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RESEARCH PAPER

Excretion kinetics of ^{13}C-urea breath test: influences of endogenous CO_2 production and dose recovery on the diagnostic accuracy of *Helicobacter pylori* infection

Suman Som · Abhijit Maity · Gourab Dutta Banik · Chiranjit Ghosh · Sujit Chaudhuri · Sunil Baran Daschakraborty · Shibendu Ghosh · Manik Pradhan

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Abstract We report for the first time the excretion kinetics of the percentage dose of ^{13}C recovered/h (^{13}C-PDR %/h) and cumulative PDR, i.e. c-PDR (%) to accomplish the highest diagnostic accuracy of the ^{13}C-urea breath test (^{13}C-UBT) for the detection of *Helicobacter pylori* infection without any risk of diagnostic errors using an optical cavity-enhanced integrated cavity output spectroscopy (ICOS) method. An optimal diagnostic cut-off point for the presence of *H. pylori* infection was determined to be c-PDR (%)=1.47 % at 60 min, using the receiver operating characteristic curve (ROC) analysis to overcome the "grey zone" containing false-positive and false-negative results of the ^{13}C-UBT. The present ^{13}C-UBT exhibited 100 % diagnostic sensitivity (true-positive rate) and 100 % specificity (true-negative rate) with an accuracy of 100 % compared with invasive endoscopy and biopsy tests. Our c-PDR (%) methodology also manifested both diagnostic positive and negative predictive values of 100 %, demonstrating excellent diagnostic accuracy. We also observed that the effect of endogenous CO_2 production related to basal metabolic rates in individuals was statistically insignificant (p= 0.78) on the diagnostic accuracy. However, the presence of *H. pylori* infection was indicated by the profound effect of urea hydrolysis rate (UHR). Our findings suggest that the current c-PDR (%) is a valid and sufficiently robust novel approach for an accurate, specific, fast and noninvasive diagnosis of *H. pylori* infection, which could routinely be used for large-scale screening purposes and diagnostic assessment, i.e. for early detection and follow-up of patients.

Keywords Breath analysis · Diagnostics · *Helicobacter pylori* · Urea breath test

Suman Som and Abhijit Maity contributed equally to this work and are considered as joint first authors.

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Introduction

Helicobacter pylori (*H. pylori*) infection is the primary cause of gastritis and peptic ulcer diseases and has been linked to the onset of various critical diseases such as stomach cancer, gastric lymphoma, and adenocarcinoma [1, 2]. It is estimated that more than half of the world's populations harbour *H. pylori* infection, with a prevalence of 80 % or more in the Indian subcontinent [3, 4]. The infection is usually acquired early in life and may remain in the stomach for the rest of the person's life if it is not properly treated [5, 6]. Most individuals harbouring *H. pylori*, however, are usually asymptomatic, and hence, they remain undiagnosed. Therefore, an accurate and

early detection of *H. pylori* infection is vital for initiation of proper treatment.

Currently, the ^{13}C-urea breath test (^{13}C-UBT) is considered to be an effective noninvasive method for detecting *H. pylori* infection by contrast with the direct invasive "gold standard" endoscopy and biopsy-based rapid urease test (RUT) [7, 8]. The ^{13}C-UBT is usually performed by ingestion of a test meal containing 75 mg of ^{13}C-enriched urea with 4 g of citric acid dissolved in 200 mL of water. Initially, a baseline breath (fasting breath) sample is collected, and subsequently, a further breath sample is collected at 30 min following the administration of the substrate. The $^{13}CO_2$ isotopic enrichments in breath samples are usually measured with a high-precision gas chromatography coupled with an isotope ratio mass spectrometer (GC-IRMS). However, the ^{13}C-UBT exploits the large amount of urease enzyme, secreted by *H. pylori* in the stomach, to hydrolyse the orally administered ^{13}C-labelled urea into ammonia and ^{13}C-labelled carbon dioxide. The ^{13}C-urea-derived $^{13}CO_2$ is then transported to the lungs through the bloodstream and is exhaled as $^{13}CO_2$ in the breath samples. The difference of $^{13}CO_2$ concentrations before and after ingestion of the labelled urea, which is reported as the delta-over-baseline (DOB) relative to a standard in per mil (‰), i.e. $\delta_{DOB}{}^{13}C‰$ ($\delta_{DOB}{}^{13}C‰=(\delta^{13}C‰)_{t=t\,min}-(\delta^{13}C‰)_{t=0\ min}$), will be exploited to detect the presence of *H. pylori* infection. Therefore, *H. pylori*-infected individuals will exhibit an increase of $^{13}CO_2$ in their breath after a certain time following ingestion of ^{13}C-enriched urea. However, to our knowledge, the time-dependent excretion patterns of the ^{13}C-UBT and the percentage dose of ^{13}C recovered per hour, i.e. ^{13}C-PDR (%/h) along with cumulative PDR, i.e. c-PDR (%) in exhaled breath samples have not been investigated in detail. The ^{13}C-PDR describes the rate of ^{13}C-enriched substrate that has been exhaled as $^{13}CO_2$ in the exhaled breath, whereas the c-PDR (%) accounts for the total amount of ^{13}C-enriched substrate metabolised at any given time. A complete evaluation of the excretion kinetic profiles, ^{13}C-PDR (%/h) and c-PDR (%) of the ^{13}C-UBT is important to understand the real emptying processes, determine the optimal sampling point, elucidate the effects of urea hydrolysis rate (UHR) and accomplish the highest diagnostic accuracy as well as the best diagnostic cut-off level for broad clinical applicability of the ^{13}C-UBT method for large-scale screening purposes.

