Oxygen-18 stable isotope of exhaled breath CO_2 as a non-invasive marker of *Helicobacter pylori* infection†

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We report for the first time the time-dependent excretion kinetics of $^{18}O/^{16}O$ isotope ratios of CO_2 in exhaled breath samples using an optical cavity-enhanced integrated cavity output spectroscopy (ICOS) method for the detection of *Helicobacter pylori* (*H. pylori*) infection in human stomach. We observed large differences in the oxygen-18 isotopic fractionations of breath CO_2 between *H. pylori* positive and negative individuals in response to orally administered, both unlabelled and labelled ^{13}C-enriched urea, suggesting a potential link between *H. pylori* infections and the ^{18}O-isotopic exchange in exhaled breath. An optimal diagnostic cut-off point of $^{18}O/^{16}O$ isotope ratios of breath CO_2 for the presence of *H. pylori* infection was determined to be 1.92‰ using the receiver operating characteristic curve (ROC) analysis, which exhibited both diagnostic sensitivity and specificity of 100% with an accuracy of 100%. Moreover, the methodology of monitoring ^{18}O in breath CO_2 manifested both positive and negative predictive values of 100%, demonstrating excellent diagnostic accuracy and suggesting that breath $C^{18}O^{16}O$ could be used as a potential marker for the identification of *H. pylori* infections. Our findings also suggest that monitoring the $^{18}O/^{16}O$ isotope ratios of breath CO_2 is a valid and sufficiently robust novel non-invasive approach for the accurate and specific detection of *H. pylori* infection in real-time, which may open new perspectives in the molecular diagnosis of *H. pylori* infection for large-scale screening purposes, early detection and follow-up of patients.

Introduction

Helicobacter pylori (*H. pylori*), the most frequent bacterial infectious disease in human stomach, has been recognized as the key risk factor for different gastric diseases, including chronic gastritis, peptic ulcer and stomach cancer.[1–4] Because the individuals harbouring these infections are often asymptomatic, an early diagnosis of *H. pylori* infections is important prior to the onset of various gastric diseases. Nowadays, the ^{13}C-urea breath test (^{13}C-UBT) is considered to be an efficient non-invasive diagnostic method for the detection of *H. pylori* infection, in contrast with the several direct invasive methods, such as endoscopy and the biopsy-based rapid urease test (RUT), bacterial culture and histopathology.[5,6] The ^{13}C-UBT is based on the principle that an orally administered ^{13}C-enriched urea ($^{13}CO(NH_2)_2$) is hydrolyzed into ammonia (NH_4^+) and ^{13}C-labelled bicarbonate (HCO_3^-) in the presence of urease enzyme secreted by *H. pylori*. The ^{13}C-labelled HCO_3^- is then transported to the lungs where it is excreted as $^{13}CO_2$ in the exhaled breath. Thus, an enrichment of $^{13}CO_2$ in exhaled breath samples will be exhibited in *H. pylori* infected individuals. An enrichment of $^{13}CO_2$ in breath, which is commonly expressed as the delta-over-baseline (DOB) value relative to a standard in per mille (‰), *i.e.* $\delta_{DOB}{}^{13}C‰$, is usually $\geq 2‰$, is strongly associated with the presence of *H. pylori* infection in human stomach.[7]

However, several reports[8,9] suggest that *H. pylori* encodes two different forms of the metalloenzyme carbonic anhydrase (α-CA and β-CA), which plays an important role in the interconversion of carbon dioxide and bicarbonate ($CO_2 + H_2O \leftrightarrow H^+ + HCO_3$), as well as for maintaining the urease activity in the human gastrointestinal tract.[10–12] Some authors[13–15] have also demonstrated that the oxygen-16 (^{16}O) isotope in $^{12}C^{16}O_2$ and the oxygen-18 (^{18}O) isotope of body water ($H_2{}^{18}O$) are rapidly exchanged during the human respiration, which is catalyzed by CA activity; suggesting the possibility of exploiting the oxygen-isotope fractionations of CO_2 in exhaled breath samples for the non-invasive diagnosis of *H. pylori* infections. Therefore, we hypothesized that monitoring stable $^{18}O/^{16}O$ isotope ratios of CO_2 in exhaled breath, expressed as DOB relative to the Vienna Pee Dee Belemnite standard, *i.e.* $\delta_{DOB}{}^{18}O‰ = [(\delta^{18}O‰)_{t=t} - (\delta^{18}O‰)_{t=basal}]$, may distinctly track the pathogenesis of *H. pylori* infections in human stomach, introducing a new

