JOURNAL OF MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Garvey *et al.*, *Journal of Medical Microbiology* 2023;72:001776

DOI 10.1099/jmm.0.001776

MICROBIOLOGY SOCIETY

OPEN ACCESS

High incidence of antibiotic resistance amongst isolates of *Helicobacter pylori* collected in Nottingham, UK, between 2001 and 2018

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Abstract

Introduction. *Helicobacter pylori* is the leading cause of peptic ulcers and gastric cancer. The most common treatment regimens use combinations of two or three antibiotics and a proton pump inhibitor (PPI) to suppress stomach acid. The World Health Organization designated clarithromycin-resistant *H. pylori* as a high priority pathogen for drug development, due to increasing antibiotic resistance globally.

Hypothesis/Gap Statement. There is no routine surveillance of *H. pylori* primary antimicrobial sensitivities in the UK, and published data are lacking.

Aim. This study aimed to characterize antimicrobial sensitivities of isolates collected in Nottingham, UK, between 2001 and 2018.

Methodology. Gastric biopsy samples were collected, with informed written consent and ethics approval, from 162 patients attending the Queen's Medical Centre in Nottingham for an upper GI tract endoscopy. Antibiotic sensitivity was assessed using E-Tests and a more cost-effective disc diffusion test.

Results. The clarithromycin, amoxicillin and levofloxacin disc diffusion tests provided identical results to E-Tests on a subset of 30 isolates. Disparities were observed in the metronidazole test results, however. In total, 241 isolates from 162 patients were tested using at least one method. Of all isolates, 28% were resistant to clarithromycin, 62% to metronidazole and 3% to amoxicillin, which are used in first-line therapies. For those antibiotics used in second- and third-line therapies, 4% were resistant to levofloxacin and none of the isolates were resistant to tetracycline. Resistance to more than one antibiotic was found in 27% of isolates. The frequency of patients with a clarithromycin-resistant strain increased dramatically over time: from 16% between 2001 and 2005 to 40% between 2011 and 2018 (P=0.011). For the same time periods, there was also an increase in those with a metronidazole-resistant strain (from 58 to 78%; P=0.05). The frequencies of clarithromycin and metronidazole resistance were higher in isolates from patients who had previously received eradication therapy, compared to those who had not (40% versus 77%, and 80% versus 92%, respectively). Of 79 pairs of isolates from the antrum and corpus regions of the same patient's stomach, only six had differences in their antimicrobial susceptibility profiles.

Conclusion. Although there was high and increasing resistance to clarithromycin and metronidazole, there was no resistance to tetracycline and the frequencies of amoxicillin and levofloxacin resistance were very low.

Received 23 August 2023; Accepted 30 October 2023; Published 14 November 2023

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Keywords: amoxicillin; antimicrobial resistance; clarithromycin; *Helicobacter pylori*; levofloxacin; metronidazole; susceptibility testing.

Abbreviations: cag, cytotoxin associated gene; EUCAST, European Committee on Antimicrobial Susceptibility Testing; GI, gastrointestinal; GP, general practitioner; MALT, mucosa-associated lymphoid tissue; MIC, minimum inhibitory concentration; NSAID, nonsteroidal anti-inflammatory drug; PPI, proton pump inhibitor; VacA, vacuolating cytotoxin A; WHO, World Health Organisation.

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One supplementary table is available with the online version of this article.

Impact Statement

Helicobacter pylori is a bacterium that infects the stomachs of almost half of people world-wide. It can cause diseases, including ulcers in the stomach and duodenum, and gastric cancer. The most common treatments use combinations of two or three antibiotics and a proton pump inhibitor (PPI) to suppress stomach acid. Specific antibiotics are needed to deliver sufficient antibiotic to the complex niche which *H. pylori* inhabits. Resistance to some of the commonly used antibiotics, including clarithromycin, levofloxacin and tetracycline, renders them ineffective. Resistance to metronidazole is partial, meaning that it is still useful in combination with other antibiotics. Over the past few decades, the infection has become much more difficult to treat as antibiotic resistance has increased dramatically. In many countries, the antibiotic resistance of circulating strains is sampled and treatment guidelines are adjusted to employ the most appropriate antibiotics. In the UK, there is no routine antibiotic resistance testing of strains when the infection is first diagnosed. Patients are therefore given antibiotic therapies without knowing if they will be effective. Thus, despite having life-threatening disease and painful symptoms, some people need to have several rounds of different therapies before *H. pylori* is successfully cleared from the stomach.