Moreover, the effect of endogenous CO_2 production associated with basal metabolic rates (BMR) may have an influence on the diagnostic accuracy of the ^{13}C-UBT. It is expected that endogenous CO_2 production varies along with age (adults>children), weight, height and sex (male>female) [9], and consequently, the DOB values are also expected to vary in accordance with these factors. Yang et al. [5] have recently demonstrated a significant effect of endogenous CO_2 production rates on the ^{13}C-UBT even after the application of urea hydrolysis rate (UHR) in children aged between 7 months and 18 years. However, to our knowledge, no investigations of the effect of endogenous CO_2 production on ^{13}C-UBT have been performed in adults aged 20–75 years until now.

Furthermore, the determination of a precise cut-off value for discriminating between *H. pylori*-positive and *H. pylori*-negative results is still the subject of debate, and subsequently, a wide range of diagnostic cut-off values between 1.3 and 11 ‰ have been suggested in several reports [7, 8]. Sometimes, it is very critical to accurately diagnose if the DOB values are very close to the selected cut-off point or at the borderline, and consequently, the results of ^{13}C-UBT remain questionable and affect the diagnostic accuracy. Some authors have also suggested a narrow spectrum of the DOB values called "grey zone" (2.0 to 5.0‰) of the ^{13}C-UBT in which the ^{13}C-UBT results are inconclusive [8]. Therefore, a DOB value within this region should be cautiously interpreted. This grey zone containing unreliable results accounts for intuitive variations of $^{13}CO_2$ in exhaled breath samples, patient's metabolism and the limits of the analytical precision of $^{13}CO_2$ measurements. Therefore, a comprehensive revaluation of the optimal diagnostic cut-off point is required to validate the widespread clinical implementation of the ^{13}C-UBT in the diagnosis of *H. pylori* infection.

In this article, we first report the time-dependent evaluation of $\delta_{DOB}{}^{13}C‰$ and ^{13}C-PDR (%/h) along with c-PDR in exhaled breath samples after ingestion of ^{13}C-labelled urea for the detection of *H. pylori* infection by means of an optical cavity-enhanced integrated cavity output spectroscopy (ICOS) system. We have employed the cavity-enhanced optical spectroscopy method in the present study because of its fast analysis time (about 30 s) compared to the MS-based method (typically 2 min) [10]. We also investigated the influences of endogenous CO_2 production and urea hydrolysis rate on the diagnostic accuracy of ^{13}C-UBT. Finally, we determined statistically sound several diagnostic parameters such as the optimal diagnostic cut-off value, sensitivity, specificity, accuracy, the risk of false-positive and false-negative results of the ^{13}C-UBT using the ICOS system against the defined gold-standard endoscopic biopsy tests.

Materials and methods

Subjects

Eighty-three subjects (61 male, 22 female, aged 21–72 years with mean age 38.33±11.69 years) with a variety of gastrointestinal disorders such as chronic gastritis, duodenal and gastric ulcer, and non-ulcer dyspepsia were included in the present study (Electronic Supplementary Material (ESM) Table S1). On the basis of gold-standard reports, such as gastrointestinal endoscopy and biopsy-based rapid urease tests (RUTs), subjects were classified into two different groups, as

either *H. pylori* positive or *H. pylori* negative. For this purpose, 34 controls negative for *H. pylori* and 49 patients positive for *H. pylori* were considered for the ^{13}C-UBTs. Subjects who had taken antibiotics, proton-pump inhibitors or bismuth-containing compound prior to 4 weeks of study and subjects having previous gastric surgery were excluded from the study. The current protocol was approved by the Ethics Committee Review Board of AMRI Hospital, Salt Lake, India (Study No. AMRI/ETHICS/2013/1). Informed consent was obtained from each patient prior to enrolment in the study.

Breath sample collection and ^{13}C-UBT

After an overnight fast, subjects performed the ^{13}C-UBT within 1–2 days after endoscopy according to the standard procedure as previously mentioned in several articles [5, 7, 8, 11]. A baseline breath sample was collected in a 750-mL breath collection bag (QT00892, QuinTron Instrument Co., USA). Subjects were first instructed to ingest 200 mL of citric acid solution (4.0-g citric acid in 200-mL water), and then a drink containing 75-mg ^{13}C-labelled Urea (CLM-311-GMP, Cambridge Isotope Laboratories, Inc., USA) dissolved in 50-mL water was orally administered as per the standard procedure. Post-dose breath samples were collected subsequently at 15-min intervals up to 90 min. During breath sample collection, subjects were instructed to hold their breath for 3 s and blow smoothly through a mouth piece directly into the breath collection bag provided. All breath samples were repeated and analysed by the ICOS system as described in the following section. Figure 1 depicts a diagram presenting the steps of the procedure and analytical protocol used in the study.