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strategy for the non-invasive diagnosis of *H. pylori* infections. Furthermore, to the best of our knowledge, the potential links between $^{18}O/^{16}O$ isotope ratios of breath CO_2 and *H. pylori* infections in response to unlabelled and labelled ^{13}C-enriched urea have not yet been fully elucidated. Therefore, the aim of the present study was to explore whether breath $C^{18}O^{16}O$ can act as a potential marker for the early detection of *H. pylori* infections.

In this article, we report for the first time, the potential links between the ^{18}O-isotope of breath CO_2 and *H. pylori* infections by exploiting the time-dependent excretion kinetics of the $^{18}O/^{16}O$ isotope ratios of CO_2 in breath samples from individuals with and without *H. pylori* infections. We utilized a laser-based high-resolution cavity enhanced absorption technique called integrated cavity output spectroscopy (ICOS) to study the breath ^{18}O kinetics. In addition, we determined several diagnostic parameters, such as an optimal diagnostic cut-off value, sensitivity and specificity, along with the risk of false positive and false negative results of breath ^{18}O in CO_2 to obtain an insight into the diagnostic effectiveness of detecting *H. pylori* infection.

Materials and methods

Human subjects

In this study, we included 109 human subjects (65 male and 44 female with a mean age of 41.10 ± 12.26 years) with various gastrointestinal disorders such as duodenal and gastric ulcer, chronic gastritis and non-ulcer dyspepsia. Detailed information of the subjects is provided in the ESI (Table S1†). The Ethics Committee Review Board of AMRI Hospital, Salt Lake, India (Study no.: AMRI/ETHICS/2013/1) approved the protocol of the current study. We also received institutional administrative approval from the S. N. Bose Centre, India, to work on human subjects pertinent to this project (Ref. no.: SNB/PER-2-6001/13-14/1769). Each patient gave written consent to participate in the study. The subjects were classified into two different groups: *H. pylori* positive patients ($n = 66$) and *H. pylori* negative patients ($n = 43$) on the basis of "gold-standard" invasive and non-invasive reports, including endoscopy and biopsy-based rapid urease test (RUT) and ^{13}C-UBT. An increase of $\delta_{DOB}{}^{13}C‰ \geq 2‰$ in the ^{13}C-UBT was considered to be indicative of *H. pylori* infection. In this study, a patient was considered to be infected with *H. pylori* only when both the test results were positive; if there were any disagreements between the test results, the patient was excluded from the study. We also excluded patients from the study if they were taking any antibiotics or proton pump inhibitors in four weeks prior to the endoscopic examination or those who had a previous history of diabetes.

Exhaled breath sample collection

The UBT was performed after overnight fasting in all the instances within 1–2 days following the endoscopic examination. On the day of the breath analysis, the end-expiratory basal breath sample was initially collected from each patient in a breath collection bag (QT00892, QuinTron Instrument Co. US, 750 ml) before ingestion of the substrate. Then, the post-dose breath samples were collected at 15 min intervals for up to 60 min following the ingestion of a drink containing 75 mg ^{13}C-labelled urea (CLM-311-GMP, Cambridge Isotope Laboratories, Inc. US) with 4.0 g citric acid dissolved in 200 ml of water. All the breath samples were subsequently analysed to determine both the $^{18}O/^{16}O$ and $^{13}C/^{12}C$ isotope ratios of CO_2 in real-time by a high-precision laser-based ICOS system, as described in the following section.

