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VALIDATION OF ^{14}C-UREA BREATH TEST FOR DIAGNOSIS OF *Helicobacter pylori*

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SUMMARY

The aim of this study was to validate the ^{14}C-urea breath test for use in diagnosis of *Helicobacter pylori* infection. Thirty *H. pylori* positive patients, based on histologic test and thirty *H. pylori* negative patients by histology and anti-*H. pylori* IgG entered the study. Fasting patients drank 5 μCi of ^{14}C-urea in 20 ml of water. Breath samples were collected at 0, 5, 10, 15, 20 and 30 min. The difference of cpm values between the two groups was significant at all the time intervals, besides time 0 ($p<0.0001$). At 20 min, the test gave 100% sensitivity and specificity with a cut-off value of 562 cpm. Females were higher expirers than males ($p=0.005$). ^{14}C-urea breath test is highly accurate for *Helicobacter pylori* diagnosis. It is fast, simple and should be the non-invasive test used after treating *Helicobacter pylori* infection.

KEYWORDS: ^{14}C urea breath test; *Helicobacter pylori* diagnosis

INTRODUCTION

After the Australian Easter holiday weekend in 1982 an incubator was opened in the Microbiology Department of the Royal Perth Hospital revealing the first ever culture of a spiral-shaped bacterium from gastric biopsies of patients with gastritis. The discovery of this organism, known as *Helicobacter pylori*, by Drs. Robin Warren and Barry Marshall has revolutionized gastroenterology[7].

Helicobacter pylori is now recognized as the main risk factor in the multifactorial pathogenetic cascade of peptic ulcer disease. Clinical studies continue to provide evidence that ulcer disease can be cured by eradication of this bacterium[9].

The initial diagnostic tests for *H. pylori* relied on gastric mucosal biopsy for urease testing[22], histological examination[16], or culture[4]. There has been great interest in *H. pylori* diagnostic tests based on non-invasive techniques that are inexpensive and sensitive[17].

^{14}C- urea breath test has been considered a very sensitive (97%) and specific (95%) non-invasive test[15]. As *H. pylori* produces large amounts of urease, the enzyme that hydrolyses ^{14}C-urea to form HCO_3^- and NH_4^+, $^{14}CO_2$ is driven towards the mucosa into the bloodstream and expired through the lungs[10].

Most experts agree that the urea breath test will be the test of choice to establish *H. pylori* eradication after a course of appropriate therapy[1]. In the Hospital of Clinics of São Paulo, this was the first time that the ^{14}C-urea breath test (UBT) was performed, so the purpose of this study was to validate UBT and to determine the diagnostic ranges for *H. pylori*-positive and *H. pylori*-negative patients.

MATERIALS AND METHODS

Patients

The study was approved by the Scientific Ethics Committee and the Council of the Department. Symptomatic patients who had been determined to have *H. pylori*, based on histologic findings, rapid urease CLO test and anti-*H. pylori* IgG were studied. These consisted of thirty patients with duodenal ulcer (twelve males and eighteen females) with a mean age of 46 $\pm$ 2 yr. In addition, symptomatic patients were studied who had undergone upper endoscopy but were negative for *H. pylori*, based on histology, rapid urease CLO test and anti-*H. pylori* IgG, or patients who had previously been infected by *H. pylori* but had a successful eradication. There were twenty four patients with duodenal ulcer, two with gastric ulcer, three with dyspepsia and one with esophagitis (fifteen males and fifteen females) with a mean age of 46 $\pm$ 3 yr.

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Histology

Antral biopsies were taken for histological techniques and CLO tests. Histology samples were prepared and stained with hematoxylin and eosin (**H&E**) and with Giemsa stain. *H. pylori* was identified after examination of **H&E** and Giemsa stained biopsy sections by a histopathologist blinded to the UBT and *H. pylori* serology. The discordant CLO test, histology, or breath test patients were submitted to a second blinded histological examination.