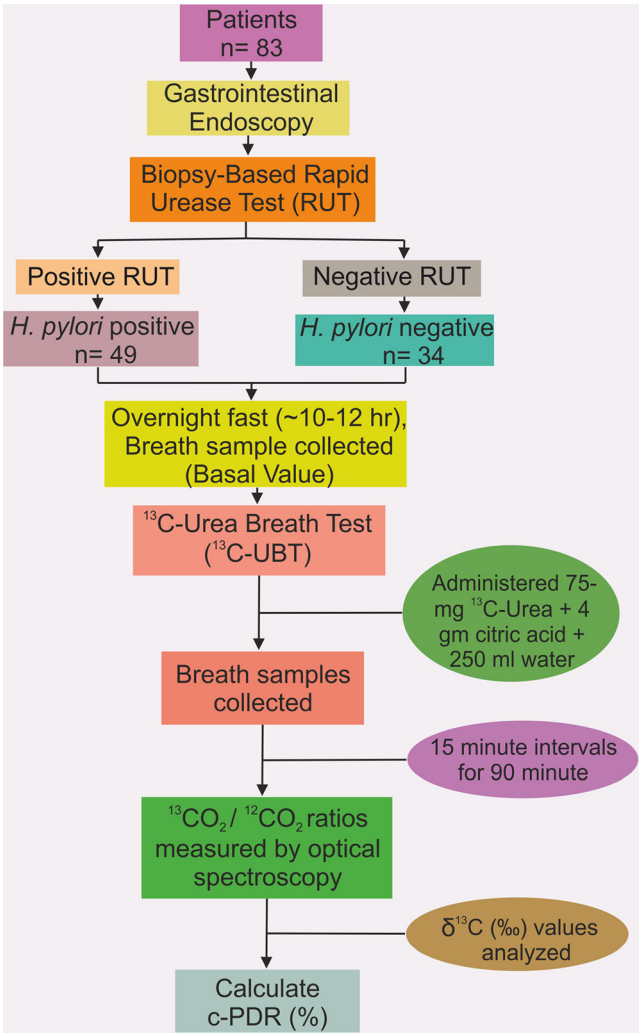

Fig. 1 Flow diagram of the methodology used in this study

Calibration and validation of ^{13}C-UBT by integrated cavity output spectrometer

We have used a high-precision isotopic CO_2 integrated cavity output spectrometer (LGR, Los Gatos Research, CA, USA) to measure simultaneously $^{12}C^{16}O^{16}O$ and $^{13}C^{16}O^{16}O$ in exhaled breath samples for *H. pylori* detection. The details of the instrument and the capability of measuring high-precision $^{13}C/^{12}C$ isotope ratios in CO_2 samples have been described elsewhere [12, 13]. In brief, a high-finesse optical cavity (59 cm long) comprised of two high-reflectivity mirrors (R~ 99.98 %) serves as an absorption measurement cell for breath sample analysis. A temperature-controlled continuous-wave distributed feedback diode laser (*cw*-DFB) operating at ~2.05 μm was coupled into the optical cavity that provided an effective optical path length of 3 km. The laser frequency of the instrument was repeatedly tuned over 20 GHz to scan over the absorption features of $^{12}C^{16}O^{16}O$ and $^{13}C^{16}O^{16}O$ at the wavenumbers of 4,874.448 and 4,874.086 cm^{-1}, respectively, every second. The two absorption features, i.e. R(28) rotational line of the $^{12}C^{16}O^{16}O$ (2,0°,13)←(0,0°,0) band and P(16) rotational line of the $^{13}C^{16}O^{16}O$ (2,0°,12)←(0,0°,0) band used to determine the $^{13}C/^{12}C$ isotope ratio, arise from the $(2\nu_1+\nu_3)$ vibrational combination band of the CO_2 molecule.

The $^{13}CO_2$ enrichment in samples is usually expressed by $\delta^{13}C$ notation in parts per thousand (or per mil, ‰) where, $\delta^{13}C‰ = (R_{sample}/R_{standard} - 1) \times 1{,}000$, where R_{sample} is the $^{13}C/^{12}C$ isotope ratio of the sample and $R_{standard}$ is the international standard Pee Dee Belemnite (PDB) value, i.e. 0.0112372. The accuracy and precision of the ICOS instrument for the $\delta^{13}C‰$ measurements of the $^{13}CO_2$-enriched breath samples were determined by measuring three calibration standards containing 5 % CO_2 in air analysed by IRMS (Cambridge Isotope Laboratory, USA). The $\delta^{13}C$ values of the calibration standards ranged from baseline level (−22.8‰) to high level (−7.33‰) including the mid level (−13.22‰). The measured $\delta^{13}C$ values by ICOS instrument were in excellent quantitative agreement with the values of calibration standards with a precision of ±0.2‰ (ESM Table S2). During

measurements, a 25-mL volume of breath sample was injected into the ICOS cell with a syringe/stopcock. High-purity dry nitrogen (HPNG10-1, F-DGSi SAS, France, purity>99.99 %) was used as the carrier gas for purging the cavity and dilution of breath samples.

Statistical analysis

One-way ANOVA test for parametric variables and the Mann–Whitney test for nonparametric variables were applied to our analysis. A two-sided p value <0.05 was considered statistically significant. A box and whiskers plot was used to illustrate the distribution of $\delta_{DOB}{}^{13}C‰$ in *H. pylori*-positive and *H. pylori*-negative subjects. Finally, a receiver operating characteristic curve (ROC) [14] was generated by plotting the true-positive rate against false-positive rate to determine the optimal diagnostic cut-off value for *H. pylori* infection. All statistical analysis was performed with Origin Pro 8.0 and Analyse-it Method Evaluation version 2.30 (Analyse-it Software Ltd., UK).