Measurements of the $^{18}O/^{16}O$ and $^{13}C/^{12}C$ isotope ratios of breath CO_2 by ICOS

To simultaneously measure both the $^{18}O/^{16}O$ and $^{13}C/^{12}C$ isotope ratios of CO_2 in exhaled breath samples, we utilized an isotopic CO_2 system (Loss Gatos Research, LGR, CCIA 36-EP) that exploits a high-finesse optical cavity-enhanced absorption technique known as integrated cavity output spectroscopy (ICOS). The ICOS system and its capability to measure high-precision isotope ratios have already been described in depth elsewhere.[16–18] Therefore, we highlight here the main features of this ICOS spectrometer. The ICOS system is coupled with a temperature controlled near infrared *cw*-DFB laser, operating at ~2.05 μm. The absorption cell (~59 cm long) consists of two high reflectivity cavity mirrors (R~99.98%) and provides an effective optical path length of 3 km. The absorption spectra of $^{12}C^{18}O^{16}O$, $^{12}C^{16}O^{16}O$ and $^{13}C^{16}O^{16}O$ at the wavenumbers of 4874.178 cm^{-1}, 4874.448 cm^{-1} and 4874.086 cm^{-1}, respectively, were recorded simultaneously by scanning the laser frequency over 20 GHz across the P(36), R(28) and P(16) ro-vibrational lines in the $(2,0^{\mathbf{0}},1) \leftarrow (0,0^{\mathbf{0}},0)$ vibrational combination band of the CO_2 molecule. Diaphragm pump and solenoid valves are used to maintain the cavity pressure (~30 Torr) and analyze the breath samples. Typically, a 35 ml amount of breath sample was injected into the cavity cell by a syringe/stopcock for the measurement. We used high-purity dry N_2 (>99.99%) as the carrier gas for purging the optical cavity, as well as to dilute the breath samples. The real-time absorption spectra were fitted with Voigt profile line shapes and, consequently, the absolute concentrations of $^{12}CO_2$, $^{13}CO_2$ and $^{12}C^{18}O^{16}O$ in breath samples were determined by the Beer's law. Furthermore, to check the accuracy and precision of the measurements for $\delta^{13}{}_{DOB}C‰$ and $\delta_{DOB}{}^{18}O‰$ values in exhaled breath samples by the ICOS system, we utilized four certified standards containing CO_2 in air with known $\delta^{13}C$ (Cambridge Isotope Laboratory, CIL, US, $\delta^{13}C = -22.8‰$, $-13.22‰$ & $-7.33‰$) and $\delta^{18}O$ (Standard NOAA air tank, Serial no. CB10073, $\delta^{18}O = -1.0‰$) values. The validation procedures for the measurements of the high-precision isotope ratios of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ by the ICOS system are described in the ESI (Tables S2 & S3†). We measured the $\delta^{13}{}_{DOB}C‰$ and $\delta_{DOB}{}^{18}O‰$ values in exhaled breath samples with a typical precision of ±0.15‰ and ±0.20‰, respectively.

Statistical analysis

We applied a Mann–Whitney test and one-way ANOVA tests for the statistical analyses and, consequently, a 'p value' of less than 0.05 was considered as statistically significant data. We also utilized Box–Whisker plots and receiver operating characteristic

curve (ROC) analysis[19] to illustrate the statistical distribution of the isotope ratios in breath samples and to determine the optimal diagnostic cut-off point of the isotope values for *H. pylori* infection, respectively. Origin Pro 8.0 (Origin Lab Corporation, USA) and Analyse-it Method Evaluation software (Analyse-it Software Ltd, UK, version 2.30) were used in the present study for all the statistical analyses.

Results and discussions

To investigate the carbon-13 and oxygen-18 isotopic enrichments of breath CO_2, we first studied the time-dependent excretion kinetics of $\delta_{DOB}{}^{13}C‰$, along with the $\delta_{DOB}{}^{18}O‰$ values, in exhaled breath samples from individuals with *H. pylori* positive and negative, using the ICOS system. The results of the excretion kinetics patterns of both infected and non-infected individuals following ingestion of ^{13}C-enriched urea are illustrated in Fig. 1. It was observed that both the $\delta_{DOB}{}^{18}O‰$ and $\delta_{DOB}{}^{13}C‰$ values in breath samples follow similar excretion kinetics. In the case of positive patients, both DOB values reached a peak value at around 30 min and then slowly decreased, whereas no significant differences of DOB values in exhaled breath samples were observed for the individuals with *H. pylori*-negative.