H. pylori serology

Sera were assayed for *H. pylori* IgG antibodies using an enzyme immunoassay (EIA- Cobas Core Roche, 2nd generation, France). Incorporation of urease into a well-characterized and highly immunogenic purified *H. pylori*- specific multicomponent antigen preparation, free of cross-reading flagella proteins, forms the basis of this test. In the first step, patient samples and controls were diluted with sample diluent and incubated with beads coated with highly purified *H. pylori* antigens. Specific antibodies were bound to the beads. After removal of the unbound material by a washing step, the antigen-antibody complex on each bead was detected with peroxidase (POD)-conjugated goat anti-human IgG antibody. After removal of unbound conjugate, the beads were incubated with a substrate solution containing tetramethylbenzidine (TMB) and hydrogen peroxide. A blue color developed, the intensity of which was proportional to the amount of *H. pylori*-specific IgG bound to the beads. The enzymatic reaction was stopped by the addition of acid, and absorbance values were determined at 450 nm. For quantitative results a standard curve was obtained by dilution series of the positive control and plotting each absorbance value versus the corresponding standard value. The concentrations of IgG antibody to *H. pylori* in patient samples were determined by interpolation from this standard curve[18].

CLO test

Antral mucosal biopsy specimen was inserted into a homemade urease test tube[4]. Urease reagent was made up to 100 ml with bacto-yeast extract 0.010g, KH_2PO_4 0.0091g, Na_2HPO_4 0.0095g, urea 2g and Phenol red 0.5% 15 drops, the pH of the solution was adjusted to 6.9. It was sterilized by filtration, dispensed into 0.5 ml aliquots and stored –20° C. If the urease enzyme of *H. pylori* was present in the gastric biopsy being tested, the resulting breakdown of urea caused the pH to rise and the color of the solution turned from yellow to bright magenta. The urease test tube was examined over the next 24 h.

^{14}C-urea breath test (UBT)

UBT was performed according to MARSHALL et al.[10] ^{14}C-urea (250 *u*Ci, Amersham) was reconstituted with 25 ml of sterile distilled water. Aliquots of 0.5 ml were frozen at -20° C until ready to use. Subjects were fasted for at least six hours prior to the test. They had to remove false teeth (if present), and cleanse their mouth with antiseptic solution (thymol, salol, menthol, saccharin, fuchsin, water and ethanol) provided by the Pharmacy Division of the Hospital; a baseline breath sample was collected and identified as Time 0. Then, they swallowed 5 *u*Ci of ^{14}C- urea dissolved in 20 ml of water. According to MARSHALL et al.[10], when ^{14}C-urea solution was given as a mouthwash, caused an immediate peak of $^{14}CO_2$ excretion (urease from commensal mouth organisms) in a two min breath sample, reaching baseline levels after 12 min. However, when was given directly into the esophagus in **Hp-** subjects, isotope excretion did not increase more than 50 cpm above the baseline value. Therefore, patients rinsed their mouth again two min after they had swallowed ^{14}C-urea, spitting the water out and making sure that none was ingested. Breath samples were collected at 5, 10, 15, 20 and 30 min. Patients were instructed to blow through tubing attached to a safety trap into a scintillation vial containing 2.5 ml of 400 mM Hyamine (Sigma) in methanol with 15 mg/l thymolphthalein (blue alkaline color). They had to blow until the solution became colorless indicating the collection of 1 mmol of CO_2. Once the breath samples had been collected, scintillation fluid (10 ml- 5.5 g PPO/ 0.2g POPOP of 2:1 v/v Toluene/Triton-X) was added to the vial; counting proceeded for 5 min per vial, and the results were expressed as cpm/mmol CO_2. Counting efficiency of the Beckman LS 100C was 93%. Patients were not allowed to take antibiotics or bismuth-containing compounds within 30 days before the test and proton pump inhibitors, sucralfate or H2-RAs within a minimum of 7 days before the test.

Determination of *H. pylori* status

Patients were considered *H. pylori* negative (**Hp -**), if histology and anti-*H. pylori* IgG were negative. Patients *H. pylori* positive (**Hp +**) were those with positive histology. The CLO test was used to provide corroborative evidence of *H. pylori* status.

Statistical analysis

The results were analyzed by GraphPad prism software version 2.0.

RESULTS

Breath samples were collected at 0, 5, 10, 15, 20 and 30 min after 5 *u*Ci of ^{14}C-urea had been swallowed by twenty seven **Hp -** patients and twenty two **Hp +** patients. With the exception of time 0, at all other times of breath collection there was a striking difference of $^{14}CO_2$ excretion between the two groups ($p < 0.0001$, Fig. 1).

