The European ^{13}C-urea breath test for the detection of *Helicobacter pylori*

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As there are no generally agreed standard methods for the detection of *Helicobacter pylori*, a standardized protocol for the ^{13}C-urea breath test (^{13}C-UBT) was proposed and evaluated. A novel 100 ml liquid fatty test meal to maximally delay gastric emptying was taken 10 min before ^{13}C-urea (100 mg in 50 ml of water). Two-litre expired breath samples were collected, one every 5 min, from 10 min (t = 10) up until 40 min (t = 40) (after ingestion of the ^{13}C-urea in water) into a large reservoir collecting bag, from which at the end of the test a single 20 ml sample was taken for analysis by mass spectrometry. In addition, a single point sample was taken directly into a vacutainer 30 min after ^{13}C-urea ingestion (t = 30). The results were compared with antral histology (two biopsies haematoxylin and eosin and Gimenez method), culture (two biopsies, microaerophobic culture using Oxoid SR147), CLO-test and enzyme linked immunosorbent assay (ELISA) serology using double antigen (120 kD and ultracentrifuged cell sonicate) coated wells. The 'gold standard' was defined by the result of any two of the biopsy based methods. *H. pylori* was detected by the 'gold standard' in 149 out of 195 (76%) patients undergoing routine upper gastrointestinal endoscopy. The ^{13}C-UBT has a specificity of 98%, with a sensitivity of 99%, in comparison with ELISA serology (83%, 95%), CLO-test (100%, 92%), histology (97%, 95%) and culture (98%, 83%), respectively.The new European standard method for the ^{13}C-UBT is as good as, and may be better than, other techniques for the detection of *H. pylori*.

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Introduction

Helicobacter pylori is now recognized as the major cause of non-autoimmune gastritis [1] and is an important factor in the aetiology of duodenal ulceration [2]. Routine detection of *H. pylori* is usually performed by histological or bacteriological examination of endoscopic antral biopsies [3]. Biochemical urease biopsy tests are also available either as commercial (CLO-test) or in-house methods [4].

H. pylori may be detected non-invasively by either urea breath test or serology. Enzyme linked immunosorbent assay (ELISA) serology, although well established as a technique [5], detects *H. pylori* indirectly and does not measure the extent or activity of *H. pylori* infection. In contrast the ^{13}C-UBT, a direct and semi-quantitative test for *H. pylori*, is an ideal method for rapidly assessing response to treatment and for serial tests and epidemiological studies.

Since the original description of the ^{13}C-UBT [6], several alternative but similar methods have been reported [7–10]. These have used different test meals, doses of ^{13}C-urea, collection techniques and different ways of expressing results. Adoption of a standard method for the ^{13}C-UBT would allow direct comparisons of results from different studies to be made, as well as facilitating the running of multicentre clinical trials. The aim of the study was to evaluate such a stan-

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dard method, as proposed by some leading European centres with previous experience of the technique for the ^{13}C-UBT.