It was previously reported that the internal urease activity of *H. pylori*, *i.e.* the urease-catalyzed hydrolysis of ^{13}C-urea, is strongly associated with the enrichments of $^{13}CO_2$ in breath samples.[3,20,21] As a result, an increase in the $\delta_{DOB}{}^{13}C‰$ values within 30 min and the subsequent gradual decrease in the $\delta_{DOB}{}^{13}C‰$ values in the excretion kinetics are most likely to be the results of the change in the internal urease activity of the bacterial environment. Nevertheless, it has also been demonstrated in some earlier reports that carbonic anhydrase (CA) enzymes are essential to maintain the urease activity of *H. pylori* infections.[8,10] The metalloenzyme CA catalyzes the interconversion of CO_2 and HCO_3^-, which is important for urease-mediated acid resistance in the gastric environment.[9,11] Moreover, as the oxygen isotopes of CO_2 (^{16}O) and H_2O (^{18}O) are rapidly exchanged during this catalyzation process in response to CA activity, an increase in the $\delta_{DOB}{}^{18}O‰$ values in exhaled breath samples for *H. pylori* positive individuals is possibly attributed to the effects of the oxygen-isotope fractionations of CO_2 in the urease-mediated bacterial environment. Therefore, the large difference in the $\delta_{DOB}{}^{18}O‰$ values in excretion kinetics exhibited a marked distinction between *H. pylori* positive and negative individuals. In view of this result, our findings suggest a potential link between *H. pylori* infections in stomach and ^{18}O-isotopic exchange in exhaled breath.

We next investigated the statistical distribution of $C^{18}O^{16}O$ enrichments in breath samples at 30 min in *H. pylori* positive and negative individuals. We utilized a Box and Whisker plot of the $\delta_{DOB}{}^{18}O‰$ values to illustrate the distribution of $C^{18}O^{16}O$ enrichments, as shown in Fig. 2. We observed that the mean, median and interquartile ranges (IQRs) indicating the mid-spread of the statistical dispersion of the $\delta_{DOB}{}^{18}O‰$ values for positive patients were 3.79‰, 3.28‰, and 2.62‰ to 3.97‰, respectively. Conversely, for the negative patients, the mean, median and IQRs of the $\delta_{DOB}{}^{18}O‰$ values were −0.66‰, −0.81‰, and −1.23‰ to 0.23‰, respectively. There was a statistically significant difference of the $\delta_{DOB}{}^{18}O‰$ values ($p < 0.01$) between the two groups of *H. pylori* positive and negative individuals, thus suggesting that ^{18}O in breath CO_2 could be used as a potential marker for the non-invasive detection of *H. pylori* infection.

We further explored whether the unlabelled urea ($CO(NH_2)_2$) (*i.e.* with no ^{13}C-enriched substrate), has any effect on the excretion kinetics of the $\delta_{DOB}{}^{13}C‰$ and $\delta_{DOB}{}^{18}O‰$ values in exhaled breath samples for the *H. pylori* infected persons. Fig. 3a and b depict the typical excretion kinetic patterns of the $\delta_{DOB}{}^{13}C‰$ and $\delta_{DOB}{}^{18}O‰$ values for 15 *H. pylori* infected individuals. We observed that when the unlabelled urea was orally administered in positive patients, the $\delta_{DOB}{}^{13}C‰$ values in breath samples did not change significantly over time, whereas the $\delta_{DOB}{}^{18}O‰$ values in breath manifested a significant change

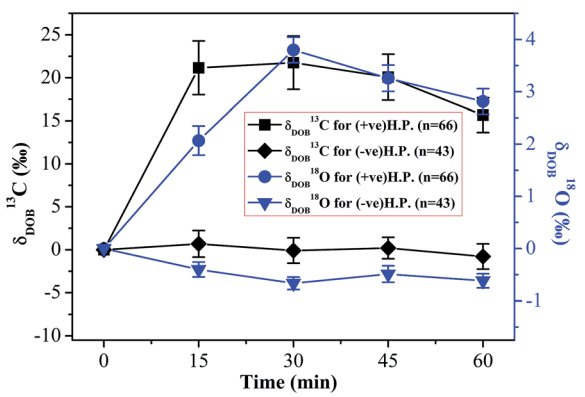

Fig. 1 Excretion kinetic profiles of the $\delta_{DOB}{}^{13}C‰$ and $\delta_{DOB}{}^{18}O‰$ values of *H. pylori* positive and *H. pylori* negative individuals for the ^{13}C-urea breath test. *n* is the number of subjects and error bars correspond to the standard error of the mean. (+ve) H.P. and (−ve) H.P. stand for *H. pylori* positive and *H. pylori* negative patients, respectively.

