Frequency and molecular characteristics of ciprofloxacin- and rifampicin-resistant *Helicobacter pylori* from gastric infections in the UK

Stephanie A. Chisholm and Robert J. Owen

Correspondence

Robert J. Owen

robert.owen@hpa.org.uk

Gastrointestinal, Emerging and Zoonotic Infections Department, Centre for Infections, Health Protection Agency, 61 Colindale Avenue, London NW9 5HT, UK

Treatment failure with standard *Helicobacter pylori* eradication regimes may require the use of 'rescue' therapies containing fluoroquinolones or rifamycins. The susceptibilities of *H. pylori* in the UK to such antimicrobials are unknown; therefore, this study aimed to determine the frequencies and molecular markers of resistance. Ciprofloxacin and rifampicin susceptibilities were determined by Etest and/or disc diffusion for 255 isolates of *H. pylori*, including 171 isolates from adult dyspeptic patients with refractive infections. Mutations in known resistance-determining regions of *gyrA* and *rpoB* were determined. The ciprofloxacin resistance rate was 7.5 %, and *gyrA* mutations, predominantly at codon position 91, were identified in most resistant isolates. One isolate (<1 %) had an unequivocal rifampicin-resistant phenotype by Etest yet had no associated mutations in the *rpoB* gene. As resistance rates were low in *H. pylori* isolates, including those from patients with refractive infections, it was concluded that fluoroquinolones or rifamycins might be considered in the UK for inclusion in 'rescue' therapies.

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INTRODUCTION

Helicobacter pylori infection can lead to the development of peptic ulcer disease, lymphoproliferative disorders and early-stage gastric carcinoma (Go, 2002; Suerbaum & Michetti, 2002). Current guidelines recommend a triple therapy comprising a proton pump inhibitor and clarithromycin and either metronidazole or amoxicillin as a first-line regimen, and a quadruple therapy comprising a proton pump inhibitor plus bismuth, metronidazole and tetracycline as a second-line strategy (Malfertheiner *et al.*, 2007). Antibiotic resistance, particularly to clarithromycin and metronidazole, is a major contributing factor in treatment failure (Dore *et al.*, 2000; Jenks, 2002), and infection can persist in as many as 20 % of patients following treatment (Gisbert & Pajares, 2005). Such limitations have led to the development of alternative 'rescue' therapies using agents not used in first- or second-line therapies, such as the fluoroquinolones moxifloxacin and levofloxacin, and rifabutin, a rifamycin derivative commonly used in the treatment of mycobacterial infections but with high efficacy against *H. pylori* (Gisbert & Pajares, 2005; Toracchio *et al.*, 2005).

Regimes containing these alternative antimicrobial agents have proved successful for the management of persistent *H. pylori* infections. Studies in Europe, the USA, Asia and several other countries have monitored fluoroquinolone resistance rates in different patient groups including

children and have reported rates of up to 33 % (Cabrita *et al.*, 2000; Tankovic *et al.*, 2003; Falsafi *et al.*, 2004; Fujimura *et al.*, 2004; Kim *et al.*, 2004; Sherif *et al.*, 2004; Bogaerts *et al.*, 2006; Kumala & Rani, 2006; Miyachi *et al.*, 2006; Carothers *et al.*, 2007; Cattoir *et al.*, 2007; Glocker *et al.*, 2007b; Zullo *et al.*, 2007; Boyanova *et al.*, 2008; Lee *et al.*, 2008). In contrast, information on rifampicin/rifabutin resistance in *H. pylori* is more limited, as no examples of resistance were found in studies of German (Heep *et al.*, 1999), Japanese (Fujimura *et al.*, 2002) or Italian (Toracchio *et al.*, 2005) isolates, although a subsequent German study reported a resistance rate of 1.4 % (Glocker *et al.*, 2007a).

