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Validation of a simplified ^{13}C-urea breath test method for the diagnosis of *Helicobacter pylori* infection

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RESUMEN

Objetivo: validar un método simplificado de la prueba en aliento con urea-^{13}C (PAU-^{13}Cs) para el diagnóstico de infección por *Helicobacter pylori* (*H. pylori*), con administración simultánea de 50 mg de urea-^{13}C y 2 g de ácido cítrico.

Material y métodos: se estudiaron 88 pacientes (49 mujeres y 39 hombres); con promedio de edad 45 ± 15 años, referidos para endoscopia gastrointestinal y toma de biopsias. La PAU-^{13}Cs se realizó en ayuno. Se recolectaron las muestras de aire espirado en tubos de cristal de 10 ml, antes y 30 minutos después de administrar simultáneamente 50 mg de urea-^{13}C y 2 g de ácido cítrico disueltos en 200 ml de agua. Las muestras se analizaron por espectrometría de masas. El diagnóstico de infección se consideró cuando el cultivo y/o la biopsia y serología fueron positivas para *H. pylori*.

Resultados: cincuenta y un pacientes (57,95%) fueron positivos, 30 (34,10%) negativos para *H. pylori* y 7 (7,95%) casos se consideraron indeterminados. La sensibilidad, especificidad, valor predictivo positivo y negativo de PAU-^{13}Cs fue de 90,2, 93,3, 95,8 y 84,8%, respectivamente. Con exactitud de 91,4%.

Conclusión: la administración simultánea de 50 mg de urea-^{13}C y 2 g de ácido cítrico, representa una alternativa para el diagnóstico no invasivo de infección por *H. pylori*, debido a que conserva la certeza diagnóstica de la PAU-^{13}C.

Palabras clave: *Helicobacter pylori*. Urea-^{13}C. Prueba en aliento. Ácido cítrico.

ABSTRACT

Objective: to validate a simplified ^{13}C-urea breath test (^{13}C-UBT) method for the diagnosis of *H. pylori* infection.

Material and methods: patients referred for gastrointestinal endoscopy and biopsy were included, and a ^{13}C-UBT was performed after a 6-hour fast. Breath samples were collected in 10 ml glass tubes before and 30 min after the simultaneous administration of 50 mg of ^{13}C-urea and 2 g of citric acid in 200 ml of water. All breath samples were analyzed using isotope ratio mass spectrometry. The diagnosis of *H. pylori* infection was established with a positive culture and/or positive histology and serology.

Results: eighty-eight patients were included, 49 female and 39 male with a mean age of 45 ± 15 yrs. Fifty-one patients (57.95%) were positive and 30 (34.1%) negative for *H. pylori*. Seven cases (7.95%) were considered undetermined. The sensitivity, specificity, positive predictive value, and negative predictive value for ^{13}C-UBT were 90.2, 93.3, 95.83, and 84.8%, respectively. Accuracy was 91.4%.

Conclusions: the simultaneous administration of 50 mg of ^{13}C-urea and 2 g of citric acid represents an alternative for the non-invasive diagnosis of *H. pylori* infection.

Key words: *Helicobacter pylori*. ^{13}C-urea. Breath test. Citric acid.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is the main cause of gastritis and peptic ulcer disease in adults, and has been associated with the development of cancer and gastric lymphoma (1). Different diagnostic methods are available: invasive ones require endoscopy and biopsy for histology, culture and/or PCR; non-invasive methods in-

clude the determination of serum antibodies, stool antigens, and urea breath tests (UBT) with ^{14}C or ^{13}C (1-3).

In 1987, the ^{13}C-UBT was introduced based on the urease activity of *H. pylori*, which hydrolyzes ^{13}C-urea to ammonia and labeled carbon dioxide ($^{13}CO_2$) (3,4). ^{13}C-UBT is currently considered a highly reliable method for the non-invasive diagnosis of *H. pylori* infection; it is the ideal test for those in whom endoscopy is not required or for follow-up in the post-treatment evaluation of *H. pylori* infection. ^{13}C-UBT is easily and safely performed without exposing the patient to radioactivity (5-9).

After the original description different modifications have been described to simplify, optimize, and refine this test with the purpose of improving its cost-effectiveness ratio. This has been attempted by decreasing the dose of urea and/or by modifying the shape and composition of the test food (5-15) (Table I).

The objective of this study was to validate a simplified method for ^{13}C-UBT with the simultaneous administration of 50 mg ^{13}C-urea and 2 g of citric acid test meal.