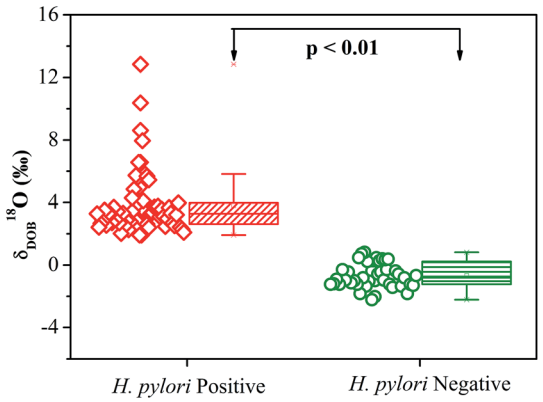

Fig. 2 Statistical comparison of the measured $\delta_{DOB}{}^{18}O‰$ values at 30 min for *H. pylori* positive and *H. pylori* negative individuals utilizing a Box–Whisker plot. Scattered points represented by the open-diamond & open-circle symbols correspond to the experimental data points measured by ICOS.

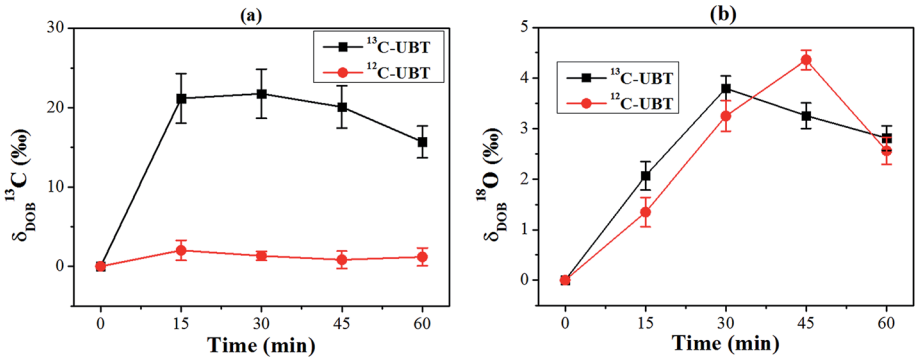

Fig. 3 Comparison of the excretion kinetic patterns of (a) $\delta_{DOB}{}^{13}C‰$ and (b) $\delta_{DOB}{}^{18}O‰$ values between the ^{13}C-urea breath test and unlabelled-urea breath test for 15 *H. pylori* positive patients.

with time and followed similar excretion kinetics with that of ^{13}C-enriched labelled urea. It is therefore noteworthy that the excretion kinetics of $\delta_{DOB}{}^{18}O‰$ followed a similar pattern regardless of the isotopic labelled substrate. This observation is possibly due to the fact that the whole mechanism, *i.e.* the urease-catalysed hydrolysis of urea to form bicarbonate and the subsequent CA-mediated inter-conversion of bicarbonate and CO_2 to finally produce $^{12}C^{16}O^{18}O$, solely depends on the substrate (urea), irrespective of its isotopic nature. In the case of ^{13}C-UBT, ^{13}C-labelled urea is essential to observe the $^{13}CO_2$ isotopic enrichments in exhaled breath samples for *H. pylori* infected individuals. Therefore, unlabelled urea as a substrate did not contribute to the enhancement of $\delta_{DOB}{}^{13}C‰$ values for *H. pylori* positive patients, and, hence, no significant changes of $\delta_{DOB}{}^{13}C‰$ values in breath were observed, as shown in Fig. 3(a).