Results and discussion

Figure 2 depicts the typical excretion kinetic patterns of $\delta_{DOB}{}^{13}C‰$ values in exhaled breath samples for three *H. pylori*-positive and three *H. pylori*-negative individuals after ingestion of ^{13}C-enriched substrate. It was observed that in *H. pylori*-positive patients, the $\delta_{DOB}{}^{13}C‰$ values in breath samples reached a maximum at around 30 min and then slowly decreased, whereas no significant differences of $\delta_{DOB}{}^{13}C‰$ values in breath samples were observed for the *H. pylori*-negative individuals.

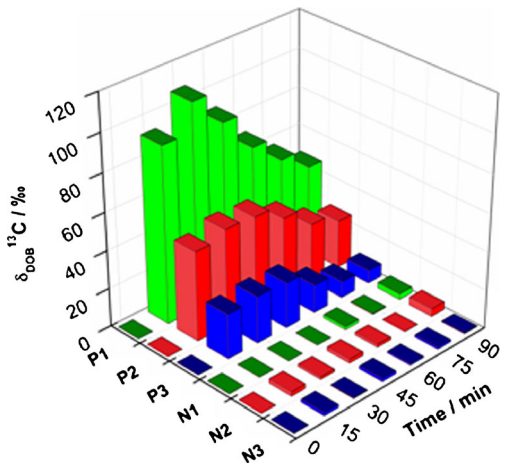

Fig. 2 Typical excretion kinetic profiles of $\delta_{DOB}{}^{13}C‰$ values in exhaled breath samples for three *H. pylori*-positive (*P1*, *P2* and *P3*) and three *H. pylori*-negative (*N1*, *N2* and *N3*) individuals as an illustration

It was previously reported by in vitro biopsies analysis that the internal urease activity of *H. pylori* strongly depends on the medium pH values, and the activity is maximum at a low pH of 3.0 and is minimum at pH 7.0 or 8.0 [15]. When ^{13}C-enriched urea is administered with citric acid, the internal urease activity is stimulated [16] at a pH of <6.5. The activity is increased with acidifications of the medium owing to increased ^{13}C-urea permeability through the urea channel in the inner membrane of *H. pylori*, allowing a large increase in ^{13}C-urea access to intra-bacterial urease enzyme [15, 17]. It was also shown before that the major effect of acidification of the medium occurs within 30 min [15]. Consequently, an increase in the rate and quantity of $\delta_{DOB}{}^{13}C‰$ values in exhaled breath within 30 min is most likely to be the result of increased urease activity, i.e. urease-catalysed hydrolysis of ^{13}C-urea increases and thus results in enrichment of $^{13}CO_2$. The gradual decrease in $\delta_{DOB}{}^{13}C‰$ values in exhaled breath samples later on is a sign of lower urease activity because of alkalinisation of the bacterial environment by production of NH_3. Consequently, the large difference in the $\delta_{DOB}{}^{13}C‰$ values in breath samples demonstrated a clear distinction between *H. pylori*-infected and *H. pylori*-uninfected individuals.

A box and whisker plot of $\delta_{DOB}{}^{13}C‰$ to illustrate the distribution of $^{13}CO_2$ enrichment at 30 min in *H. pylori*-positive and *H. pylori*-negative individuals is shown in Fig. 3. The mean, median and interquartile ranges (IQRs) (i.e. mid-spread of statistical dispersion) for positive and negative patients were 22.66, 12.64 and 4.32‰ to 31.71‰, respectively and 1.44, 1.24 and 0.56‰ to 2.06‰, respectively. It was observed that the median values of $\delta_{DOB}{}^{13}C‰$ increased significantly for the group with *H. pylori*-positive individuals compared to that of the group with *H. pylori*-negative individuals. There was a statistically significant difference between the $\delta_{DOB}{}^{13}C‰$ values (p<0.0001) obtained for the

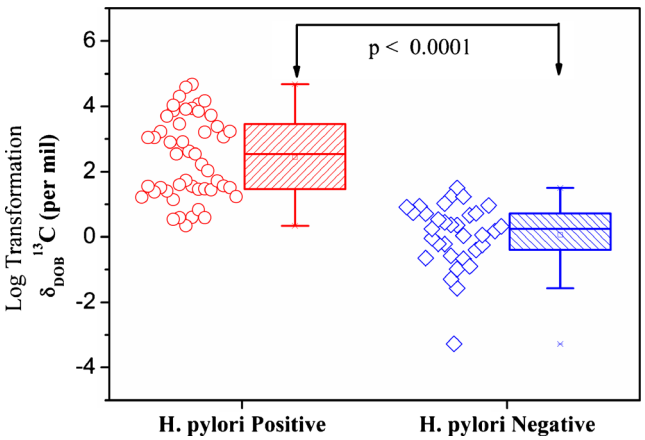

Fig. 3 Box and whiskers plot of $\delta_{DOB}{}^{13}C‰$ illustrating the statistical distribution of $^{13}CO_2$ enrichment at 30 min in *H. pylori*-positive and *H. pylori*-negative individuals. The scattered points represented by *open circle* and *open diamond symbols* correspond to experimental data points obtained by the ICOS instrument

two different types of groups, and therefore, they can be distinguished.