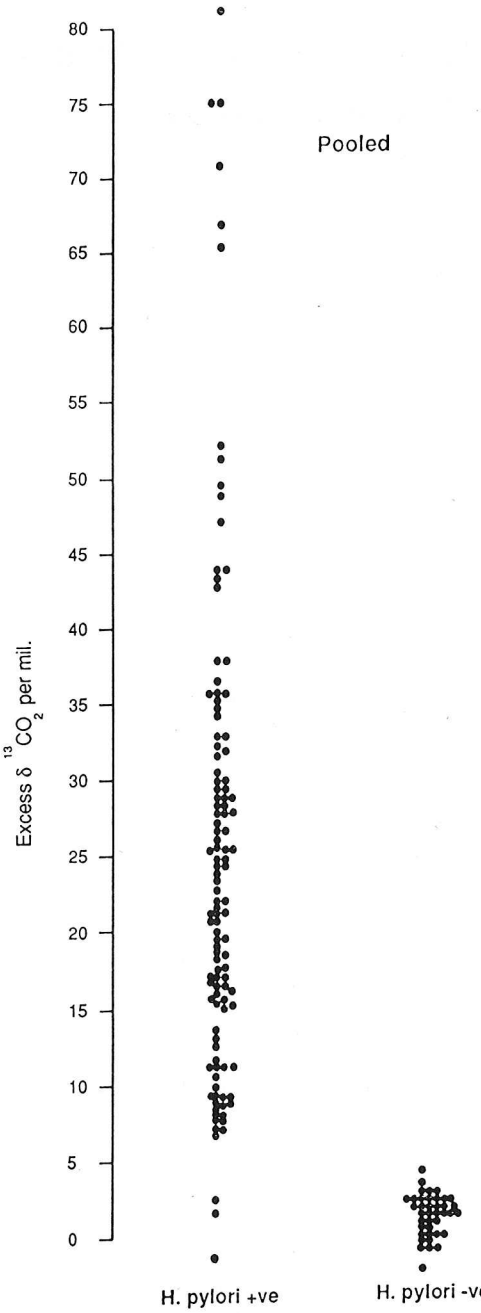

Fig. 1. Discrimination of the European Standard ^{13}C-UBT using the 'pooled' technique for breath collection. Results are expressed as excess $\delta^{13}CO_2$ excretion per mil of exhaled breath by subtraction of the baseline pre-^{13}C-urea breath result.

Patients and methods

Patients attending for routine upper gastrointestinal endoscopy were invited to enter the study, which had the approval of the local ethical committees of the participating centres. The patients gave written informed consent. Height, weight, occupation, indication for endoscopy, current and recent medication were recorded. Exclusion criteria included previous gastric surgery, known bleeding diathesis, use of oral anticoagulant medication or recent (within 2 months) bismuth compounds or antibiotic combinations known to be active against *H. pylori*.

Endoscopy

Endoscopy was performed with intravenous sedation (midazolam or diazepam) and endoscopic appearances recorded. Five biopsies were taken from the antrum for the detection of *H. pylori*. The endoscopes were sterilized after each examination according to local standard guidelines; the biopsy forceps were sterilized by autoclaving. At endoscopy 10 ml of blood was taken and the serum stored at −70°C.

^{13}C-urea breath test

The ^{13}C-UBT was performed within 2 days of the endoscopy in all patients recruited into the study. To determine the reproducibility of the test, 12 patients had two further tests within 5 days of the first.

Method

A baseline exhaled breath sample was taken using a disposable plastic straw, the tip of which was approximately 1 cm from the bottom of an opened 20 ml vacutainer. Patients gently exhaled, displacing the air in the vacutainer, until condensation appeared in the tip of the vacutainer. The straw was removed immediately and the vacutainer resealed as described previously [8].

Patients were then given a fatty test meal consisting of 76% lipid, (57% oleic and 23% palmitic fatty acids), 19% carbohydrate and 5% protein to maximally delay gastric emptying. Ten minutes later they drank 100 mg ^{13}C-urea dissolved in 50 ml of water, (time zero). Distribution of the ^{13}C-urea within the stomach was aided by turning the patients on to each side for 2 min.

Pooled breath was collected into a small collecting bag, as described previously [9], every 5 min from t = 10 to t = 40 with two-litre expired breath samples. The entire contents of the small bag were repeatedly expelled into a large reservoir bag, from which at the end of the test a 20 ml sample was taken for analysis. For comparison a single point breath sample was taken, 30 min after ingesting the ^{13}C-urea, (t - 30) in an identical manner to the baseline sample.

All samples were taken in duplicate and the ratio (δ) of $^{13}C:^{12}C$, expressed as $\delta^{13}CO_2$ (per mil), in the expired breath measured by ratio mass spectrometry. Results were expressed as excess $\delta^{13}CO_2$ excretion per mil by subtraction of the baseline pre-^{13}C-urea breath sample result.