This study tested the susceptibility of 241 *H. pylori* strains, gathered from 162 patients in Nottingham between 2001 and 2018, to the commonly used therapeutic antibiotics. We found a worrying trend of high and increasing resistance to clarithromycin and metronidazole, which are frequently used in initial treatments. There was no resistance to tetracycline, and the frequencies of amoxicillin and levofloxacin resistance were very low. We recommend regional *H. pylori* sensitivity sampling of strains in the UK, to guide selection of appropriate antibiotics. This would avoid unnecessary treatment failures and improve patient outcomes. Given the high rate of resistant strains that we, and other UK and international research groups, have found in patients who have previously been treated, we suggest that patients with failed first-line treatments undergo antibiotic sensitivity testing so that they can be given optimal treatments.

DATA SUMMARY

The authors confirm all supporting data and protocols have been provided within the article. A table containing the patient demographic and antimicrobial susceptibility of all isolates is provided (Table S1, available in the online version of this article).

INTRODUCTION

Helicobacter pylori is a widespread human pathogen, which usually causes asymptomatic chronic inflammation of the mucosal lining of the stomach [1]. The bacterium is a common part of the gastric microbiota, and its global prevalence is thought to be around 40% [2]. Colonization with this organism is usually established in early childhood and persists lifelong in the absence of effective treatment. Approximately 10–20% of *H. pylori*-positive individuals will develop gastric or duodenal ulcer disease, and around 1–2% of colonized individuals develop gastric cancer [1, 3]. The circumstances leading to disease are not yet fully known, however virulence of the colonizing strain, as well as host and environmental factors such as diet and smoking all contribute. The virulence factors that are most reported to influence the risk of disease are the *cag* pathogenicity island (encoding a type-IV secretion system that translocates CagA) and the active s1/i1 form of vacuolating cytotoxin A (VacA) [1].

Effective *H. pylori* eradication therapy is needed to heal and prevent recurrence of peptic ulcers, to treat low-grade gastric B cell mucosa-associated lymphoid tissue (MALT) lymphoma, and treat *H. pylori*-associated chronic dyspepsia, and also as an important part of a strategy to prevent gastric adenocarcinoma in high-risk groups [4]. The World Health Organisation (WHO) and International Agency for Research on Cancer consensus group classified *H. pylori* as a biological carcinogen [5]. Around 80% of gastric cancer cases can be attributed to *H. pylori* infection [6]. Approximately one million new cases of gastric cancer occur worldwide each year, and the prognosis is often poor because symptoms may only first become apparent when gastric cancer has reached an advanced stage [7]. In the UK, 54% of those diagnosed with gastric cancer die within a year and the 5-year survival rate is just 21% [8]. Gastric cancer is more common in males, and incidence rates vary in different countries, with the highest incidence (>30 per 100 000 males) and the highest mortality (21 per 100 000 males) in Eastern Asia. The annual global burden of gastric cancer is predicted to increase to approximately 1.8 million new cases and 1.3 million deaths by 2040 [9]. Despite decades of research, there is no effective vaccine against *H. pylori* infection [10], meaning that it is not possible to avoid using antibiotic treatment to prevent *H. pylori*-associated gastric cancer.

In the UK, *H. pylori* is usually treated with a triple therapy consisting of two antibiotics (clarithromycin, with either amoxicillin or metronidazole) and a proton pump inhibitor (PPI) drug such as omeprazole to suppress gastric acid secretion [11]. Antimicrobial resistance has become a serious issue world-wide over the past few decades, with the WHO designating clarithromycin-resistant *H. pylori* as a high-priority pathogen for drug development [12, 13]. In a survey from Vietnam, which has the highest incidence of gastric cancer in Southeast Asia, 33% of isolates collected in 2008 were resistant to clarithromycin, and 70% to metronidazole. A 2015 study from the same region found that 85.5% of isolates were resistant to clarithromycin [14]. Patients who have had a

recent clarithromycin treatment, for *H. pylori* or other infections, are at increased risk of harbouring a resistant strain, and this drug should not be used [4]. Amoxicillin-resistant strains are much less common, although one *H. pylori* strain was reported to express beta-lactamase [15], which is the main mechanism for resistance to penicillin antibiotics in other types of bacteria [16]. Individuals who are allergic to penicillins need to be prescribed other treatment options, which may not be as effective due to drug resistance. When first-line treatments fail, tetracycline and levofloxacin are commonly used as part of second-line therapy, but resistance to these is also increasing globally [17].