There is currently no information on either fluoroquinolone or rifamycin resistance of *H. pylori* from patients in the UK, so the aim of our study was to report on the frequencies of resistance to ciprofloxacin and rifampicin using isolates from adult patients, including those with refractive *H. pylori* infection. We also detailed the gene mutations associated with such resistances because of their importance in providing the basis of future rapid molecular diagnostic tests – an approach already successfully applied to clarithromycin for which the molecular basis of resistance in *H. pylori* is well understood (Chisholm & Owen, 2008). Ciprofloxacin inhibits subunit A of DNA gyrase (Reece & Maxwell, 1991), and in *H. pylori*, resistance is reported to arise from mutations in the 'quinolone resistance-determining region' (QRDR) of *gyrA* (Moore *et al.*, 1995) with a number of mutations associated

Abbreviation: QRDR, quinolone resistance-determining region.

with higher resistance levels (Bogaerts *et al.*, 2006; Miyachi *et al.*, 2006). Rifampicin, by contrast, exerts its antibacterial effect by binding to the β -subunit of the DNA-directed RNA polymerase encoded by the RNA polymerase subunit B gene (*rpoB*) gene, with resistance in *H. pylori* developing as a result of specific mutations in *rpoB* (Heep *et al.*, 1999; Wang *et al.*, 2001). Here, we present information on the presence of mutations associated with antibiotic-resistant phenotypes.

METHODS

Bacterial isolates. *H. pylori* isolates cultured over a 6-year period (2001–2006) from gastric biopsies of 255 adult dyspeptic patients were included in this study. The majority of isolates (171/255, 67%) were cultured from gastric biopsies referred to our laboratory for routine testing by previously described methods (Chisholm *et al.*, 2007). The biopsies were referred from 76 different hospitals located across England, Scotland and Wales, and were from patients undergoing gastroendoscopy as part of a local routine clinical investigation. Available information indicated that 70% ($n=121$) of these patients had at least one failed eradication therapy attempt. The treatment histories of the remaining 50 patients with culture-positive biopsies were unknown. An additional 84 isolates (33%) in the study were received as cultures after primary isolation had been performed in local microbiology laboratories, as described previously (Elviss *et al.*, 2004, 2005). Available information indicated that these isolates were from gastric biopsies from patients attending endoscopy clinics for routine investigation prior to specific anti-*H. pylori* eradication therapy. The history of any previous treatment regimens used was unknown. All 255 isolates were preserved at -80°C .

Antibiotic susceptibility testing. The isolates had been tested previously for susceptibility to metronidazole, clarithromycin, tetracycline and amoxicillin using established methods used longitudinally in our accredited laboratory (Chisholm *et al.*, 2007). For the present study, susceptibilities to ciprofloxacin and rifampicin were tested by Etest and/or disc diffusion methods. Briefly, exponential (48 h) bacterial growth was suspended in maximum recovery diluent (Oxoid) to a concentration of $\sim 10^8$ c.f.u. ml^{-1} (MacFarland standard 4) and inoculated onto pre-dried Columbia agar base plates containing defibrinated horse blood (10%, v/v). Ciprofloxacin and rifampicin Etests (AB Biodisk) and/or antibiotic discs (Oxoid) containing 1 μg ciprofloxacin or 5 μg rifampicin were applied to the surface of these plates and any inhibition of bacterial growth was recorded following 48 h microaerophilic incubation (4% O_2 , 5% CO_2 , 86% N_2 , 5% H_2) at 37°C in a MACS VA500 Microaerophilic Workstation (Don Whitley Scientific).

Initially, MICs for ciprofloxacin and rifampicin for 115 isolates were determined by Etest. Isolates were classified as ciprofloxacin resistant ($\geq 1 \text{ mg l}^{-1}$) or sensitive ($< 1 \text{ mg l}^{-1}$) and rifampicin resistant ($\geq 4 \text{ mg l}^{-1}$) or sensitive ($< 4 \text{ mg l}^{-1}$) based on criteria indicated by previous investigators (Cabrita *et al.*, 2000; Heep *et al.*, 2000a; Tankovic *et al.*, 2003). Thirty-three of these 115 isolates were tested additionally by the disc diffusion method to establish appropriate zone size cut-offs to define resistant and sensitive isolates. A further 139 isolates received in 2006 were screened prospectively by the disc diffusion method and MICs were then determined by Etest for any isolates resistant by disc diffusion.