Accuracy analysis of UBT was shown in Table 1. Cut-off value (Mean + 3 SD) was established at all the time points, except at time 0. The specificity of the test was the percent of **Hp -** patients presenting $^{14}CO_2$ excretion below the cut-off value and the sensitivity of the test was the percent of **Hp +** patients with $^{14}CO_2$ excretion above the normal range. The specificity of the test was 96% at all the times, except at 10 and 20 min when it was 100%; the sensitivity of the test was 59% at 5 min and 100% at all other times of breath collection.

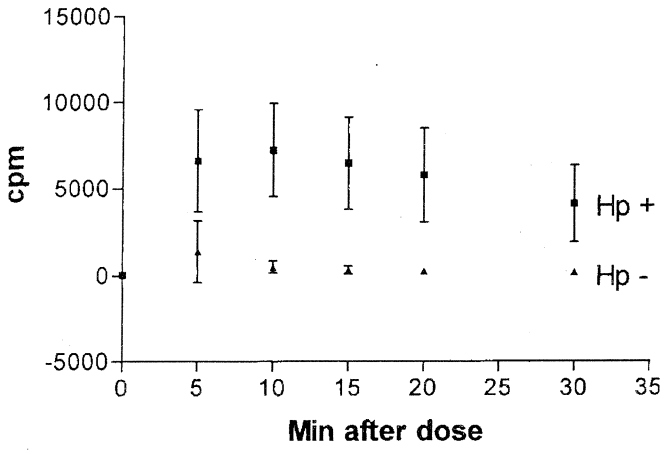

Fig. 1- $^{14}CO_2$ excretion for patients who were **Hp +** and **Hp-** . Graph shows means and bars represent SD of breath samples collected at 0, 5, 10, 15, 20 and 30 min after 5 uCi of ^{14}C-urea intake. Values are presented as cpm.

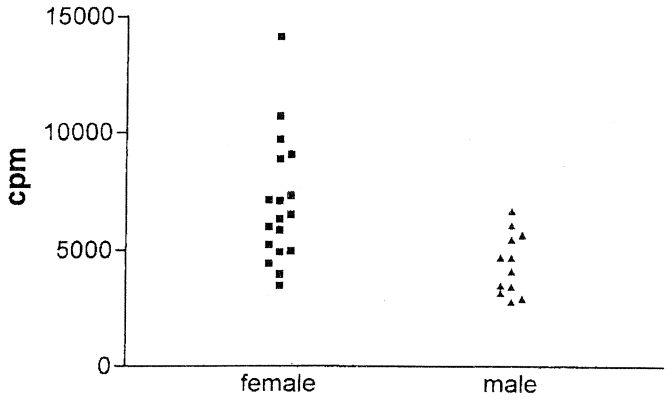

Fig. 3- ^{14}C-urea breath test from 30 **Hp +** patients, 18 females and 12 males, after 20 min of ^{14}C-urea intake. Each dot represents a single value in cpm.

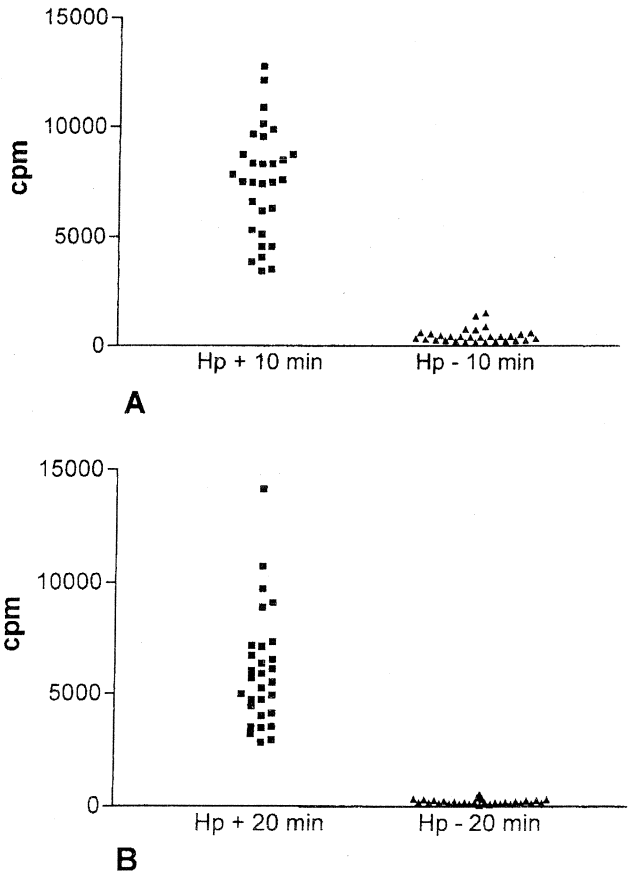

Fig. 2- ^{14}C-urea breath tests from 30 **Hp+** patients and 30 **Hp -** patients after 10 min (**A**) and 20 min (**B**) of 5uCi of ^{14}C-urea intake. Each dot represents a single value in cpm.

