

The aims of the present study were:

1. To validate the Delta cut-off of 8.5‰, which we previously proposed as a threshold for considering the UBiT positive, instead of the Delta cut-off of 2.5‰ that is proposed by the manufacturer¹; and
2. To rule out the possibility that these ‘false-positive’ results were due to low-density *H. pylori* infection that was missed by the reference tests.

Patients and methods

Outpatients sent to the Endoscopy Unit of the ‘Hospital de Sabadell’ in Barcelona, Spain, for dyspeptic symptoms from February 2008 to November 2011 were prospectively recruited for our study. Patients were contacted prior to the endoscopy. Those who agreed to participate in the study were instructed to avoid anti-secretory drugs in the 2 weeks before the test. We excluded the patients whom were unable to stop anti-secretory drugs, those whom had received antibiotics in the 4 weeks before the endoscopy and

those whom had previous *H. pylori* treatment. Before the endoscopy, the patient provided signed informed consent and was administered a UBT (UBiT-100 mg, Otsuka, Barcelona, Spain). During endoscopy, four antral biopsies were obtained: Two for histology, one for the urease test and culture, and one for molecular analysis. We included 272 patients in the study. The patients’ clinical and demographic data are shown in Table 1.

Diagnostic tests

Sample collection and analysis. Different operators who were unaware of the results of the other assessments performed the diagnostic tests (histology, RUT, microbiological culture, UBiT and PCR).

Urea Breath Test. The UBT was performed with UBiTest 100 mg (Otsuka Pharmaceutical Europe, Barcelona, Spain). Determinations were performed in accordance with the manufacturer’s specifications. A basal breath sample was collected by blowing into a specially-designed bag. After this, patients took a pill of 100 mg of ¹³C-labelled urea in 100 ml of water and 20 minutes later, filled a second breath bag. Samples were immediately processed by non-dispersive infrared spectrophotometry (NIRS) (POCone™ Infrared Spectrophotometer, Otsuka Pharmaceutical, Tokyo, Japan). In accordance with the manufacturer’s specifications, an increase in the proportion ¹³C/¹²C ($\Delta^{13}\text{CO}_2$ (‰)) of 2.5‰ or more after urea intake was considered as indicative of *H. pylori* infection.

Rapid urease test and histology. RUT was performed after mucosal sampling using the JATROX HP test (CHR Heim Arzneimittel GmbH, Darmstadt, Germany) and was read according to the manufacturer’s specifications. We collected biopsies for histology in formalin, stained them with Giemsa, and then they were evaluated by two pathologists whom are digestive disease specialists. The pathologists were blinded to the results of the other diagnostic tests.

Table 1. Characteristics of the patients

Gender (male/female) (n)	153/119
Age (mean $\pm$ SD)	49.3 $\pm$ 13.4
Endoscopic diagnosis (n)	
Duodenal ulcer/erosive duodenitis	48
Gastric ulcer	5
Erosive gastritis	28
Esophagitis	38
Normal	153

Microbiological culture. Isolation, culture and identification of *H. pylori* were performed: After a positive RUT test, the biopsy was plated on Pylori Agar (Biomérieux, Spain) in microaerophilic conditions (CampyGenTMOXOID, England). After a maximum of 1 week, the *H. pylori* isolates grown were sub-cultured in Columbia plates (Biomérieux) and identified by colony morphology and Gram-negativity; and test positivity for urease, catalase and oxidase.

***H. pylori* detection by real-time PCR.** Total deoxyribonucleic acid (DNA) from the endoscopic biopsies was isolated with MasterPure (Epicentre, USA). Isolated DNA was quantified with a NanoDrop spectrophotometer (Nano-Drop Technologies, USA).