However, to investigate the diagnostic accuracy of the present ^{13}C-UBT for distinguishing *H. pylori*-positive and *H. pylori*-negative individuals, a ROC was constructed by plotting the true-positive rate (sensitivity) against the false-positive rate, i.e. (1-specificity) at 30 min according to the standard procedure as shown in Fig. 4. The sensitivity, specificity and false-positive and false-negative results of ^{13}C-UBT were calculated at various cut-off values (ESM Table S3). An optimal cut-off point for ^{13}C-UBT was defined as the point with the highest sensitivity, specificity and diagnostic accuracy to identify individuals with and without *H. pylori* infection.

The optimal diagnostic cut-off value was determined to be $\delta_{DOB}{}^{13}$C‰ (cut-off)=3.14‰, exhibiting 87.8 % sensitivity (95 % confidence interval (CI) 75.2–95.4) and 91.2 % specificity (95 % CI 76.3–98.1) with an accuracy of 89.16 %. The calculated cut-off value as determined by the ROC analysis correlated well with cut-off values of between 2 and 5‰ mentioned in the grey zone [7, 8]. The area under the ROC curve referred to as AUC was also determined to be 0.95 (95 % CI 91.0–99.0). At a cut-off value of 3.14‰, it was therefore possible to correctly diagnose 43 of 49 patients as positive (i.e. 6 false-negative patients) and 31 of 34 patients as negative (i.e. 3 false-positive patients). It is noted that when the DOB value is close to the cut-off point and the cut-off point is within the grey zone, the risk of a false-positive or false-negative response of the ^{13}C-UBT is extremely high. It should be emphasised here that the grey zone of the ^{13}C-UBT was not hitherto well addressed. The evaluation of the grey zone is very important for the early diagnosis of *H. pylori* infection because many individuals harbouring the infection fall in this region. In this study, we therefore, have explored a possible way to overcome the grey zone by exploiting the time-dependent evaluation of $\delta_{DOB}{}^{13}$C‰ values in the exhaled breath samples.

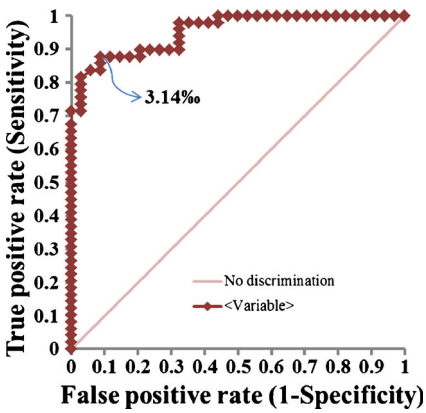

Fig. 4 Receiver operating characteristic curve (ROC) analysis for the ^{13}C-UBT. The optimal diagnostic cut-off value was determined to be $\delta_{DOB}{}^{13}$C‰=3.14‰ at 30 min

However, there are several possibilities which can affect the results of ^{13}C-UBT within the grey zone. One such possibility is to investigate the effect of endogenous CO_2 production associated with basal metabolic rates (BMR) in individuals. To study this effect, we applied the Mifflin–St Jeor equations to calculate the BMR based on age, weight and height of either sex [18]. The effect of the endogenous CO_2 production rates between the two groups of *H. pylori*-positive and *H. pylori*-negative individuals (10.57 vs 10.66 mmol/min) was statistically insignificant (p=0.78). However, the effect of urea hydrolysis rate (UHR) at 30 min on ^{13}C-UBT between H. *pylori*-positive and *H. pylori*-negative individuals (81.49 vs 5.30 μg/min) was statistically significant (p<0.0001) which thus showed the presence of *H. pylori* infection (ESM Fig. S1a, b). To study the UHR, we applied the Schofield equations [19] and used the experimentally determined $\delta_{DOB}{}^{13}$C‰ values as shown below [5]:

$$\mathrm{UHR} = \text{endogenous}\,CO_2\ \text{production} \times\ \delta_{DOB}{}^{13}\text{C‰} \times 0.346294\,(\mu g/\mathrm{min}) \quad (1)$$

We subsequently investigated the optimal diagnostic cut-off value of 10.48 μg/min by means of UHR at 30 min, but no significant improvement in the results of diagnostic sensitivity, specificity and accuracy was observed (ESM Table S4), demonstrating the insignificant effect of endogenous CO_2 production on ^{13}C-UBT.

Figure 5 depicts the excretion patterns of $\delta_{DOB}{}^{13}$C‰ values within the grey zone for the two groups (20 *H. pylori*-positive and 17 *H. pylori*-negative individuals) of patients. It was observed that the $\delta_{DOB}{}^{13}$C‰ values at each time point for the two groups were very close or sometimes overlap each other, demonstrating that the accurate detection of *H. pylori* infection in the grey zone is often incorrect and uncertain. We also observed that there were sudden rises or abrupt falls of

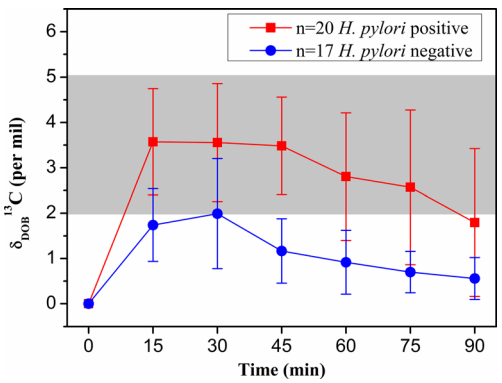

Fig. 5 The time evaluation of $\delta_{DOB}{}^{13}$C values for the two groups of 20 *H. pylori*-positive and 17 *H. pylori*-negative individuals within the grey zone. The *shaded region* corresponds to grey zone, and *n* is the number of patients. The error bar corresponds to 1 standard deviation (SD)

$\delta_{DOB}{}^{13}C‰$ values at 30 min in the individual excretion kinetic patterns, which have essentially reflected in the outcomes of ^{13}C-UBT for producing false-positive and false-negative results. The detailed results are shown in the Electronic Supplementary Material (ESM Tables S5, S6 and S7).