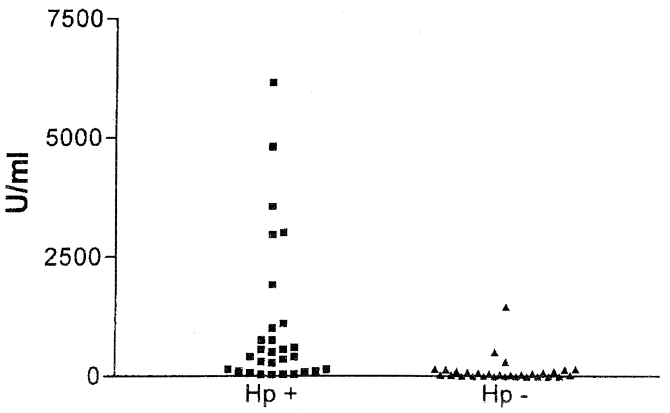

Fig. 4- Concentration of IgG anti-*H. pylori* (U/ml) in **Hp +** and **Hp -** patients. Each dot represents a single value.

As the specificity of the test was 100% at 10 and 20 min, sixty patients (thirty **Hp -** and thirty **Hp +**) entered the study to establish the best time for breath collection. The differences between **Hp -** and **Hp +** were larger at 20 min (Fig.2), consequently this was the time point found as the best for breath collection with a cut-off of 562 cpm.

Females **Hp +** were higher expirers than males at 20 min, this difference was significant by t test (p=0.0051, Fig.3). **Hp +** patients had higher concentrations of antibodies IgG anti-*H. pylori* than **Hp -** patients (p=0.0019 by t test, Fig. 4).

TABLE 1
Accuracy of ^{14}C-urea breath test

PARAMETER	Time (min)				
	5	**10**	**15**	**20**	**30**
M± SD Hp -	1368± 1764	497± 343	292±233	229±111	178±113
M ± SD Hp +	6639± 2881	7199±2624	6402± 2616	5728± 2638	4081±2168
Cut-off value	6660	1526	991	562	517
Sensitivity	59%	100%	100%	100%	100%
Specificity	96%	100%	96%	100%	96%

DISCUSSION

Thirty **Hp +** patients and thirty **Hp -** patients entered the study to validate ^{14}C-urea breath test. Breath was collected at 0, 5, 10, 15, 20 and 30 min, and unexpectedly the difference between the two groups was significant ($p<0.0001$) at all these time points, except time 0. The sensitivity and specificity of the assay at 20 min was 100%; therefore, as was described previously[1, 10], this was the time of choice for breath collection after 5 *u*Ci of ^{14}C-urea intake. When a capsule containing ^{14}C-urea (1 *u*Ci) was given, a single time point of 10 min was chosen for accuracy analysis[15]; nonetheless, in other authors' [1] experiments the 20-min sample gave the most consistent and reproducible data.

According to MARSHALL et al.[10], counting until 10 min corresponded to bacterial urease of the oropharynx, therefore it is important to perform a mouthwash before taking ^{14}C-urea, cleansing the mouth again 2 min later and spitting the water out. Even though at 5 and 10 min, most probably there was an addition of the mouth bacteria and *Helicobacter pylori* urease activities, the difference between the **Hp -** and **Hp +** groups was too significant ($p<0.0001$) to consider the possibility of false positive results. In **Hp-** patients at the 10 min the level of $^{14}CO_2$ started to fall reaching almost baseline values at 30 min, however in **Hp+** patients, the $^{14}CO_2$ count was still high at this time point.

We obtained a cut-off value of 562 cpm at 20 min, different from MARSHALL et al.[10] which was 928 cpm. Sensitivity and specificity depend on the chosen cut-off point[11]; ours gave 100% of sensitivity and specificity.