Histology

Two antral biopsies were place in formalin 10% before routine processing to paraffin wax and staining with haematoxylin and eosin and by the Gimenez technique [3]. Every biopsy was examined by the same experienced histopathologist (M.M.W.) without knowledge of the results of the other tests. The gastritis was classified according to the Whitehead system [11] and the number of *H. pylori* assessed and scored as 0 (none), 1+ (few), 2+ (moderate), 3+ (many) and 4+ (teeming).

Culture

Two antral biopsies were placed in normal saline before prompt culture using non-selective and selective media (Oxoid SR147). Plates were incubated microaerophobically for up to 7 days. *H. pylori* was identified by typical colony appearance and positive oxidase and urease tests. The growth of the colonies was graded as 0 (none), 1+ (few), 2+ (moderate) or 3+ (many).

Table 1. Comparison of sensitivity and specificity of the methods of detection of *H. pylori*.

Method of detection	Number	Sensitivity (%)	Specificity (%)
^{13}C-UBT			
(pooled)	195	99	98
(t = 30)	167	98	92
Histology	195	95	97
Culture	195	83	98
CLO-test	190	92	100
Serology			
high 'cut-off'	156	91	87
low 'cut-off'	156	98	80

Biopsy urease test

One antral biopsy was processed using a biopsy urease test (CLO-test, Delta West Ltd, Western Australia), the colour change at 24 h was recorded.

Serology

At endoscopy 10 ml of clotted blood was taken, spun down, and serum stored at −20°C for serology. Frozen samples were transported to Austria where they were also kept at −20°C. Serology was performed by ELISA with double coated antigen wells using the 120 kD and ultrasonicated antigens as described previously [12]. Results were expressed as reciprocal titres with the sensitivity and specificity calculated for the upper (1500) and lower (500) limits of the 'cut off' determined by previous studies.

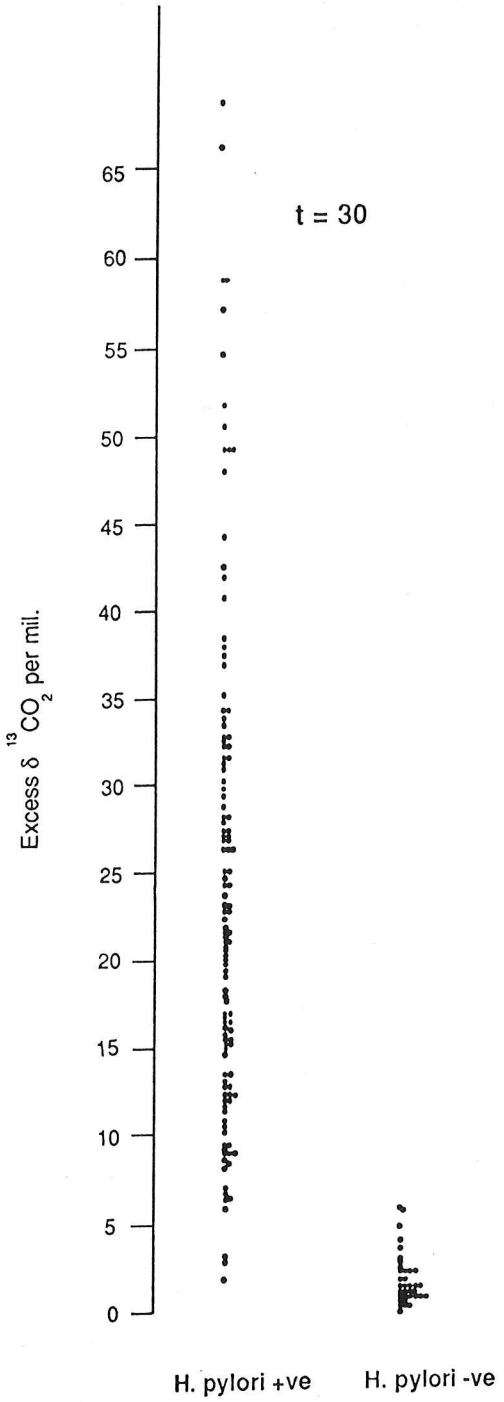

Fig. 2. Discrimination of the European Standard ^{13}C-UBT using a single sample at t = 30 for breath collection. Results are expressed as excess $\delta^{13}CO_2$ excretion per mil of exhaled breath by subtraction of the baseline pre-^{13}C-urea breath result.