European clinical guidelines for the diagnosis and treatment of *H. pylori* infection [4], and the UK Health Securities Agency (formerly Public Health England) [11], recommend that patients with dyspepsia, peptic ulcer disease and MALT lymphoma should be tested for *H. pylori. H. pylori* treatment guidelines, from several clinical consortia, agree that the local antibiotic treatments around the world should be designed to work as effectively as possible, based on local antibiotic sensitivity data. In most of the world, antibiotic resistance profiles of circulating *H. pylori* strains are sampled, and this has led most regional and national guidelines to recommend a move away from clarithromycin-based triple therapy to other regimens [4, 13, 18–21]. Unfortunately, this information is not available in the UK because there is no routine culture or screening of isolates. The UK guidelines do state that antimicrobial sensitivity testing should be done when a patient has undergone two failed rounds of eradication therapy, however this culture-based work is often not carried out. Unfortunately, the approach often involves exposing patients to multiple rounds of combination antibiotics.

One large previous UK cohort study of *H. pylori*-infected adult patients, between February 2000 and May 2001, revealed that the overall success in eradicating *H. pylori* infection after one, two and three rounds of therapy was 73, 94 and 98%, respectively [22]. Several different first-line therapy regimens had been used, the most common being combinations of a PPI with amoxicillin and metronidazole (which achieved 65% successful eradication), PPI with amoxicillin and clarithromycin (72%), or PPI with metronidazole and clarithromycin (66%). Recent data on *H. pylori* antimicrobial sensitivity in the UK, however, are lacking. We therefore aimed to characterize antimicrobial sensitivities of isolates collected in Nottingham between 2001 and 2018. These isolates were cultured from gastric biopsies donated by patients attending the Queen's Medical Centre in Nottingham for an upper GI tract endoscopy. Antibiotic sensitivity was assessed using E-Tests, and a more cost-effective disc diffusion test method was also employed.

METHODS

Sample collection and *H. pylori* isolation

Gastric biopsy samples were collected from 162 patients attending Queens Medical Centre (Nottingham, UK) for a routine upper gastrointestinal endoscopy between 2001 and 2018. The Nottingham Research Ethics Committee 2 granted ethical approval for the use of the clinical samples and patient information for this study (reference 08/H0408/195). The patients all gave written, informed consent for collection of additional gastric biopsies for the purposes of research, and *H. pylori* status was determined in the clinic using a rapid biopsy urease test. Patients regularly taking high-dose non-steroidal anti-inflammatory drugs (NSAIDs) or antibiotics in the preceding 2 weeks were excluded from the study. Anonymized demographic information gathered included age, sex and smoking habits. *H. pylori* infection, and gastro-duodenal disease status was collected (Table 1).

All patients had been referred for an endoscopy by their general practitioner (GP), with the most common indication being chronic dyspepsia. The endoscopist's notes indicated whether patients had previously received *H. pylori* eradication therapy, and the number of rounds of treatment. Unfortunately, information about which specific antibiotics had previously been prescribed was held by GPs and it was not possible for us to access this.

H. pylori isolates were cultured from biopsy samples taken from the antrum and corpus regions of each patient's stomach, as previously described [23]. Upon recovery from the patient, the biopsies were immediately placed into Iso-sensitest Broth (Oxoid) containing 15% (vol/vol) glycerol, then swabbed onto the surface of blood agar plates, which were incubated for up to 5 days. The small colonies that grew were confirmed as *H. pylori* using Gram staining and urease testing, and were combined together. The number of colonies originating from each biopsy varied, and was not recorded. The colonies were suspended in Iso-sensitest Broth (Oxoid, UK) with 15% (vol/vol) glycerol (Courtin and Warner, UK) for storage at −80°C.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using E-Test strips (all 0.016–256 μg ml^{-1}, except levofloxacin 0.002–32 μg ml^{-1}) (bioMérieux, France) to evaluate the MICs for amoxicillin, clarithromycin, metronidazole, tetracycline and levofloxacin. Breakpoints indicative of resistance and susceptibility from the E-Test strips were taken from the EUCAST guidelines [24]. A disc diffusion method as described by Lang *et al.* [25], was also developed further and compared to the E-Tests. The breakpoints for antibiotic discs were taken from the published literature (Table 2).