MATERIAL AND METHODS

Study population

We designed a prospective study performed between February 2004 and March 2005 that included patients with 18-75 years of age programmed for upper endoscopy due to dyspepsia, peptic acid disease, and upper GI hemorrhage. All subjects voluntarily agreed to participate and signed a written consent. The following were excluded: patients that had received previous treatment (antibiotics and or proton-pump inhibitors 30 days prior to endoscopy, or H_2 antagonists one day prior to endoscopy); patients with an endoscopic diagnosis of grade C or D esophagitis (Los Angeles classification); patients with complicated ulcers (Forrest classification I and II, perforated and/or penetrated); patients with a history of partial or total gastrectomy, and of eradication treatment for *H. pylori*. Patients with incomplete data for the final analysis were also excluded. The investigation was approved by the research committee of our hospital, and carried out according to the protocol, good medical practices, and the General Health Law Regulation for Health Research.

DIAGNOSTIC METHODS

Upper gastrointestinal endoscopy and biopsy

All endoscopies were performed and interpreted by the same group of endoscopists. A Fujinon® EG 201FP video-endoscope and BF2416SF biopsy forceps (Fujinon® Inc, Japan) were used. Seven gastric biopsies were taken: three from the pre-pyloric region (1-2 cm from pylorus), two from the corpus (greater curvature and posterior side), one from incisura angularis, and one from the cardia. Five biopsies were placed in culture tubes; one from the body and one from the antrum were immediately fixed in 10% formalin for histopathology (16,17).

Histology

Both formalin-fixed samples were routinely stained with hematoxylin-eosin (H&E) and modified Giemsa (17,18). Slides were randomly and blindly assigned to

Table I. Clinical validation studies of the urea-^{13}C breath test for the diagnosis of *H. pylori* infection

Author, year (reference)	*N*	*Urea-^{13}C (mg)*	*Time (min)*	*Test food*	*SE (%)/ES (%)*
Current study	81	50	30	2 g citric acid	90,4/96,6*
Peng N-J, 2005 (5)	50	100 (caps.)	15-30	No	100/100*
	50	50 (caps.)	6-15	No	96/100*
Levine, 2004 (14)	72	75	30	4,5 g citric acid	–
Canete A, 2003 (15)	100	50	40	4,2 g citric acid	79-96/86-99*
Wong WM, 2003 (13)	200	50 (tab.)	20	456 mg citric acid	100/100*
Gatta L, 2003 (12)	200	100 (tab.)	10	912 mg citric acid	96,7-100/93,7-99,8*
Liao CC, 2002 (11)	152	50	15	200 ml of fat rich beverage	99/97*
Graham DY, 2001(8)	249	75	15	2,5 g citric acid	92-99,4/96,2-100*****
Leodolter A, 1999 (10)	233	75	30	4,4 g citric acid	94,7/97,8*
	320	75	40	4,4 g citric acid	92,2/98,8*
Domínguez-Muñoz, 1997 (9)	90	75	30	4,4 g citric acid, H_2O 200 ml	95,6/96,6*
Klein PD, 1996 (7)	120	125	30	Vanilla Ensure®	95,4-95,3/87,9-95,5**
Logan RPH, 1991 (6)	195	100	> 40	Beverage with 76% fat (57% oleic acid and 23% palmitic acid), 19% carbohydrates and 5% protein	98/92* 95/97** 83/98*** 95/83****
Graham DY, 1987 (3)	65	350 (5 mg/kg)	> 45	Fat-rich pudding	90-100/95

Standard criteria for diagnosis of *H. pylori* infection: *Culture or histology and serology, and/or CLO-test; **Histology; ***Culture; ****Serology; *****Other versions of breath test .SE = sensitivity; ES = specificity; caps = capsules; tab. = tablets.

experienced pathologists, and interpreted based on Sydney's current classification of gastritis (19). A histopathological diagnosis of infection was established when curved or S-shaped bacterial structures compatible with *H. pylori* were found in any of the samples.

Cultures

Specimens were transported independently in *Brucella* broth containing 30% glycerol and frozen at -70 °C for 24-48 hours until culture. Biopsies were homogenized and inoculated in trypticasein-soy agar supplemented with 7.5% lamb blood. Plates were incubated at 35-37 °C up to ten days in microaerobic conditions (5% O_2, 100 ml CO_2, and 85% N_2). Cultures were considered positive when Gram-, urease-, catalase-, and oxidase-positive colonies with a typical morphology developed within 7-10 days.

Serology

ELISA tests were performed to determine IgG anti-*H. pylori* antibodies in the serum obtained from a 10-ml venous blood sample from each patient. A concentration equal to or greater than 15 U/ml was considered positive.

