Helicobacter pylori antibiotic resistance and [¹³C]urea breath test values

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A correlation between δ over baseline (DOB) values of the [¹³C]urea breath test (UBT) and *Helicobacter pylori* clarithromycin resistance has been reported, suggesting a possible predictive role of UBT in therapeutic outcome. However, available data are limited and conflicting. This study aimed to clarify this issue, assessing the possible relationship between *H. pylori* resistance towards different antibiotics (clarithromycin, metronidazole and levofloxacin) and UBT values. The data showed similar DOB values between susceptible and resistant strains for clarithromycin (46.9 ± 32.3 vs 45.7 ± 30.6 ; $P=0.8$), metronidazole (46.4 ± 29.6 vs 47.4 ± 37.9 ; $P=0.8$), and levofloxacin (45.0 ± 30.2 vs 54.2 ± 38.4 ; $P=0.08$). Likewise, comparable DOB values were observed between susceptible and multidrug-resistant strains (45.4 ± 29.6 vs 54.8 ± 44.8 ; $P=0.1$). In conclusion, our data failed to find a significant correlation between UBT values and *H. pylori* antibiotic resistance.

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INTRODUCTION

It is widely accepted that antibiotic resistance is the main factor affecting the efficacy of therapy for *Helicobacter pylori* infection (Megraud, 2004). Currently, bacterial susceptibility towards different antibiotics is performed by using either an Etest on cultures or culture-free techniques, such as PCR (Owen, 2002). Undeniably, both culture and PCR-based methods have some limitations. Indeed, *H. pylori* is a quite fastidious bacterium so that the sensitivity of the culture may be reduced, even in expert hands (Zullo *et al.*, 2003), whilst PCR-based techniques are available only in selected centres (De Francesco *et al.*, 2006a, b, c, 2007). Therefore, to have a non-invasive, widely accessible and cheap method for anticipating the presence of *H. pylori* resistance would be a definite advantage for the infection management in clinical practice. It has been suggested that the [¹³C]urea breath test (UBT) may predict clarithromycin resistance and, consequently, *H. pylori* eradication therapy outcome. In detail, a study reported a significant correlation between the δ over baseline (DOB) values of UBT and the presence of primary antibiotic resistance (Kawai *et al.*, 2008). Moreover, increased DOB values have been observed in eradication failure patients harbouring clarithromycin-resistant strains as compared to those with susceptible strains. These data suggest that UBT may predict secondary clarithromycin

resistance (Kao *et al.*, 2005), although data are conflicting (Buzás & Széles, 2008). Other studies failed to confirm the correlation between DOB values and primary clarithromycin resistance – the key antibiotic for *H. pylori* therapy – suggesting the need of further studies on this topic (Zullo *et al.*, 2008). We designed the present study with the aim of assessing whether DOB values are associated either with primary *H. pylori* resistance towards different antibiotics, as determined by culture and Etest, or with different point mutations of clarithromycin resistance, assessed by TaqMan real-time PCR.

METHODS

Patients. This was a post hoc analysis of a large study we recently published (Zullo *et al.*, 2007). Briefly, the study population consisted of patients with dyspeptic symptoms and positive UBT, who were never previously treated for *H. pylori* infection. All patients underwent endoscopy with biopsies for histology (two samples from the antral gastric mucosa and two samples from the body gastric mucosa), and a rapid urease test (one sample from the antral gastric mucosa). Two further biopsies were taken for bacterial culture. Overall, data regarding 240 out of 255 patients enrolled in the previous trial were considered for the present post hoc study. Nine patients of the initial study were not considered because of a lack of levofloxacin resistance assessment, whilst six patients were excluded due to a lack of UBT values.

[¹³C]UBT. All UBTs were carried out after an overnight fast. A baseline breath sample was obtained and then 75 mg [¹³C]urea with citric acid

Abbreviations: DOB, δ over baseline value; UBT, urea breath test.

(1.5 g) was administered in water solution. Another breath sample was collected 30 min after administration of the test solution and the test was considered positive if there was a difference between the baseline sample and the 30 min sample that exceeded 4.5 parts per 1000 of $^{13}\text{CO}_2$. All the breath samples were analysed using a single gas isotope ratio mass spectrometer (Finnigan) in the Department of Internal Medicine and Gastroenterology, University of Bologna. The accuracy of the UBT was previously validated with sensitivity and specificity values of 94.7 and 95.7 %, respectively (Vaira & Vakil, 2001).