Therefore, to avoid the risk of diagnostic errors in the grey zone and consequently to achieve the highest diagnostic accuracy of the ^{13}C-UBT, we have explored the percentage dose of ^{13}C recovered per hour, i.e. ^{13}C-PDR (%/h), and cumulative percentage dose of ^{13}C recovered, i.e. c-PDR (%), in exhaled breath samples for the present ^{13}C-UBT.

To investigate the ^{13}C-PDR, we applied the following formula of Wu and colleagues [20]:

$$^{13}\mathrm{C{-}PDR\,(\%/h)} = \frac{\delta_{DOB}{}^{13}C \times R_{PDB} \times 10^{-3} \times V_{CO_2}}{\left(\frac{D}{M_t}\right) \times \left(p \times \frac{n}{100}\right)} \times 100 \quad (2)$$

where $\delta_{DOB}{}^{13}C$ is the DOB values, R_{PDB} is equal to 0.0112372, D is the dose of substrate administered, M_t is molecular weight of the substrate, p is ^{13}C atom % excess, n is the number of labelled carbon positions and V_{CO_2} is the CO_2 production rate per hour. The c-PDR (%) values were calculated using a trapezoidal rule [21] from the ^{13}C-PDR values. Figure 6 shows the time profiles of c-PDR (%) for the two groups of patients within the grey zone. A clear distinction between the groups of *H. pylori*-positive and *H. pylori*-negative individuals was observed after 45 min (0.75 h) in the kinetic profiles. Thus, the measurement of c-PDR (%) is more advantageous compared to a single-point $\delta_{DOB}{}^{13}C$ measurement because it accounts for the cumulative effect of $\delta_{DOB}{}^{13}C‰$ at any given time.

However, a clear disadvantage of the c-PDR (%) methodology compared to the standard single-point $\delta_{DOB}{}^{13}C‰$ measurement is that at least five to six breath samples need to be measured. In several reports [8], many authors have also suggested that a repeat ^{13}C-UBT or invasive endoscopy and biopsy tests would be an appropriate choice to have a conclusive result for the grey-zone patients. Nevertheless, this is much more expensive and time consuming than a single test regardless of requiring breath samples at multiple time points.

We subsequently explored the diagnostic accuracy of the ^{13}C-UBT by constructing another ROC curve using the c-PDR (%) values, and Fig. 7 depicts the ROC curve. An optimal diagnostic cut-off level was estimated to be c-PDR= 1.47 % at 60 min, exhibiting 100 % diagnostic sensitivity (95 % CI 92.7–100), 100 % specificity (95 % CI 89.7–100) and 100 % accuracy of the ^{13}C-UBT for the detection of *H. pylori* infection (ESM Tables S8 and S9).

We also investigated whether the current c-PDR (%) methodology is statistically robust compared to the standard one for the grey-zone patients. Figure 8 illustrates a simple statistical test, demonstrating that the optimal cut-off point of the standard $\delta_{DOB}{}^{13}C‰$ test, i.e. 3.14‰ lies within the overlapping areas between the two groups of patients, considering only 1 standard deviation (SD) of the mean values, whereas in the case of c-PDR (%) methodology, the cut-off point (1.47 %) is quite far away from the overlapping areas of both types of patients even by considering 2 SD. It is also noted that the cut-off value of the c-PDR (%) could be decreased to 1.29 % without compromising the sensitivity and specificity of this methodology.

Thus, the c-PDR (%) methodology appears to be sufficiently robust for an accurate diagnosis of *H. pylori* infection, avoiding a repeat endoscopic biopsy test, as some authors have previously suggested this invasive test [8] when the DOB values are inconclusive within the grey zone. Moreover, we determined the positive and negative predictive values (i.e. PPV and NPV) of the present ^{13}C-UBT. The PPV and NPV essentially indicate the probabilities that the infection is truly positive and negative, respectively, among the total test outcome positives and test outcome negatives, respectively [22]. The present ^{13}C-UBT demonstrated both PPV and NPV of

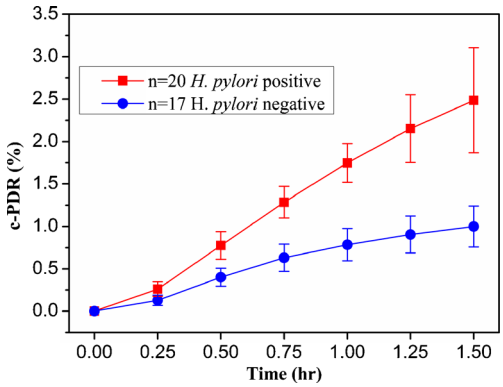

Fig. 6 Time profiles of c-PDR (%) for the two groups of 20 *H. pylori*-positive and 17 *H. pylori*-negative individuals within the grey zone. The error bar corresponds to 1 standard deviation (SD)