Table 1. Demographics of the 162 patients from whom the *H. pylori* isolates were derived

Gender	Male 50.0% (*n*=81) Female 50.0% (*n*=81)
Age	Mean 53.2 years (range 19–86 years)
Gastro-duodenal disease status identified at endoscopy	Duodenal ulcer 38.9% (*n*=63) Gastric ulcer 11.1% (*n*=18) Gastric cancer 0.6% (*n*=1) Gastritis/duodenitis 6.8% (*n*=8/3) None 42.6% (*n*=69)
Previous failed *H. pylori* treatment	25.3% (*n*=41)
Isolates recovered from antral and corpus gastric biopsies	46 isolates from the antrum only (single-site isolates from 46 patients) 37 isolates from the corpus only (single-site isolates from 37 patients) 158 isolates, from both the antrum and corpus (paired isolates from 79 patients) Total of 241 isolates recovered from 162 patients
Smoking, % (*n/N*)	Non-smoker 72%(116/162) Smoker 15%(25/162) Ex-smoker 9%(14/162) Unknown 4%(7/162)
Virulence factor genotype of all isolates, % (*n/N*)	*cagA* status: positive 72%(166/232); negative 28%(66/232) (nine isolates removed due to ambiguous data) *vacA* type: i1 66%(152/231); i2 34%(79/231) (10 isolates removed due to ambiguous data)

Standardization of plates

Sterile petri dishes of 30 ml Mueller–Hinton agar (Oxoid, UK), supplemented with 7.5% defibrinated horse blood (Thermo Scientific) were poured and allowed to dry. Each batch was tested for consistency, using a control strain of *E. coli* DH5-α (Thermo Fisher Scientific, UK) suspended in sterile 0.85% saline to a standard of 3±0.2 on a Den1 McFarland Densitometer (Grant Instruments, Cambridgeshire, UK). A sterile cotton swab was soaked with the suspension, and then it was spread over the surface of the agar and allowed to sink in. A 10 µg amoxicillin disc (Oxoid Ltd, UK) was placed in the centre of the plate, which was then inverted and incubated for 16 h at 37°C, under normal atmospheric conditions. The diameter of the zone of clearing crossing the centre of the disc was then measured in millimetres using callipers. Batches of plates were used only if the zone diameter on the control plate was 12–20 mm. Plates were stored at 4°C for use within a maximum of 7 days after being poured.

Testing of *H. pylori* isolates

H. pylori strains were inoculated onto 5% (vol/vol) horse blood agar base no. 2 plates (Oxoid, UK), with incubation at 37°C for 48 h under microaerobic conditions (10% carbon dioxide, 5% oxygen, 85% nitrogen). The growth was suspended in sterile 0.85% saline, up to a standard of 3±0.2 on a McFarland Den1 Densitometer. A sterile cotton swab was then used to spread this suspension onto the prepared plates of Mueller–Hinton agar with 7.5% defibrinated horse blood (Thermo Scientific) and allowed to dry. One E-Test strip or antibiotic disc [clarithromycin (15 µg), amoxicillin (10 µg), levofloxacin (5 µg), tetracycline (30 µg), or metronidazole (5 µg) (all Oxoid, UK)] was then placed onto the centre of the plate using sterile forceps. For each set of sensitivity tests performed, a control of *H. pylori* strain NCTC 11637 was also plated and tested in the same manner for consistency of results.

Table 2. The breakpoints used to calculate the minimum inhibitory concentration (MIC) of antibiotic from E-Tests [24], and the diameters of the zone of clearing from disc diffusion tests indicative of resistance and susceptibility

Antibiotic	E-Test strip breakpoints – MIC (µg ml^{-1})		Disc diffusion test breakpoints – diameter of zone of clearing (mm)*		
	Resistant	Susceptible	Resistant	Susceptible	Reference
Clarithromycin	>0.5	≤0.25	≤28	>28	[51]
Amoxicillin	>0.125	≤0.125	≤25	>25	[25]
Levofloxacin	>1	≤1	<12	≥12	[52]
Tetracycline	>1	≤1	<25	>25	[25]
Metronidazole	>8	≤8	<16	≥21	[53]

*Strains that had a zone size between these breakpoints were classed as being of intermediate resistance.

Plates were incubated at 37°C under microaerobic conditions as described above for 5 days, after which the MICs and zones of clearing were measured in millimetres using callipers. All tests were performed in duplicate, and repeated independently by two researchers with consistent results.