TaqMan PCR was used to amplify fragments of the 16S ribosomal ribonucleic acid (rRNA) and ureA genes of *H. pylori*. Real-time PCR was carried out in an ABI 7500 thermocycler (Life Sciences, USA) with the following thermal cycling conditions:

1. TaqManPCR for the 16S rRNA gene: Initial uracil-DNA glycosylase (UNG) incubation at 37°C for 10 min., followed by denaturation at 95°C for 10 min., and then 40 cycles consisting of denaturation at 92°C for 15 s., followed by annealing/extension at 64°C for 1 min.
2. TaqManPCR for the ureA gene: Initial UNG incubation at 37°C for 10 min., followed by denaturation at 95°C for 10 min., and then 40 cycles consisting of denaturation at 95°C for 15 s, with touchdown annealing from 60°C to 57°C for 10 s (temperature decrements of 0.2°C) and extension at 72°C, for 35 s.

The 25 µl PCR reaction mixture was prepared with 12.5 µl of 2 x SensiMix (dU) (Quantace, UK), 0.5 µl of 50 x UNG, and 2.5 µl of DNA for all genes. Magnesium chloride (MgCl₂) concentrations were 5.5 mM, 4.0 mM, and 3.0 mM for the 16S, ureA and vacA (s) gene, respectively. Primer concentrations were 0.1 µM for the 16S, 0.25 µM for ureA and 0.4 µM for vacA genes, respectively. The TaqMan probe concentrations

were 40 nM for 16S and 20 nM for the urea PCRs, respectively.

We labeled the TaqMan probe with the fluorescent dyes 6-carboxyfluorescein (6-FAM) on the 5' end and with 6-carboxytetramethylrhodamine (TAMRA) on the 3' end; we purified the probes by high performance liquid chromatography (HPLC). Primers and probe were purchased from Stab vida, Lda. (Oeiras, Portugal). Corresponding sequences are listed in Table 2.

All tests were performed by an experienced molecular biologist, whom was blinded to the *H. pylori* status of the samples and to the results of the other tests.

Histological predictors of infection. A specialized pathologist who was unaware of the final *H. pylori* status analyzed the biopsy specimens and recorded the presence of *H. pylori* infection, and those of active gastritis, a finding that is shown to predict active *H. pylori* infection.⁸

Statistical methods

Sample size calculation. Assuming a prevalence of *H. pylori* infection of 75% in the population with dyspepsia,¹¹ and an UBiT specificity of 78%, we calculated that a sample size of 265 patients should obtain an estimation of UBiT's sensitivity and specificity with 10% precision and a 95% confidence level.

Statistical analysis. *H. pylori* infection status was determined on the basis of the combined results of culture, RUT and histology. Patients with a positive culture or both positive RUT and histology were considered infected; patients with no positive reference tests were considered not infected. In this regard, in a previous evaluation the combination of RUT and histology was shown to be extremely reliable, with a sensitivity of 97% and a specificity of 99% for diagnosing *H. pylori* infection.¹ Patients with only a positive RUT or a positive histology were considered undetermined and excluded from the analysis.

Table 2. Primers and probes used to amplify the target genes

Gene	Forward primer	Reverse primer	Ref.
16S rRNA	5'-CTCATTGCGAAGGCGACCT-3'	5'-TCTAATCCTGTTTGCTCCCA-3'	19
ureA	5'-CGTGGCAAGCATGATCCAT-3'	5'-GGGTATGCACGGTTACGAGTTT-3'	20
TaqManprobe	Sequence		
16S rRNA	5'- (6-FAM)-ATTACTGACGCTGATTGCGGAAAGC-TAMRA-3'		
ureA	5'- (6-FAM)-TCAGGAAACATCGCTCAATACCACTT-TAMRA-3'		

FAM: fluorescent dye 6-carboxyfluorescein; Ref: reference number; rRNA: ribosomal RNA; TAMRA: fluorescent dye 6-carboxytetramethylrhodamine; ureaA: urease A

Figure 1. Distribution of UBT values in patients with positive and negative *H. pylori* infection according to the Gold Standard. *H. pylori*: the bacteria *Helicobacter pylori*; UBiT: the specific name given to a commercial UBT; UBT: urea breath test