For this study to overcome the inherent sampling error of biopsy based methods the 'gold standard' was taken as the result of any two of the three biopsy based tests. A normal range for the ^{13}C-UBT (taken as ± 3 s.d. from the mean) was defined for those patients without

evidence of *H. pylori* on histology, culture and CLO-test.

Analysis of results

All procedures were done by the operators without the knowledge of the patients' *H. pylori* status by other methods. The variables recorded were subjected to statistical correlation and multivariate analysis (SAS). Reproducibility was determined by calculating the co-efficient of variation within subjects using an analysis of variance.

Results

One hundred and ninety-five patients (160 men), mean age 41 years, (range 14–82), were recruited into the study. Indications for endoscopy included epigastric pain (53%), previous duodenal ulcer (22%) and abdominal pain (15%). The most frequent endoscopic findings were duodenal ulcer (31%), gastritis (20%), normal (18%) and duodenitis/erosions (16%).

H. pylori was detected by the 'gold standard' in 149 out of the 195 patients (76%).

^{13}C-urea breath test

In the 46 patients without *H. pylori* as defined by the gold standard, the ^{13}C-UBT had a mean exhaled excess $\delta^{13}CO_2$ of 1.3 per ml (standard deviation 1.3) which at ± 3 s.d. gave an upper limit of excess $\delta^{13}CO_2$ excretion of 5 per ml. This was taken as the upper limit of normal (*H. pylori* negative) patients.

Pooled breath collection

The pooled ^{13}C-UBT correctly identified 147 of the 149 patients positive for *H. pylori* by the 'gold standard' (sensitivity 99%, specificity 98%). In those patients with a positive breath test, the median excess $\delta^{13}CO_2$ excretion in the expired breath for the pooled method was 26.8 per ml (range 6.5–86.5 per mil, Fig. 1). The pooled method gave three false negative results.

Single t = 30 breath collection

The single sample breath (166 tests carried out) collection gave similar results to the pooled method (Fig. 2, Table 1), but there were two false negatives (both of whom had a positive pooled breath test) and three false positives (sensitivity 98%, specificity 92%).