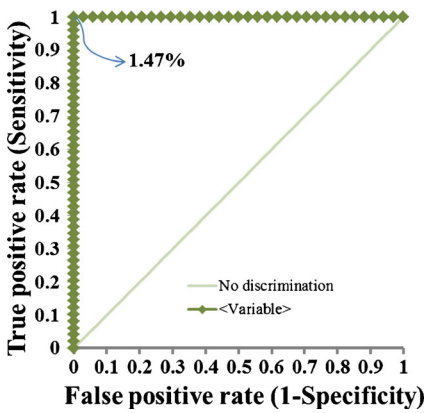

Fig. 7 Receiver operating characteristic curve (ROC) analysis for the c-PDR (%). The optimal diagnostic cut-off value was determined to be c-PDR (%)=1.47 % at 60 min (1.0 h)

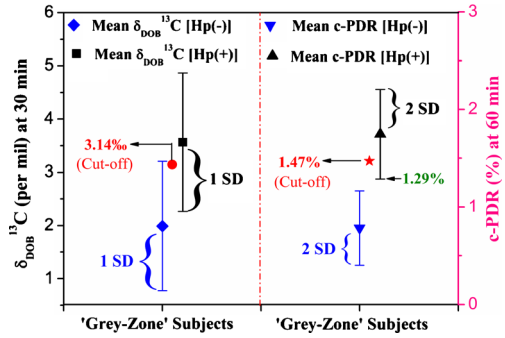

Fig. 8 A statistical comparison between single-point $\delta_{DOB}{}^{13}C‰$ measurement and c-PDR (%) methodology for grey-zone individuals. The *error bar* corresponds to 1 SD and 2 SD respectively for $\delta_{DOB}{}^{13}C‰$ and c-PDR (%) measurements. Hp(+) and Hp(−) stand for *H. pylori*-positive and *H. pylori*-negative individuals, respectively, and SD is the standard deviation

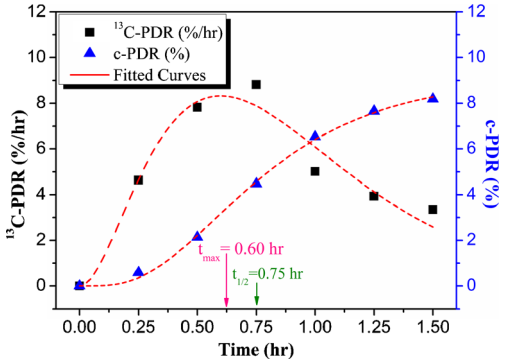

Fig. 9 Time profiles of percentage dose of ^{13}C recovered per hour, ^{13}C-PDR (%/h) and cumulative percentage dose of ^{13}C recovered, c-PDR (%). Median values of 49 *H. pylori*-positive patients are used to plot. *Red lines* are the fitted curves. t_{max} and $t_{1/2}$ indicate the time of maximal emptying rate and half emptying time, respectively

100 %, manifesting excellent diagnostic accuracy for large-scale screening purposes. Table 1 shows the comparison of various diagnostic parameters for ^{13}C-UBT in three different methodologies.

Finally, we have investigated the real emptying process of the present ^{13}C-UBT for *H. pylori*-infected individuals. Figure 9 illustrates the time profiles of ^{13}C-PDR (%/h) and c-PDR (%) of *H. pylori*-infected individuals. The median data for the ^{13}C-PDR (%/h) of 49 *H. pylori*-positive patients were fitted by the following mathematical formula for the determination of the time of maximal emptying rate [15]:

$$y = at^b e^{-ct} \tag{3}$$

where y is the percentage of $^{13}CO_2$ excretion per hour, t is the time, and a, b and c are constants. From the fitting constants, the time of maximal emptying rate of the current ^{13}C-UBT was calculated to be $t_{max}=(b/c)=0.60$ h (36 min) as shown in Fig. 9.

The calculated value also supported the maximum $\delta_{DOB}{}^{13}C‰$ values at 30 min as previously observed in Fig. 2 for *H. pylori*-positive patients. The c-PDR (%) data were also fitted to another three-parameter model with

$$y = m\left(1-e^{-kt}\right)^{\beta} \tag{4}$$

where m, k and β are constants to determine the half emptying time [15] of the current protocol, and it was estimated to be $t_{1/2}=(-1/k)\ln(1-2^{-1/\beta})=0.75$ h(45 min). From Fig. 9, it was also observed that about 8 % of the total ^{13}C dose recovered over 1.5 h which in turn exhibited the existence of *H. pylori* infection.

Conclusion

We have demonstrated for the first time the excretion kinetic patterns of $\delta_{DOB}{}^{13}C‰$, ^{13}C-PDR (%/h) as well as c-PDR (%) for the ^{13}C-UBT in the diagnosis of *H. pylori* infection. We subsequently investigated the effect of endogenous CO_2 production and urea hydrolysis rates on the excretion kinetic curves. The present study clearly demonstrates how to overcome the grey zone in the ^{13}C-UBT for the accurate determination of the infection without any risk of diagnostic errors and consequently introduces a new diagnostic cut-off value of c-PDR (%)=1.47 % at 60 min. Moreover, the current c-PDR methodology exhibited both sensitivity (true-positive rates) and specificity (true-negative rates) of 100 % and a diagnostic accuracy of 100 % compared with endoscopic biopsy tests, thus making it a sufficiently robust and novel method for an

Table 1 Comparisons of diagnostic parameters for ^{13}C-UBT in three different methodologies