Sequencing of resistance-associated gene regions. Genomic DNA was extracted from exponential (48 h) cultures using a MagNA Pure System LC Total NA Isolation kit (Roche Diagnostics), according to the manufacturer's instructions. To test for markers of

ciprofloxacin resistance, a 246 bp fragment of the *gyrA* gene containing the QRDR was amplified by PCR as described previously (Fujimura *et al.*, 2004). To test for markers of rifampicin resistance, two fragments of *rpoB* were amplified using previously described primers: the first fragment contained the amino acid V149 region (Heep *et al.*, 2000b), and the second fragment contained cluster regions I and II (Heep *et al.*, 1999). All amplicons were purified by using a Whatman 96-Well PCR Cleanup kit (VWR International), according to the manufacturer's protocol, and quantified by gel electrophoresis. The purified fragments were sequenced using a CEQ800 Genetic Analysis System (Beckman Coulter). Sequences were trimmed and analysed using Bionumerics version 2.0 and GeneBase version 1.0 software (Applied Maths).

RESULTS AND DISCUSSION

Characterization of the susceptibility of the study population to ciprofloxacin

The MICs for ciprofloxacin of 115 isolates of *H. pylori* had a bimodal distribution (Fig. 1a), with a clear separation between sensitive (MICs ranging from 0.016 to 0.5 mg l^{-1}) and resistant (MICs ranging from 4 to 32 mg l^{-1}) isolates at the 1 mg l^{-1} breakpoint. The MIC_{90} and MIC_{50} values of the isolates examined were 0.38 and 0.094 mg l^{-1} , respectively, with median and modal MICs of 0.094 mg l^{-1} , indicating a predominantly susceptible population. Zones of inhibition observed by disc diffusion testing ranged from 20 to 54 mm (mean 41 mm, mode 42 mm) for ciprofloxacin-sensitive isolates ($n=24$) and from 0 to 19 mm (mean 6 mm, mode 0 mm) for resistant isolates ($n=10$). With one exception, all zone sizes of ≤ 14 mm were associated with MICs of 12 mg l^{-1} or more, whilst larger zones of 19 mm were associated with lower MICs (2 and 4 mg l^{-1}) (Table 1).

To date, most studies have monitored fluoroquinolone resistance in *H. pylori* by Etest or to a lesser extent by the agar dilution method; the single study that used disc diffusion did not state the cut-off for resistance (Falsafi *et al.*, 2004). The data we obtained for 1 μg ciprofloxacin discs demonstrated that zones of inhibition of ≤ 14 mm by disc diffusion were usually indicative of ciprofloxacin MICs of $\geq 12 \text{ mg l}^{-1}$. Larger disc zones of 19 mm were associated with lower-level ciprofloxacin resistance, but these were less reliable indicators of resistance, as zones of a similar size (20 mm) were observed for two isolates that were sensitive according to MIC by Etest. Whilst our results indicated a cut-off zone size of ≤ 15 mm for resistance by the 1 μg ciprofloxacin disc diffusion test using an inoculum of MacFarland standard 4, it is important that isolates that display intermediate zone sizes of > 15 to ≤ 20 mm should be tested by an alternative method, such as Etest, to confirm full susceptibility. Whilst Etest and agar dilution are the preferred method for susceptibility testing of *H. pylori*, disc diffusion nevertheless offers an economical alternative to Etest that can be applied as a screening method in laboratories with insufficient throughput to justify agar dilution.

Fig. 1. MICs determined for 115 isolates of *H. pylori* for (a) ciprofloxacin and (b) rifampicin.

Frequency of resistance to ciprofloxacin

Of the 255 isolates tested by Etest and/or disc diffusion, 7.5 % ($n=19$) were ciprofloxacin resistant. This study is the first to have surveyed ciprofloxacin susceptibility of isolates of *H. pylori* from adult patients in the UK, and our results showed that the overall resistance based on the data from patients with reported failures by standard eradication regimens was 7.5 %, even when isolates were resistant to both metronidazole and clarithromycin (dually resistant). In the treatment failure patients, dual resistance was a feature of 88 % (106/121) of isolates, whereas we reported previously that dual resistance was less than 10 % for isolates from UK patients that were considered unlikely to have received previous eradication therapy based on the available clinical information (Chisholm & Owen, 2008). Furthermore, ciprofloxacin resistance was not a feature of the 10 exclusively metronidazole-resistant isolates and the two exclusively clarithromycin-resistant isolates from the treatment failure patients. Overall, the findings indicated that failed *H. pylori* eradication by standard treatment regimens had not selected specifically for ciprofloxacin resistance.