Culture and Etest. Biopsies collected for bacterial culture were streaked onto Columbia agar enriched with 5 % horse blood, containing 100 µg vancomycin ml^{-1} , 200 µg trimethoprim ml^{-1} , 10 µg polymyxin B ml^{-1} and 10.7 µg nalidixic acid ml^{-1} to inhibit the growth of microbes other than *H. pylori*. The plates were incubated in a microaerobic environment at 37 °C for 7 days, and inspected daily from the third day. The isolates were identified by Gram stain, and by oxidase, catalase and urease tests. Suspensions from primary plates were prepared in sterile saline solution (0.9 % NaCl) at approximately 10^8 c.f.u. ml^{-1} , an amount sufficient for performing the Etest. A blood agar plate (Muller–Hinton with 5 % horse blood but without any antibiotics) was streaked in three directions with a swab dipped into each bacterial suspension to produce a lawn of growth. An Etest strip (AB Biodisk) was placed onto a separate plate that was immediately incubated in a microaerobic atmosphere at 37 °C for 72 h. Isolated strains were tested for clarithromycin, metronidazole and levofloxacin resistance using >1, >8 and >1 mg l^{-1} as break point the MIC, respectively.

TaqMan real-time PCR. For the present study, paraffin-embedded antral biopsy specimens were retrieved and used for clarithromycin-resistance assessment by TaqMan PCR. The technique was performed in blind in a single centre (Section of Gastroenterology, Department of Medical Sciences, University of Foggia). DNAs were extracted from the same antral samples used for histology using a NucleoSpin tissue kit (Macherey-Nagel) applied on paraffin-embedded sections (at least five sections of 10 µm thickness), which are largely accepted to constitute a reliable substrate for DNA analysis like fresh material (De Francesco *et al.*, 2006a, b, c, 2007). The A2142C, A2142G and A2143G point mutations were analysed by a novel method (TaqMan real-time PCR) that was first used for *Mycobacterium tuberculosis* detection, and then successively for *H. pylori* DNA sequencing on paraffin-embedded samples, as we have recently reported (De Francesco *et al.*, 2007). Briefly, real-time PCR was performed in 96-well plates on the ABI Prism 7900HT sequence detection system (Applied Biosystems); data collection and analyses were carried out using the specific software (from the same provider). The final reaction volume (25 µl) was analysed in triplicate (three samples for each patient) and all experiments were repeated twice. Primers and probes for allelic discrimination are listed in previous works (De Francesco *et al.*, 2006a, b, c, 2007). The analysis yielded three major clusters corresponding to the three genotypes: homozygous wild-type, homozygous and heterozygous mutated type or heteroresistant status (combination of susceptible and resistant strains). The operator who performed all the PCR procedure was working blind of the culture results. In our experience, the variability between duplicates and triplicates within the same run or different runs was usually between 0 and 2 %, in agreement with that reported in other studies (Gerard *et al.*, 1998).

Statistical analysis. The statistical analysis was performed using Student's *t*-test for paired and unpaired data as appropriate. The results are expressed as means $\pm$ SD. Differences were considered significant at the 5 % probability level. The analyses were performed with Statsoft 7.1 program for Windows XP.

RESULTS AND DISCUSSION

The baseline demographic characteristics are reported in Table 1. Overall, 119 (49.6 %) patients were infected with an antibiotic-susceptible strain, whilst a bacterial resistance towards at least one of the tested antibiotics was present in the remaining 121 (50.4 %) patients (Table 2). Overall, there were 39, 65 and 44 patients infected with either clarithromycin-, metronidazole- or levofloxacin-resistant strains, respectively, whilst 26 patients were infected with multidrug-resistant (double or triple drug) strains. By considering antibiotic resistance assessed by Etest, comparable DOB values were observed between susceptible and single-drug-resistant strains (45.4 ± 29.6 vs 46.1 ± 30.8 ; $P=0.8$). In detail, comparable values emerged between susceptible and resistant strains for clarithromycin (46.9 ± 32.3 vs 45.7 ± 30.6 ; $P=0.8$), metronidazole (46.4 ± 29.6 vs 47.4 ± 37.9 ; $P=0.8$) and levofloxacin (45.0 ± 30.2 vs 54.2 ± 38.4 ; $P=0.08$). Similarly, comparable DOB values were observed between susceptible and multidrug-resistant strains (45.4 ± 29.6 vs 54.8 ± 44.8 ; $P=0.1$).