Urea ^{13}C breath test

The simplified ^{13}C UBT was performed 3 to 5 days after endoscopy with a minimum 6-hour fast. A test beverage containing 2 g of citric acid (Citra-LP, San Miguel de Proyectos Agropecuarios S.P.R, México) and 50 mg of 99% urea-^{13}C (Isotec Inc®, Ohio, USA), dissolved in 200 ml of water was given to patients. Samples were collected before (baseline) and 30 min after the ingestion of the test beverage. Patients were instructed to blow through a straw directly into a 10-ml Exetainer® tube (LabCo Limited®, Buckinghamshire, UK). All samples were duplicated and analyzed by isotopic mass spectrometry (Breath-Mat plus Finningan, Bremen, Germany) in the Gastroenterology Laboratory.

Results were expressed in delta units (δ), which refer to the post-baseline and baseline relation of ^{13}C/^{12}C in delta units by 1,000 (‰).

Diagnosis of H. pylori infection

Infection by *H. pylori* was diagnosed when the culture was positive and/or histology and serology were simultaneously positive. Patients with negative cultures, biopsy, and serology were considered non-infected by *H. pylori*, and those with a contaminated culture, positive biopsy and negative serology, or positive serology and negative biopsy, were considered indeterminate (20,21).

Statistical analysis

Data were expressed as medians (intervals), means (± standard deviation), and percentages.

A receiver operating characteristic curve (ROC curve) was built using the δ of each patient, and the diagnosis of *H. pylori* was established according to standard criteria. The optimal cut-off point for ^{13}C-UBT was defined as the point with the highest sensitivity, specificity, and diagnostic accuracy to identify patients with and without *H. pylori* infection.

Sensitivity (SE), specificity (SP), positive predictive value (PPV), negative predictive value (NPP), positive likelihood ratio (PPR), negative likelihood ratio (N-R), and 95% confidence intervals (CI) were all calculated. Initially, only cases classified as positive or negative were included; thereafter, our analysis included indeterminate cases with histopathologic results as reference.

The analysis was performed using SPSS for Windows (version 12.0; SPSS, Chicago, IL, USA).

RESULTS

Eighty-eight patients, 49 females and 39 males, with a mean age of 45 ± 15 years and who completed all four diagnostic tests were included. Indications for endoscopy were peptic acid disease (69.3%), dyspepsia (26.2%), and upper GI bleeding (4.5%). Histological results were chronic active gastritis (54.5%), chronic superficial gastritis (38.6%), acute gastritis (1.2%), and normal mucosa (5.7%).

Based on the standard criteria for the diagnosis of *H. pylori* infection (19,20), 51 (57.95%) were positive, of which 26 had a positive culture (51%) and 25 (49%) had simultaneous positive histology and serology. Thirty cases (34.10%) were negative and 7 (7.95%) were classified as indeterminate. The prevalence of *H. pylori* infection was 62.96% (95% CI, 62.28-63.64)

H. pylori was identified in the gastric *corpus* and *antrum* in 17 (66%) of all 26 cases with positive culture, in 5 cases only in the antrum (19%), and in 4 cases only in the gastric corpus (15%). Cultures were reported without growth in 35 cases, and contaminated in 20. *H. pylori* was diagnosed by histopathology in the gastric corpus and/or antrum in 49 patients. Chronic active gastritis was found in 72.5%. Chronic superficial gastritis (64.5%) and chronic active gastritis (19.4%) predominated in those without *H. pylori*. Anti-*H. pylori* IgG determination was positive in 47 cases and negative in 34.

Cut-off point for the simplified breath urea ^{13}C test

The increase of 4.22 δ represented the cut-off point of greatest diagnostic accuracy (Fig. 1), with SE of 90.20% (95% CI, 89.17-91.23), SP of 93.33% (95% CI,