Our *H. pylori* resistance rate for ciprofloxacin is consistent with fluoroquinolone resistance rates of less than 10 % in various countries including Germany, where a 9 % ciprofloxacin resistance rate was reported in isolates from patients who had failed eradication therapy (Heep *et al.*, 2000a). Furthermore, a subsequent analysis provided evidence of a temporal trend in ciprofloxacin resistance, with rates for isolates from other German treatment failure patients rising from 11 % in 2003 to 22 % in 2005 (Glocker *et al.*, 2007b). The interpretation of direct comparisons between datasets needs to take into account differences in the types of patient populations studied and local treatment practices. For example, studies of ciprofloxacin resistance rates in Bulgaria (Boyanova *et al.*, 2008), Japan (Fujimura *et al.*, 2004), Egypt (Sherif *et al.*, 2004) and Iran (Falsafi *et al.*, 2004) included analyses of pre-treatment populations, and some included paediatric as well as adult patients. Interestingly, other independent studies have highlighted markedly different resistance rates within a particular country – for instance in France rates of 3 % (Tankovic *et al.*, 2003) and 17 % (Cattoir *et al.*, 2007), and likewise in Japan rates of 5.5 % (Fujimura *et al.*, 2004) and 15 % (Miyachi *et al.*, 2006).

Table 1. Characteristics of *gyrA* QRDR mutations in isolates of *H. pylori* with a ciprofloxacin (CIP)-resistant phenotype

<i>gyrA</i> mutation	CIP MIC (mg l ⁻¹)	1 µg disc zone size (mm)	Isolate no.	Biopsy*
Asp91Gly	2	19	H4281	Post-TF
Asp91Gly	4	19	H4573	Post-TF
Asp91Gly	12	14	H061240430	NK
Asp91Gly	16	0	H051660640	Post-TF
Asp91Gly	32	0	H053880517	Post-TF
Asp91Gly	>32	0	H042580533	Post-TF
Asp91Asn	16	0	H041960697	Post-TF
Asp91Asn	16	11	H064400334	NK
Asp91Asn	32	0	H060420429	NK
Asp91Asn	>32	0	H062040554	NK
Asp91Tyr	32	12	H064160518	NK
Asp91Tyr	>32	0	H064040339	NK
Asn87Lys	6	9	H050780467	Post-TF
Asn87Lys	32	0	H064640499	Post-TF
Asn87Lys	>32	0	H053140361	Post-TF
Asn87Lys	>32	0	H064160517	NK
Ala88Val	>32	0	H062160313	Post-TF
None	>32	0	H045160162	NK

*Clinical details provided indicated that gastric biopsy was taken post-treatment failure (Post-TF). Details for other cases were not known (NK). Each isolate was from an unrelated case.

It is unclear until more data are available whether these differences indicate a true temporal trend or population selection effects. Significantly higher levels of fluoroquinolone resistance have been reported in some countries, with rates as high as 20 % in Iran (Falsafi *et al.*, 2004) and 33 % in Korea (Kim *et al.*, 2004). The reason for these higher rates is unclear, but they may reflect different patterns of fluoroquinolone usage in those countries. For instance ciprofloxacin use in outpatients is lower in the UK relative to many other European countries (Muller *et al.*, 2007).

Rescue treatment regimens based on levofloxacin have been used widely as an alternative to ciprofloxacin, as levofloxacin is absorbed in tissues more efficiently and has fewer side effects. There is evidence that ciprofloxacin-resistant strains of *H. pylori* exhibit cross-resistance to levofloxacin (Bogaerts *et al.*, 2006). Although not investigated in the present study, previous investigations of levofloxacin resistance have reported rates of 8.8 % in the USA (Carothers *et al.*, 2007), 11.5 % in China (Lee *et al.*, 2008), 15 % in Japan (Miyachi *et al.*, 2006) and 19 % in Italy (Zullo *et al.*, 2007). In Italy, a levofloxacin resistance rate of 22 % was demonstrated in 67 isolates with resistance to both clarithromycin and metronidazole (Branca *et al.*, 2004). Carothers *et al.* (2007) found that levofloxacin resistance in the USA was associated with prior fluoroquinolone usage over the previous 10 years and with the total number of courses.