Sensitivity, specificity, and positive and negative predictive values were calculated for the UBiT technique; and they were expressed as percentages and 95%CI. All calculations were performed using SPSS 20.0 (SPSS Inc., USA) and Epidat 3.1 (XG and OMS, Spain) for Windows. The study was performed in compliance with the STAndards for the Reporting of Diagnostic accuracy (STARD) studies’ recommendations.^{12,13}

Results

Patients

We excluded 17 patients because the gold standard could not be calculated, for a variety of technical reasons: Out of the 17 excluded, 13 patients had only positive histology and a negative RUT (or a positive RUT and negative histology), and no culture. In the remaining four excluded patients, either the histology or the RUT results were missed, and culture was not performed. Of the remaining 255 patients, 131 were considered infected and 124 non-infected, according to the established gold standard.

UBiT sensitivity and specificity

Using the recommended Delta value of 2.5‰, UBiT had a sensitivity of 100%, but a specificity of only 83.1%, with 21 (16.9%) false-positive results (Figure 1). Increasing the Delta cut-off value to 8.5‰ did not improve the reliability of UBiT, although the specificity of the test rose to 95.2% and the sensitivity decreased to 80.2%. Figure 2 shows a STARD flow diagram for the calculations performed with both the manufacturer’s recommended Delta cut-off value of 2.5‰ and for a Delta cut-off value of 8.5‰. We show

Figure 2. STARD diagram depicting the flow of patients and the results of the analysis. Values are calculated for UBiT Delta cut-offs of 2.5‰ and 8.5‰.

GS +: positive gold standard, GS -: negative gold standard; STARD: Standards for Reporting of Diagnostic Accuracy; UBiT: a specific commercial UBT; UBT: urea breath test

Table 3. Sensitivity, specificity, PPV and NPV with 95% CI for diagnosing *H. pylori* with UBiT, using Delta cut-off values of 2.5‰ or 8.5‰ for UBiT

Test	Sensitivity (CI 95%)	Specificity (CI 95%)	PPV (CI 95%)	NPV (CI 95%)
Urea breath test Delta cut-off value of 2.5‰.	100% (99.6–100)	83.10% (76.1–90.1)	86.20% (80.4–92)	100% (99.5–100)
Urea breath test Delta cut-off value of 8.5‰.	80.20% (72.9–87.4)	95.20% (91–99.3)	94.60% (89.9–99.3)	81.90% (75.3–88.6)

H. pylori: the bacteria *Helicobacter pylori*; NPV: negative predictive value; PPV: positive predictive value; UBiT: Urea Breath Test.

UBiT sensitivity, specificity, and positive and negative predictive values in Table 3.

Assessment of occult *H. pylori* infection by gastric biopsy PCR in patients with false-positive UBiT results

We showed that 53 of the 455 patients analyzed in either this study or our previous study¹ had a false-positive UBiT test. In nine patients, the sample retrieved was insufficient for PCR analysis. The remaining 44 patients were included in the analysis. In addition, 25 positive controls with all positive tests (including UBT, RUT, histology and culture) and 25 negative control patients with all negative tests were included. Gastric biopsies of all the positive controls were positive by the two PCR techniques; in contrast, none of the 25 negative controls had two positive PCR tests. As 12 of the 44 patients

Figure 3. Number of PCR positive tests in positive and negative controls and in 'false-positive' UBiT results. The 25 positive controls with all positive tests (UBiT, RUT, histology and culture) were all positive for the two PCRs, whereas none of the 25 negative controls had two positive PCR tests; also, 27% of patients with a 'false-positive' UBiT were positive for the two PCRs. PCR: polymerase chain reaction; RUT: Rapid Urease Test; UBiT: name of the commercial urea breath test used.

(27%) with a 'false-positive' UBiT were also positive for the two PCR tests, this suggested they had a real infection (Figure 3).