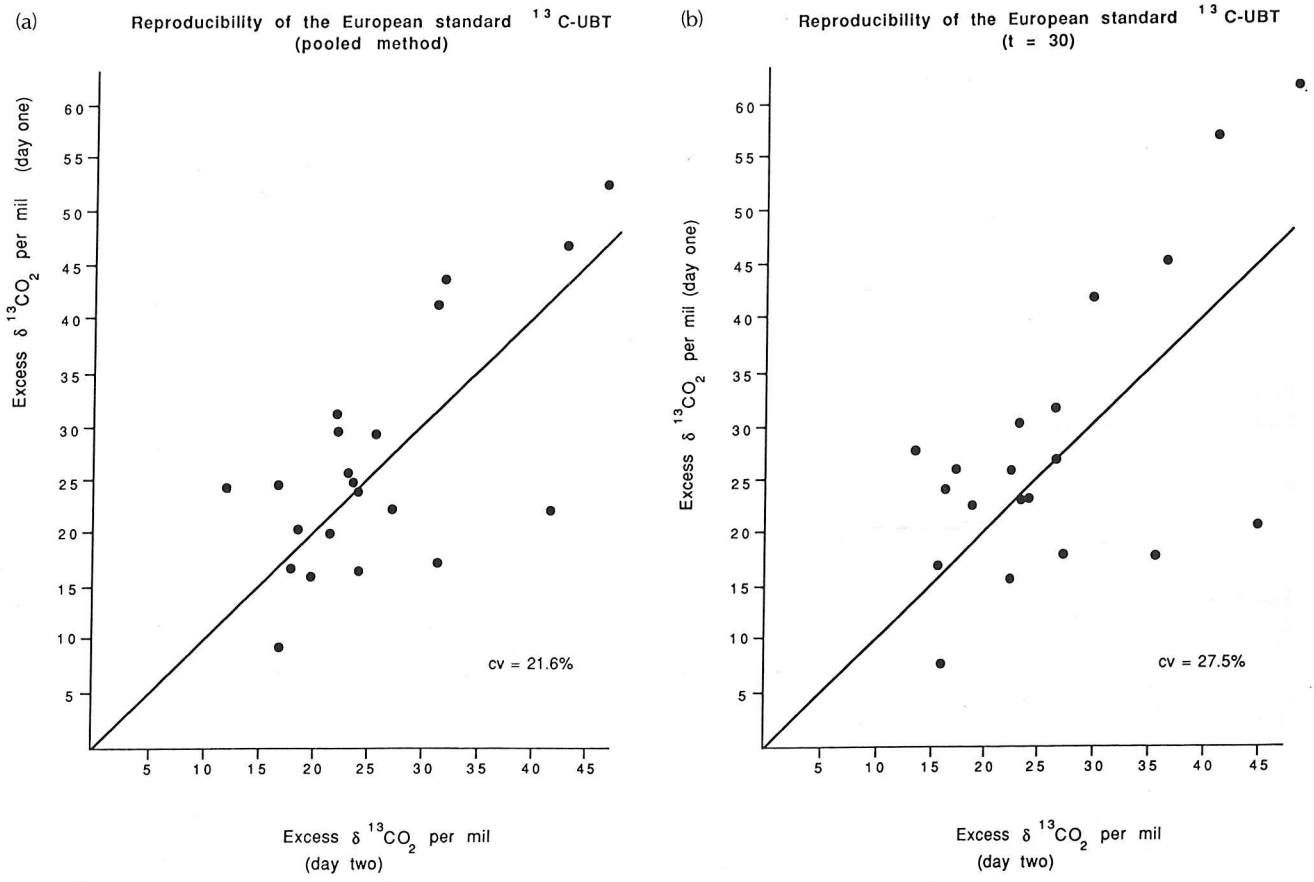

Fig. 3. (a) and (b) Scattergrams showing the reproducibility of the European standard ^{13}C-UBT for the pooled and single sample techniques for breath collection. Coefficients of variation (cv) are also given.