Characterization of *gyrA* mutations in ciprofloxacin-sensitive and -resistant isolates

The QRDR of *gyrA* was sequenced in 50 isolates, 18 of which were ciprofloxacin resistant, whilst the remaining 32

were ciprofloxacin sensitive. With one exception (strain H045160162), all of the ciprofloxacin-resistant isolates contained mutations in the QRDR of *gyrA* (Table 1) resulting in amino acid substitutions. Most (12/18, 67 %) isolates with a resistant phenotype contained mutations at aa 91, where Asp mutated to Gly ($n=6$), to Asn ($n=4$) or to Tyr ($n=2$). The *gyrA* QRDR of the remaining seven isolates contained single substitutions of Asn87→Lys ($n=4$) or Ala88→Val ($n=1$). The *gyrA* amino acid sequence was wild-type for the majority (91 %) of ciprofloxacin-sensitive isolates, and matched the QRDR sequence in GenBank (accession no. L29481; Moore *et al.*, 1995). Not all mutations in the *gyrA* QRDR were associated with resistance, as three isolates with a ciprofloxacin-sensitive phenotype (MICs ranging from 0.016 to 0.19 mg l⁻¹) contained the Asn87→Thr substitution.

Molecular markers for fluoroquinolone resistance in *H. pylori* are well documented (Cattoir *et al.*, 2007; Glocker *et al.*, 2007b) and we have confirmed that all but one of the UK isolates with a ciprofloxacin-resistant phenotype contained mutations in the QRDR of the *gyrA* gene. The particular mutations observed have all been reported previously (Moore *et al.*, 1995; Bogaerts *et al.*, 2006; Glocker *et al.*, 2007b) and were consistent with the most common mutations being located at aa 91, as found in isolates from France (Tankovic *et al.*, 2003; Cattoir *et al.*, 2007) and Japan (Fujimura *et al.*, 2004). Substitutions at aa 87 also were observed but not at the higher frequency noted in two large surveys of ciprofloxacin resistance in Belgium (Bogaerts *et al.*, 2006) and Germany (Glocker & Kist, 2004), suggesting geographical variation in *gyrA*

QRDR genotype distribution. The significance of such variations is unclear, but it has been reported that certain mutations might be linked to a higher level of resistance. For instance, analysis of Belgian isolates indicated that two specific mutations (Asn87→Ile and Asp91→Asn) were consistently associated with higher MICs ($>32 \text{ mg l}^{-1}$) (Bogaerts *et al.*, 2006). However, we were unable to correlate the mutation type of any UK isolate with MIC (Etest), and only one of the four isolates containing the *gyrA* Asp91→Asn marker also exhibited a similarly high MIC. Knowledge of these common mutations is important as it provides the basis for designing real-time PCR assays that can be used to provide a rapid susceptibility result for culture (Glocker & Kist, 2004) and offers the potential for direct application to gastric biopsies that have proved culture-negative – an approach already used successfully for clarithromycin and tetracycline testing (Chisholm & Owen, 2008).

Identification of rifampicin-resistant isolates of *H. pylori*

Rifampicin MICs were more difficult to determine as faint fading edges of growth on blood agar medium were observed for cultures of most of the 115 isolates tested. A range of MICs was observed for sensitive isolates ($0.016\text{--}2 \text{ mg l}^{-1}$) (Fig. 1b). The MIC₉₀ and MIC₅₀ values of the isolates examined were 1.0 and 0.25 mg l^{-1} , respectively (median 0.25 mg l^{-1} , mode 0.125 mg l^{-1}). One isolate (H062600603) was initially resistant (MIC 6 mg l^{-1} , zone of inhibition of 0 mm) but this was not stable on repeat testing (MIC 1 mg l^{-1}). Another isolate (H4573) was the only one in this set that was resistant by Etest (MIC 12 mg l^{-1}). Disc diffusion zones of inhibition were again difficult to demarcate due to the faint fading edges of growth, and ranged from 18 to 49 mm (mean 31 mm, mode 30 mm) for sensitive isolates ($n=31$). The range included the isolate considered resistant by Etest that gave an inhibition zone of 26 mm.