In a subgroup of 39 patients showing clarithromycin resistance by culture, a TaqMan real-time PCR was performed to search for the three specific point mutations linked with clarithromycin resistance. A point mutation was detected in 37 out of these cases with a phenotypic clarithromycin-resistant strain. In detail the A2143G point mutation was observed in 22 patients, the A2142G point mutation in 10 patients and the A2142C point mutation in 5 patients. As shown in Table 3, no significant difference in DOB values was observed between the three different clarithromycin-resistance point mutations. In the 19 patients (16 with a resistant strain and 3 with a susceptible strain) in whom bacterial eradication failed no significant modifications in DOB values emerged following therapy (56.4 ± 38.2 vs 51.0 ± 34.0 ; $P=0.5$). In detail, in infected patients with a resistant strain DOB values did not significantly change (58.6 ± 42.0 vs 54.7 ± 34.9 ; $P=0.7$), whilst a significant reduction was observed in those patients harbouring a susceptible strain (51.7 ± 24.8 vs 31.0 ± 22.8 ; $P=0.01$).

Some studies have suggested that high DOB values are associated a high bacterial load in the stomach (Perri

Table 1. Baseline, demographic and clinical characteristics of patients

Characteristic	No. of patients or age
Patients	240
Mean age $\pm$ SD (years)	48.8 ± 14.8
Sex (male/female)	84/156
Peptic ulcer/non-ulcer dyspepsia	25/215
Smoking habit (yes/no)	82/158
Alcohol intake (yes/no)	88/152

Table 2. Pattern of primary bacterial resistance towards different antibiotics

Antibiotic resistance	No. of cases
Clarithromycin	25
Metronidazole	43
Levofloxacin	27
Clarithromycin/metronidazole	9
Clarithromycin/levofloxacin	4
Metronidazole/levofloxacin	12
Clarithromycin/metronidazole/ levofloxacin	1
Total	121

et al., 1998; Zagari *et al.*, 2005; Cremonini *et al.*, 2005), as well as with some *H. pylori* virulence factors, such as CagA (Zullo *et al.*, 2004; Sarker *et al.*, 2004). Moreover, in other studies increased DOB values were found in bacterial strains harbouring antibiotic resistance (Kawai *et al.*, 2008; Kao *et al.*, 2005). Our data failed to confirm a possible predictive role of UBT for bacterial resistance. Indeed, we observed similar DOB values between patients infected with either susceptible or resistant *H. pylori* strains towards the different antibiotics tested. Clarithromycin resistance is regarded as the key factor involved in *H. pylori* therapy failure, and some studies found that the main three different point mutations implicated in such resistance are associated with different MIC values *in vitro* (Owen, 2002; Megraud, 2004). We previously found that the A2143G point mutation, but not the other two point mutations, significantly reduced the eradication rate following therapy (De Francesco *et al.*, 2006a). The present study found comparable DOB values among strains with the three different point mutations of clarithromycin resistance. Therefore, on the basis of UBT values it does not seem to be possible to predict the presence of different clarithromycin-resistance point mutations and, in turn, to anticipate the therapy success probability. Therefore, the bacterial urease activity, which correlates with DOB values, seems to mainly depend on the overall bacterial load rather than on the presence of antibiotic resistance. In conclusion, our data based on

Table 3. DOB values and different clarithromycin point mutations

No significant difference emerged between the groups ($P=0.3$ for A2143G vs A2142G mutation genotype; $P=0.6$ for A2143G vs A2142C mutation genotype).

Point mutation (no. of cases)	DOB values (mean $\pm$ SD)
A2143G (22)	54.1 $\pm$ 36.2
A2142G (10)	74.9 $\pm$ 49.2
A2142C (5)	61.6 $\pm$ 14.6

both phenotypic and genotypic testing showed that presence of primary *H. pylori* resistance towards different antibiotics is not associated with different UBT values as compared to susceptible strains.

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