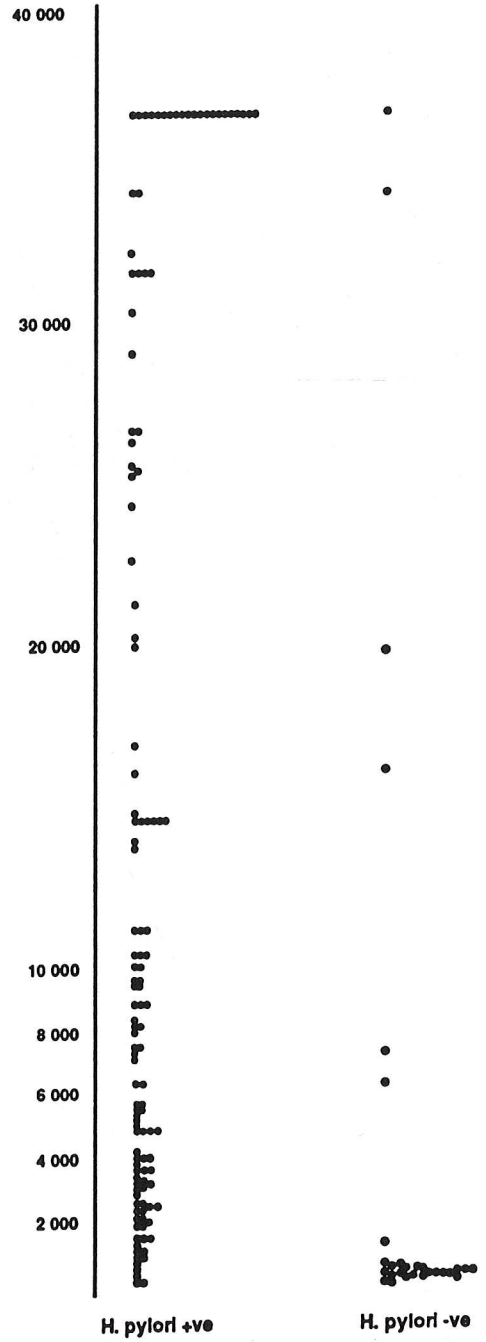

Fig. 4. Discrimination of ELISA serology using the combined 120 kD and ultrasonicated antigens [12]. Results are expressed as reciprocal antibody titres.

There was no correlation between the breath test results, symptoms or appearances at endoscopy with either technique.

Reproducibility of the ^{13}C-UBT

The pooled method for breath collection was more reproducible than the single sample method (coefficient of variation 22% n = 11, F = 8.2, $P<0.0001$ and 28% n = 11, F = 5.4, $P<0.0006$, respectively). A scattergram demonstrating the reproducibility of the two methods is shown (Fig. 3a and 3b).

Histology

H. pylori was correctly identified by antral histology in 140 patients (sensitivity 95%), but gave two false positive results (specificity 97%), when compared with the 'gold standard'. All the patients with *H. pylori* had gastritis, but the grade and type of gastritis was not related to the excess $^{13}CO_2$ excretion. In those without *H. pylori* antral histology was either normal (35%), or showed mild chronic (25%) or chronic atrophic (25%) gastritis.

One of the false positive histology results was in a patient with a previous history of duodenal ulcer disease. She was negative for *H. pylori* according to a CLO-test and culture, and with a ^{13}C-UBT excess $\delta^{13}CO_2$ 4.1 per ml, but scanty organisms and mild active gastritis were present on antral histology. Four months later the ^{13}C-UBT result was 15.6 excess $\delta^{13}CO_2$ per mil, with confirmatory histology, culture and CLO-test and a recurrent duodenal ulcer.

Microbiology

H. pylori was correctly identified by culture in 123 patients. There was one false positive result (specificity 98%, sensitivity 83%) which was the results of the definition of the 'gold standard' in this study (histology and CLO-test were negative but culture and ^{13}C-UBT were positive).

Biopsy urease test

CLO-test was positive in 137 patients (sensitivity 92%), with no false positive results (specificity 100%).

Serology

The double coated antigen ELISA results were calculated using a high and low cut-off. For a reciprocal antibody titre of 1500 the sensitivity and specificity were 91% and 87%, respectively, whilst for a reciprocal antibody titre of 500, they were 98% and 80%, respectively (Fig. 4).

Discussion

The ^{13}C-UBT for the detection of *H. pylori* infection of the gastric mucosa has many potential advantages over other methods and may be the ideal method for monitoring and following the response to treatment and for epidemiological studies.

The standard protocol for the ^{13}C-UBT used in this study is derived from the previously described methods. Four components to the breath test need to be considered: test meal, isotope, breath collection and expression of results.

It is well recognized that a test meal increases the discrimination of the ^{13}C-UBT [13] (theoretically by in-

creasing the residence time of ^{13}C-urea in the stomach). However, the components (content and form) of the chosen meals has never been validated. For the present study the composition of the meal were chosen to maximize the inhibition of gastric emptying and at the same time minimize the $^{13}CO_2$ release by small bowel bacterial digestion of carbohydrate, a potential problem with previous test meals. Measurement of the inhibition of gastric motor function by the test meal used in this study has shown a lag phase before the start of gastric emptying of at least 30 min [14]. Because of the high fat content of the meal it should eventually be possible to manufacture a solid 'chocolate bar' meal, useful in the production and distribution of ^{13}C-UBT kits.