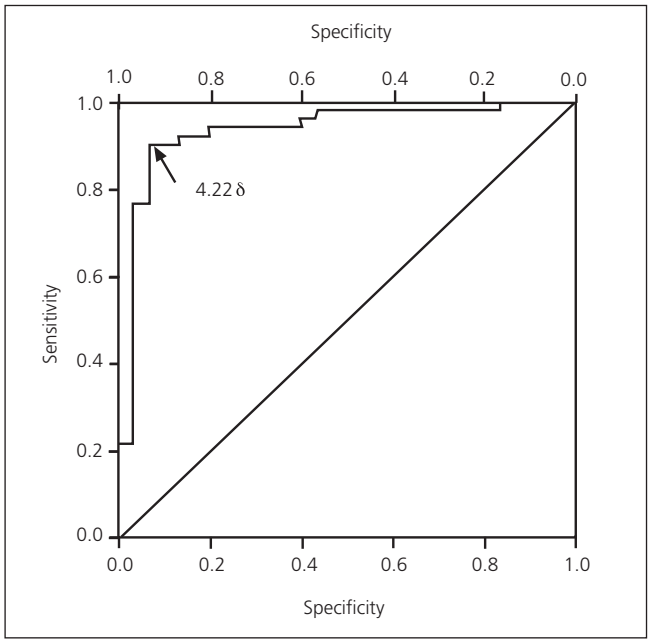

Fig.1. Illustrates the ROC curve with delta values of simplified UBT-^{13}C. The cut-off point with greatest sensitivity (SE) and specificity (SP) corresponded to an increase of ≥ 4.22 δ with SE 90.20% (CI 95%, 89.17-91.23) and SP 93.33% (CI 95%, 91.61-95.05), in relation to ≥ 3.15/≥ 6.60 δ with SE 90.20% (CI 95%, 89.17-91.23)/88.24% (CI 95%, 87.20-89.27) and SE 90.0% (CI 95%, 88.27-91.73)/93.33% (CI 95%, 91.61-95.05), respectively. The area beneath the curve is 93.3% (CI 95%, 87.0-99.6).

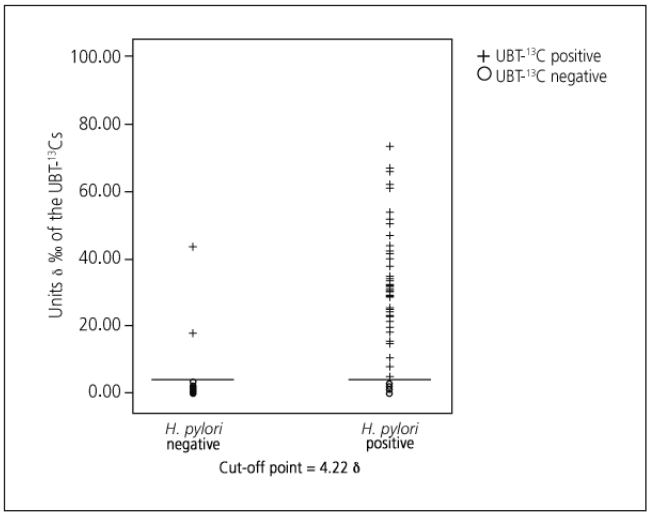

Fig. 2. Illustrates the result in δ obtained with UBT-^{13}C performed in patients with and without *H. pylori* infection. The cross represents a positive result and the circle represents a negative result. The cut-off point used was 4.22 δ, shown as a horizontal line. Median in *H. pylori* negative cases was 0.825 δ (interval 0-43.82) and in *H. pylori* positive cases 28.93 δ (interval 0.16-82.24).

91.61-95.05), PPV of 95.83% (95% CI, 94.76-96.91), NPV of 84.8% (95% CI, 83.3-86.4), positive likelihood ratio of 13.53 (95% CI, 13.42-13.64), and negative likelihood ratio of 0.11 (95% CI, 0.10-0.11), with an accuracy of 91.4%. Five false negative results and two false positive results were found (Table II).

For all 30 cases defined as negative for *H. pylori* according to standard criteria the delta median was 0.825 δ, and 28.93 δ in the 51 positive *H. pylori* cases (Fig. 2). Including the 7 indeterminate cases defined by standard criteria (Table III) and taking as a reference the histopathological study and 4.22 δ as the optimal cut-off point of ^{13}C UBT, the following values were obtained: SE of 91.23% (95% CI, 90.31-92.15), SP of 90.32% (95% CI, 88.65-92.00), PPV of 94.55% (95% CI, 93.6-95.49), NPV of 84.8% (95% CI, 83.3-86.4), and accuracy 90.9%.

The simplified ^{13}C UBT was well tolerated by all patients, and no adverse events were reported.