Methodology	Breath samples collection	Receiver operating characteristics (ROC) curve results						
		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	FP	FN	Cut-off value
$\delta_{DOB}{}^{13}C(‰)$	Basal and 30 min	87.8	91.2	93.48	83.78	3	6	*3.14‰* (at 30 min)
UHR (μg/min)	Basal and 30 min	87.8	91.2	93.48	83.78	3	6	*10.48 μg/min* (at 30 min)
c-PDR (%)	Basal and multiple breath samples	100	100	100	100	0	0	*1.47 %* (at 60 min)

PPV positive predictive value, *NPV* negative predictive value, *FP* false positive, *FN* false negative, *UHR* urea hydrolysis rate, *c-PDR* cumulative percentage dose recovery

accurate and fast noninvasive diagnosis of *H. pylori* infection for large-scale screening purposes. However, it should be emphasised that we have investigated the c-PDR (%) methodology only on limited number of samples as a preliminary test, and a much larger study would be required to confirm the general rule that c-PDR (%)=1.47 % at 60 min is always the best choices.

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References

1. Imrie C, Rowland M, Bourke B, Drumm B (2001) Is Helicobacter pylori infection in childhood a risk factor for gastric cancer? Pediatrics 107:373–380
2. Suzuki H, Marshall BJ, Hibi T (2006) Overview: Helicobacter pylori and extragastric disease. Int J Hematol 84:291–300
3. Crowe SE (2005) Helicobacter infection, chronic inflammation and the development of malignancy. Curr Opin Gastroenterol 21:32–38
4. Thirumurthi S, Graham DY (2012) Helicobacter pylori infection in India from a western perspective. Indian J Med Res 136:549–562
5. Yang HR, Ko JS, Seo JK (2008) Does the diagnostic accuracy of the ^{13}C-urea breath test vary with age even after the application of urea hydrolysis rate? Helicobacter 13:239–244
6. Perry S, Parsonnet J (2005) Commentry: H. pylori infection in early and the problem of imperfect tests. Int J Epidemiol 34:1356–1358
7. Goddard AF, Logan RPH (1997) Review article: urea breath tests for detecting Helicobacter pylori. Aliment Pharmacol Ther 11:641–649
8. Gisbert JP, Pajares JM (2004) Review article: ^{13}C-urea breath test in the diagnosis of Helicobacter pylori infection—a critical review. Aliment Pharmacol Ther 20:1001–1017
9. Klein PD, Malaty HM, Czinn SJ, Emmons SJ, Martin RF, Graham DY (1999) Normalizing results of ^{13}C-Urea breath testing for CO_2 production rates in children. J Pediatr Gastroenterol Nutr 29:297–301
10. Savarino V, Vigneri S, Celle G (1999) The ^{13}C urea breath test in the diagnosis of Helicobacter pylori infection. Gut 45(Suppl I):18–I22
11. Dominguez-Munoz JE, Leodolter A, Sauerbruch T, Malfertheiner P (1997) A citric acid solution is an optimal test drink in the ^{13}C-urea breath test for the diagnosis of Helicobacter pylori infection. Gut 40: 459–462
12. McAlexander I, Rau GH, Liem J, Owano T, Fellers R, Bear D, Gupta M (2011) Deployment of a carbon isotope ratiometer for the monitoring of CO_2 sequestration leakage. Anal Chem 83:6223–6229
13. Creedon CT, Verhulst PJ, Choi KM, Mason JE, Linden DR, Szurszewski JH, Gibbons SJ, Farrugia G (2013) Assessment of gastric emptying in non-obese diabetic mice using a [^{13}C]-octanoic acid breath test. J Vis Exp 73:e50301
14. Grzybowski M, Younger JG (1997) Statistical methodology: III. Receiver operating characteristic (ROC) curves. Acad Emerg Med 4:818–826
15. Pantoflickova D, Scott DR, Sachs G, Dorta G, Blum AL (2003) ^{13}C urea breath test (UBT) in the diagnosis of Helicobacter pylori; why does it work better with acid test meals? Gut 52:933–937
16. Scott DR, Weeks D, Hong C, Postitus S, Melchers K, Sachs G (1998) The role of internal urease in acid resistance of Helicobacter pylori. Gastroenterology 114:58–70
17. Rektorschek M, Weeks D, Sachs G, Melchers K (1998) Influence of pH on metabolism and urease activity of Helicobacter pylori. Gastroenterology 115:628–641
18. Mifflin MD, St Jeor ST, Hill LA, Scott BJ, Daugherty SA, Koh YO (1990) A new predictive equation for resting energy expenditure in healthy individuals. Am J Clin Nutr 51:241–247
19. Schofield WN (1985) Predicting basal metabolic rate, new standards and review of previous work. Hum Nutr Clin Nutr 39:S5–S41
20. Wu IC, Ohsawa I, Fuku N, Tanaka M (2010) Metabolic analysis of 13C-labeled pyruvate for noninvasive assessment of mitochondrial function. Ann N Y Acad Sci 1201:111–120
21. Evenepoel P, Hiele M, Geypens B, Geboes KP, Rutgeerts P, Ghoos Y (2000) ^{13}C-egg white breath test: a non-invasive test of pancreatic trypsin activity in the small intestine. Gut 46:52–57
22. Akobeng AK (2006) Understanding diagnostic tests 1: sensitivity, specificity and predictive values. Acta Paediatr 96:338–341