Different doses of isotope have been used previously, with no apparent detrimental effect on the sensitivity, or specificity of the test; indeed even doses lower than those used in our protocol have been used successfully [8]. However, if the ^{13}C-UBT is to be an indicator of total urease activity *in vivo*, then it is important to ensure that the concentration of ^{13}C-urea within the stomach is near or slightly above the optimum for the *H. pylori* urease.

Previously the ^{13}C-UBT has relied upon serial sample breath collection and the construction of excretion curves for each patient. However, analysis of up to 10 samples of $^{13}CO_2$ by ratio mass spectrometry is expensive and limits the use of the ^{13}C-UBT as a method for the detection of *H. pylori*. To overcome this major drawback two simpler and cheaper methods of breath collection were assessed. First, the quantitative pooled collection of breath samples averages the excretion of exhaled $^{13}CO_2$ over the collection period and is known to approximate to the area under the curve [9]; the second method depends on a single diagnostic sample taken 30 min after the isotope has been ingested. In the present study both methods have been shown to have a similar high sensitivity and specificity, but the single 30-min sample was less reproducible than the pooled technique (Fig. 3a and 3b). The 30-min sample should therefore not be used for research when repeat serial assessments of *H. pylori* status are required, but because of its simplicity it is ideal for routine clinical use, when only pre- and posttreatment assessments of *H. pylori* status are required.

The measurement of $^{13}CO_2$ by isotope ratio mass spectrometry is a standard technique with results usually expressed as a ratio of $^{13}C:^{12}C$ parts per thousand relative to CO_2 produced from PDB calcium carbonate [15] which has a ratio of $^{13}C:^{12}C$ close to the natural abundance of ^{13}C (PDB, Belmnitella americana, a mineral from the Pedee formation of South Carolina, USA); baseline samples of $^{13}C:^{12}C$ usually have negative values. With the amount of ^{13}C-urea used in the breath test the ratio of $^{13}C:^{12}C$ relative to PDB is often exceeded in those patients with *H. pylori*, thus giving a positive value. By expressing the results as excess $\delta^{13}CO_2$ excretion (i.e. difference between before and after $\delta^{13}C$-urea) this possible source of confusion, and any variation between mass spectrometers in their standardization with PDB, are avoided.

The ^{13}C-UBT compared favourably with endoscopic biopsy based methods, which have the major drawback of sampling error, because *H. pylori* can be a patchy infection. To detect low levels of infection the 'gold standard' against which the ^{13}C-UBT was compared was taken as the result of two of the three biopsy based methods. In three instances *H. pylori* was detected by only one of the three methods, by histology in two and by culture alone in one, accounting for the specificities of 97% and 98%, respectively. Defining the 'gold standard' as a positive result from any of the biopsy based method as opposed to positive results from two biopsy based methods would have given specificities of 100% for both histology and culture, but sensitivities of only 90% and 88%, respectively.

The sensitivity of the ELISA serology used in this study is similar to that reported previously [12]. None of the patients with false positive serology had taken bismuth compounds or antibiotic regimens known to be active *in vivo* against *H. pylori*. These false positive results suggest that natural clearance of *H. pylori* may occur in some patients, in line with the earlier report from Meyer *et al.* [16]. This important preliminary finding, if confirmed by larger studies on non-selected populations, will lead to a radical re-appraisal of sero-epidemiological studies on *H. pylori* infection.

These results represent the first standardized protocol for the detection of *H. pylori* infection. They demonstrate the inherent advantages of the ^{13}C-UBT over other methods of detection and show it to be a much cheaper and easier method than previous protocols for the ^{13}C-UBT.

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