Taken together, these findings indicate that ^{13}C-UBT has a clinical applicability only when a patient ingests a ^{13}C-enriched labelled urea and accordingly clinicians are then able to utilize the $\delta_{DOB}{}^{13}C‰$ values in breath to correctly diagnose the infection. Whilst, measuring the $\delta_{DOB}{}^{18}O‰$ values in exhaled breath may provide a useful way of monitoring the status of *H. pylori* infection, and, furthermore, ^{18}O in breath CO_2 could be used as a potential biomarker of this infection regardless of the unlabelled urea. These data also indicate the potential of using the $\delta_{DOB}{}^{18}O‰$ values in exhaled breath as an alternative, cost-effective and robust non-invasive diagnostic approach for *H. pylori* infection, and hence this may open a new route to the diagnosis of *H. pylori* infections.

We finally determined the optimal diagnostic cut-off point of the $\delta_{DOB}{}^{18}O‰$ values in exhaled breath samples for precisely distinguishing *H. pylori* positive and negative individuals. We utilized a receiver operating characteristics curve (ROC) analysis by plotting the false positive rate (1-specificity) *vs.* the true positive rate (sensitivity), as shown in Fig. 4. The statistically sound diagnostic cut-off point of the $\delta_{DOB}{}^{18}O‰$ values was defined as the point where we obtained the highest level of diagnostic sensitivity, specificity and accuracy to correctly identify individuals harbouring *H. pylori* infections. In our study, using the ROC analysis, the optimal diagnostic cut-off point was determined to be $\delta_{DOB}{}^{18}O‰ = 1.92‰$. Therefore, individuals with $\delta_{DOB}{}^{18}O‰ \geq 1.92‰$ were considered to be *H. pylori* positive, and this corresponded to the diagnostic sensitivity and specificity of 100% (95% CI 94.6–100) and 100% (95% CI 91.8–100), respectively, along with a diagnostic accuracy of 100%. Furthermore, we also determined the risk of false positive and false negative results in terms of positive and negative predictive values, *i.e.* PPV and NPV,[22] using the cut-off of $\delta_{DOB}{}^{18}O‰ = 1.92‰$. In our study, both PPV and NPV were estimated to be 100%, demonstrating a superior diagnostic accuracy, as well as the capability of the present ^{18}O-isotopic methodology for large-scale screening purposes in community population. We therefore posit that monitoring ^{18}O in breath CO_2 may distinctly track the evolution of *H. pylori* infection prior to the onset of various gastric disorders related to this infection. These findings may also have a broad clinical efficacy for the accurate assessment of *H. pylori* infection by human breath analysis and, therefore, provide a unique approach to treat the world's most common bacterial infective disease in human stomach.

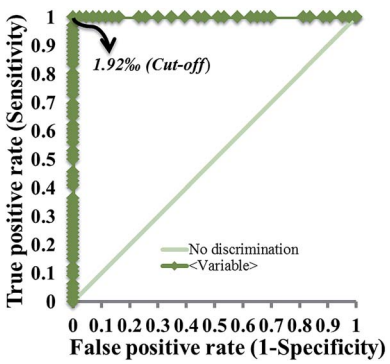

Fig. 4 Receiver operating characteristic curve (ROC) analysis for the $\delta_{DOB}{}^{18}O‰$ values. The optimal diagnostic cut-off value was determined to be $\delta_{DOB}{}^{18}O‰ = 1.92‰$ at 30 min.

Conclusion

In conclusion, we have extensively demonstrated for the first time, the time-dependent excretion kinetics of high-precision $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotope ratios of CO_2, in terms of $\delta_{DOB}{}^{13}C‰$

and $\delta_{DOB}{}^{18}O‰$ values, respectively, using an optical cavity enhanced spectroscopy technique in exhaled breath samples from individuals harbouring *H. pylori* infection. We have also taken an important step towards the potential link between the $^{18}O/^{16}O$ isotope ratios of breath CO_2 and *H. pylori* infections, thus suggesting that ^{18}O in breath CO_2 could be used as a potential molecular biomarker for the identification of *H. pylori* infections in a non-invasive method. The ^{18}O in breath CO_2 methodology demonstrated a diagnostic sensitivity of 100% and a specificity of 100%, along with 100% accuracy, thus making it a powerful and novel alternative diagnostic method for the non-invasive assessment of *H. pylori* infection in real-time. In addition, our findings may open new perspectives in the molecular diagnosis of *H. pylori* infection with large-scale screening purposes, early detection, and for follow up of patients.

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