Table II. Results of diagnostic tests and UBT^{13}C in false negative and false positive subjects

Case No.	*Clinical diagnosis*	*Histological diagnosis*	*Culture*	*Biopsy*	*Serology*	*UBT-^{13}C (δ)*
21603	PAD	GCS, Hp +	Neg	Pos	Pos	Neg (2.02δ)
46703	Dyspepsia	CAG, Hp +	Pos	Pos	Pos	Neg (2.72δ)
48003	UGIB (U.G.)	CAG, Hp +	Neg	Pos	Pos	Neg (0.16δ)
52103	Dyspepsia	CSG	Pos	Neg	Pos	Neg (1.03δ)
54404	PAD	CAG, Hp +	Neg	Pos	Pos	Neg (1.18δ)
23903	PAD	CSAG	Neg	Neg	Neg	Pos (43.82δ)
46103	PAD	CAG, Hp +++	Neg	Pos	Neg	Pos (18.01δ)

PAD: peptic acid disease; IGIBUGIB: upper GI bleeding; GU: gastric ulcer; CSG: chronic superficial gastritis; CAG: chronic active gastritis, CSAG: chronic superficial active gastritis; Hp+: few *Helicobacter pylori* escaso; Hp+++: abundant *Helicobacter pylori*; Neg: negative; Pos: positive; T-^{13}C: breath test with urea-^{13}C.

Table III. Result of UBT-^{13}C in indeterminate cases of *H. pylori* by standard criteria

Case No.	*Clinical diagnosis*	*Culture*	*Biopsy*	*Serology*	*UBT-^{13}C*
22103	Dyspepsia	Contaminated	Pos	Neg	Pos
22503	PAD	Contaminated	Neg	Pos	Pos
22703	PAD	Contaminated	Pos	Neg	Pos
23203	PAD	Contaminated	Pos	Neg	Pos
23803	PAD (GU)	Contaminated	Neg	Pos	Pos
48103	PAD (GU)	Contaminated	Pos	Neg	Pos
54703	PAD	Contaminated	Neg	Pos	Neg

Neg: negative; Pos: positive; UBT-^{13}C: simplified breath test with urea-^{13}C; PAD: peptic acid disease; GU: gastric ulcer.

DISCUSSION

This study confirms the utility of the simplified breath test using smaller doses of urea-^{13}C and citric acid for the diagnosis of *H. pylori* infection, since it preserves the diagnostic accuracy previously reported with higher doses (SE 90.20% and SP 93.33%) (7-10,14).

Since the original description by Graham et al., who used 350 mg (5 mg/kg) of urea-^{13}C and sampled expired

air every 10 min for 3 h (3), several modifications have been made including the use of different urea doses, changes in the composition of test foods, simplified expired air sampling techniques, and different methods for measuring carbon 13. These modifications have been proposed to simplify and optimize the test with the purpose of decreasing costs and expanding applications (3-15).

In 1991 Logan et al. standardized the breath test by administering 100 ml of a fat-rich beverage and obtaining a breath sample 10 minutes later, after which 100 mg of urea-^{13}C in 50 ml of water were immediately administered. Thirty minutes later a second sample was obtained. This test had a sensitivity of 98% and a specificity of 92% (6). Initially the test food was thought to improve results because it induced delayed gastric emptying, thus increasing the distribution of substrates in the stomach and the duration and contact area of urea and bacteria (6-8). In 1997 the test was validated with 75 mg of urea-^{13}C and 4.5 g of citric acid, with a sensitivity and specificity of 95.6 and 96.6%, respectively (9). The effect of citric acid on CO_2 excretion was attributed to delayed gastric emptying (9,10). However, it has been reported that citric acid can produce an increase in intra-bacterial urease, and depends on the medium pH; recent studies suggest that its effect could also be related to the properties of nickel (22-24). Test validation studies are currently being published using different doses of urea and citric acid, showing that diagnostic accuracy is preserved and costs are reduced by decreasing the dose of substrates (5,8-15).

We validated the test using the simultaneous administration of 50 mg of urea-^{13}C and 2 g of citric acid. The 50 mg of urea-^{13}C dose has been used by different authors with a sensitivity and specificity greater than 90%. In 1999, Graham et al. (24) described that 1, 2 and 4 g of citric acid increased the activity of the *H. pylori* urease with a significant increase of $^{13}CO_2$ in expired air; they suggested that the dose of citric acid could be reduced (> 1 g/200 ml) to simplify and improve the palatability of the test. However, they also commented that further studies were required to support their findings. The dose of citric acid was then reduced to 2 g to improve palatability and patient compliance. Diagnostic accuracy was preserved with these modifications (sensitivity and specificity greater than 90%). Other authors suggested that the test could be performed with lower doses of urea^{13}C (25 mg and 15 mg) (25).

According to ROC curves the cut-off point with the greatest diagnostic accuracy for ^{13}C-UBTs was 4.22‰. Values between 2-5 δ have been suggested by other authors, depending on test conditions and methods (5,6,10,26).

Our results suggest that the simplified breath test using urea-^{13}C with a dose of 50 mg of urea-^{13}C and 2 g of citric acid preserves diagnostic accuracy and is an adequate alternative for the non-invasive diagnosis of *H. pylori* infection.

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