Role of histology for detecting 'occult' *H. pylori* infection

Although all 25 positive controls and none of the negative controls had active gastritis (mucosal neutrophil infiltration) in the histological assessment, the presence of active gastritis was not useful in assessing our patients with 'occult' infection. In the group of 44 patients with a false-positive UBiT result, active gastritis was present only in 2 of the 12 (16.7%) with both PCR tests that were positive; and was also detected in 6 of the 24 patients (25%) with both PCR tests that were negative. In addition, of the 12 patients with 'occult' infection, only one had gastric atrophy and another had intestinal metaplasia, both mild-to-moderate.

Discussion

This study confirms previous data regarding the low reliability of UBiT. The false positive rate of 16.9% was similar to the rate observed in our previous study (22%).¹ Our results lend additional support to the recommendation that UBiTest 100 mg should be modified to include citric acid co-administration and re-evaluated to confirm good sensitivity and specificity. This measure will probably improve the reliability of the

test. In fact, citric acid is already incorporated in the kits marketed by Otsuka in the US.¹⁴

The present study adds another interesting finding to the previous data: Many of the discordant results between diagnostic tests (around one-quarter, in the current analysis) are attributable to low-density 'occult' infection. So, 'occult' infection may occur in clinical practice; and in an undetermined number of patients, the infection may pass undetected after one or more of the conventional tests. Our study adds evidence to the rather scattered data in the literature suggesting that undetectable infection is not exceptional in many different settings. For example, it is well known that antisecretory drugs or antibiotics could reduce the density of the infection to levels undetectable by conventional tests; and for this reason, it is recommended that these drugs should be stopped before diagnostic tests are carried out^{15,16}; however, the use of proton pump inhibitors (PPI) does not seem to explain the occurrence of 'occult' infection in the present study: Patients were interviewed by phone before inclusion in the study and again before performing the UBiT and endoscopy, and those taking these drugs were excluded. As another example, Bik et al.⁷ evaluated the gastric microbiota of 23 healthy volunteers. They detected *H. pylori* infection in 12 individuals by conventional tests and in 19 of the 23, by molecular tests. Furthermore, Raderer et al.⁵ report six patients with gastric MALT lymphoma with negative results for all *H. pylori* conventional tests, whom presented complete resolution of the disease after *H. pylori* treatment. The finding was attributed to 'occult' infection.⁴ Finally, Guell et al.⁶ report that 79% of the patients with peptic ulcer bleeding whom tested negative for *H. pylori* during the bleeding episode had active infection, which was only detected when the tests were repeated a few weeks after the acute bleeding episode.

The prevalence and relevance of 'occult' *H. pylori* infection in dyspeptic or in ulcer patients is currently unknown. It is possible, for example, that 'occult' infection may account for a proportion of idiopathic peptic ulcers, an entity that seems to be increasingly frequent worldwide.^{17,18} It may be worth investigating the prevalence of 'occult' infection in these cases.

In conclusion, our study confirmed previous reports suggesting that some of the UBT that are commercialized in Europe present an unusually high rate of false-positive results. Modifying the cut-off for positive results did not improve the test performance. For this reason, it seems advisable to modify current diagnostic protocols (probably by including citric acid pre-administration) and then re-evaluate the modified test. However, our study also shows that in 27% of these patients with 'false-positive' UBiT results, molecular

methods were able to show there was an 'occult' low-density infection, which was not detected by the remaining reference tests.

Funding

This work was supported by the Instituto de Salud Carlos III (grant numbers PI 05/1157 and PI 05/0664; as well as a research grant from their Programa de Intensificación en Investigación, for Xavier Calvet) and the Societat Catalana de Digestologia. CIBEREHD is also supported by the Instituto de Salud Carlos III.

Conflict of interest

In the past 5 years, the group signed research agreements with Dako and Oxoid Ltd. for evaluating the reliability of newly-developed stool tests for *H. pylori*; however, these agreements were not related to the present study. None of the authors had any conflicts of interest regarding the development or results of the present study.

Acknowledgements

We are indebted to Michael Maudsley for his help with the English.

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