The study of metabolic fate of the radioactive carbon in the ^{14}C-urea breath test showed that the mean combined urinary and breath recovery for high expirers in 72 h was 86% (SD=7%) and for low expirers it was 97% (SD=3%), thus the long-term retention of ^{14}C was low[12], consequently the radiation exposure was trivial[10,15].

Females **Hp +** were higher expirers than males at 20 min, this difference was significant ($p=0.005$), authors[10] that had the same result accounted for the lower endogenous $^{12}CO_2$ production by females. Furthermore, there was a significant correlation between ^{14}C-urea breath test result and the number of *H. pylori* colonies in culture[2,17] . In addition, a direct correlation between the ^{13}C-urea breath test values and the density of *Helicobacter pylori* in antrum biopsies was seen[20, 21].

We hypothesize that there is a direct correlation between cpm values of ^{14}C-urea breath test and the density of *Helicobacter pylori* on the gastric mucosa, and females have a higher number of bacteria, perhaps explaining their worse response to treatment. However, we are planning to prove whether this hypothesis is correct or incorrect in the near future. If this was true, ^{14}C-urea breath test could predict treatment success. Otherwise, the percentage of women in therapeutic failures was greater than expected as they harbored metronidazole-resistant strains more than twice as often as men. The explanation for this was the previous use of antibiotics for gynecologic complaints[3,19].

Serology is of limited use, in that it is only good for the diagnosis of pre-treatment patients, as antibodies do not fall immediately after *H. pylori* has been eradicated. In the present study, even though many patients of **Hp -** group were previously infected, the concentration of antibodies in this group was lower than of **Hp+** group ($p<0.0001$). Therefore, sequential serological samples could be used to determine if *H. pylori* has been eradicated.

The original version of the urea breath test was reported by GRAHAM et al.[5] with the stable isotope ^{13}C that can be used in children and pregnant women. Otherwise, ^{13}C-urea breath test requires patients to drink a test meal (50 ml) to delay gastric emptying, holding the isotopic urea in the stomach, also a relatively large dose of ^{13}C-urea substrate is necessary to measurably increase $^{13}CO_2$ excretion[8]. The ^{14}C isotope is cheaper and more sensitive[6], as it does not require a test meal, allowing ^{14}C-urea to be fully exposed to gastric mucosal urease[10].

In nature, ^{14}C is produced by the collision of cosmic radiation and nitrogen, therefore, a small fraction is present in the air, being 1: 10^{12} the proportion of ^{14}C to ^{12}C. Plants, human tissues and bones, normally have ^{14}C in such a way to allow archeologists to identify the age of fossils by measuring this long living isotope in bones[13].

In conclusion, the ^{14}C-urea breath test should be the non-invasive test for choice for *H. pylori* infection diagnosis. It is highly sensitive and specific at 20 min of breath collection, and is also fast, simple and inexpensive.

RESUMO

Validação do teste expiratório com ^{14}C-uréia para o diagnóstico do *Helicobacter pylori*

O teste expiratório com ^{14}C-uréia diagnostica infecção pelo *Helicobacter pylori* no estômago. A urease, produzida por esta bactéria, quebra a ^{14}C-uréia, resultando em HCO_3^- e NH_4^+, sendo expirado $^{14}CO_2$ pelos pulmões e quantificado, então, por contador Beta. O objetivo do nosso estudo foi validar o teste expiratório com ^{14}C-uréia, determinando o tempo ideal de coleta da amostra e o valor de corte. Foi definido o tempo de 20 min para coleta do exame após a ingestão de 5 μCi de ^{14}C-uréia com valor de corte de 562 cpm. A sensibilidade e a especificidade do teste foram de 100% aos 20 min. As diferenças de contagens entre os grupos *Helicobacter pylori* negativos e *Helicobacter pylori* positivos foram estatisticamente significativas ($p<0,0001$). As pacientes do sexo feminino, aos 20 min apresentaram contagens superiores às dos pacientes do sexo masculino ($p=0,005$). É preciso verificar se as mulheres têm maior densidade bacteriana, ou se é pelo fato dos homens expirarem mais CO_2, diluindo a amostra. O teste expiratório com ^{14}C-uréia é método diagnóstico não invasivo, muito sensível, específico e barato.

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