Frequency of resistance to rifampicin

H. pylori isolates were tested for susceptibility to rifampicin, as rifamycin derivative-based therapies have been shown to provide a highly effective and well-tolerated alternative eradication strategy (Gisbert & Pajares, 2005; Gisbert *et al.*, 2003; Toracchio *et al.*, 2005). Although there are no reports on the use of rifamycin-based therapies in the UK, our findings show that *H. pylori* resistance to this drug was $<1\%$, even when isolates from treatment failures by conventional therapies were included. Only two isolates (2/255, 0.8%) displayed possible resistance to rifampicin in the Etest and/or disc diffusion assays used, so we concluded that prevalence of resistance to this agent is currently very low in the UK. There is a general paucity of information on rifampicin/rifabutin resistance of isolates from other European countries, but a recent study in Germany reported an overall resistance rate of 1.4% and noted that the majority of resistant strains were isolated from patients after treatment failure (Glocker *et al.*, 2007a).

The technical aspects of testing the susceptibility of *H. pylori* to rifampicin have not hitherto been critically assessed, as inclusion of the drug in *H. pylori* eradication regimens is a relatively recent development. We observed that the precise MICs of the two rifampicin-resistant isolates were difficult to define, as cultures exhibited a faint fading edge of growth on agar plates. Repeat MIC testing of the isolates failed to resolve the problem encountered with interpretation, particularly as the disc diffusion test indicated resistance in only one of these isolates. As rifampicin susceptibility testing by Etest has been described to date in only two studies (Fujimura *et al.*, 2002; Heep *et al.*, 2002), it is important to develop standardized laboratory test methods and interpretative criteria. The possibility that difficulties in determining susceptibility in the current study could be overcome by alternative methods using different media or inocula cannot be excluded. Other less widely used methods of susceptibility testing such as agar dilution (Wang *et al.*, 2001) provide an alternative means of identifying resistant isolates and of validating the disc diffusion and Etest.

Investigation of *rpoB* for mutations associated with rifampicin resistance

The *rpoB* sequences at the 5' end of *rpoB* and in the *rpoB* cluster I and II regions of four isolates of *H. pylori* that were either resistant (two isolates with MICs of 6 and 12 mg l^{-1}) or potentially had reduced rifampicin susceptibility (two isolates with MICs of 1.5 and 2 mg l^{-1} but disc zone sizes of 12 and 13 mm) and five randomly selected rifampicin-susceptible isolates (disc zone sizes of 22–28 mm) were examined. All of the sequences had the wild-type amino acid sequence and no substitutions were present at any of the amino acid positions in the regions previously reported to be associated with rifampicin/rifabutin resistance in *H. pylori* (Heep *et al.*, 2000b, 2002; Wang *et al.*, 2001; Glocker *et al.*, 2007a). The use of molecular tests merits further investigation, as mutations in *H. pylori rpoB* at codons 525–545 and 586 have been shown to arise as a result of resistance induction (Heep *et al.*, 1999; Wang *et al.*, 2001). However, observations based on a single rifampicin-resistant clinical isolate from Germany identified only one mutation (V149F) at the 5' end of *rpoB* that was able to transform susceptible isolates to the resistant phenotype (Heep *et al.*, 2000b). None of these documented mutations in *rpoB* were found in either of the isolates with reduced susceptibility to rifampicin described in the current study, suggesting that the complete *rpoB* genes of a larger number of isolates with resistant phenotypes need to be sequenced and that the potential contribution of other mechanisms to this resistance should be explored.

Conclusions

As the study included isolates of *H. pylori* from patients who had failed eradication attempts by standard regimens, we conclude that such 'problem' patients could potentially

benefit from inclusion of ciprofloxacin/levofloxacin or rifampicin in future eradication therapy, as resistance rates are low in the UK. The isolates investigated were from gastric biopsy samples referred for routine testing and were not part of a systematic surveillance study; consequently, it is not possible to draw any conclusions about the general situation on *H. pylori* resistance to these antibiotics in the UK. However, future studies need to aim to include isolates from a more representative selection of cases and to ensure that resistance data on levofloxacin are available.

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