PCR genotyping for *cagA*, and *vacA* i1 and i2 types

H. pylori isolates were genotyped for *vacA* and *cagA* using PCR, as previously described [26, 27].

Statistical analysis

Data was collected and recorded in Microsoft Excel. Statistical analysis was performed using GraphPad Prism 9.5.1 software. A Fisher's exact test was used to analyse frequencies of resistance between patients who had or had not previously undergone eradication therapy. For analysis of antibiotic resistance by year, a Chi-square test for a trend was used. *P*-values of $\leq$0.05 were considered statistically significant.

RESULTS

Isolates tested for antimicrobial susceptibility

Of the 162 infected patients in the study, 63 had duodenal ulceration, 18 had gastric ulceration and one had gastric cancer (Table 1). Forty-one patients in the study had previously undergone rounds of *H. pylori* eradication therapy and, upon return for further investigation, were still found to be *H. pylori*-positive. The number of previous rounds of therapy was not always specified in the endoscopy notes but, where this information was provided, the highest number was five. Altogether, 121 of the patients were infected with a more virulent *cagA*+ strain, and 102 patients had *H. pylori* of the more virulent *vacA* i1 type.

Gastric biopsies were collected from the antrum and corpus regions of each patient's stomach during endoscopy. Isolates that combined multiple colonies were cultured from both antral and corpus biopsies from 79 patients. Overall, 46 patients yielded an isolate only from the antrum, and isolates were recovered only from the corpus of a further 37 patients. This yielded a total of 241 isolates for characterization; 83 were single isolates from individual patients, and 158 were paired isolates from the antrum and corpus. The latter enabled investigation of whether the isolates from the two gastric regions of the same patient had different antibiotic sensitivities.

For the isolates tested using E-Test strips, the mean values and total range of MICs are summarized in Table 3. When isolates grew immediately adjacent to the E-Test strip, with no zone of clearance, the MIC was recorded as the highest concentration on the test strip (32 μg ml^{-1} for levofloxacin, 256 μg ml^{-1} for all others). This was the case for 15/19 clarithromycin resistant isolates, all 10 levofloxacin-resistant isolates, and 26/45 metronidazole-resistant isolates. Of the six amoxicillin-resistant isolates, the MICs ranged between 0.19 and 0.75 μg ml^{-1}.

Comparison of E-Test and disc diffusion methods

Because the E-Test method is more expensive than the previously used antibiotic disc diffusion protocol, we looked to develop the disc method and assess the results against E-Tests. For this, 30 isolates were tested side by side, and the categorical results (resistant, sensitive and intermediate) were compared (Table 4). The results showed 100% agreement between the tests for clarithromycin (20 resistant, 10 sensitive), amoxicillin (four resistant, 26 sensitive) and levofloxacin (six resistant, 24 sensitive). When testing metronidazole susceptibility using disc tests, there is the complicating possibility for an intermediate result, therefore an additional 16 isolates were tested and the results compared.

There were 36 resistant, four intermediate and six sensitive results from metronidazole disc diffusion tests, compared with 25 resistant and 21 sensitive results using E-Tests. Where the disc test indicated that the isolate was sensitive to metronidazole (zone of inhibition $\geq$21 mm; n=6), this result was completely replicated by the E-Test (mean MIC 0.31 μg ml^{-1}, range 0.016 to 0.5 μg ml^{-1}). As anticipated, where there was no clearance around the antibiotic discs (n=22), the E-Tests provided the same resistant determination (mean MIC 213.5 μg ml^{-1}, range 12 to >256 μg ml^{-1}). Problems arose, however, where the diameters of

Table 3. Summary of MIC data from E-Tests

	Clarithromycin	Amoxicillin	Tetracycline	Levofloxacin	Metronidazole
Mean MIC (μg ml^{-1})	80.0	0.055	0.118	5.1	60.9
Range (μg ml^{-1}) (minimum and maximum values)	0.016 to >256	0.016 to 0.75	0.016 to 0.565	0.036 to >32	0.016 to >256
No. of isolates tested	59	100	58	63	126
Resistant/sensitive	23/36	6/94	0/58	9/54	45/81

Table 4. Comparison of results from disc diffusion and E-Test assays

Antibiotic	Test method	Resistant	Sensitive	Intermediate
Clarithromycin	**Disc**	*n*=20	*n*=10	N/A
	E-Test	*n*=20 mean MIC 174.8 μg ml^{-1} (range 1.5 to >256 μg ml^{-1})	*n*=10 mean MIC 0.027 μg ml^{-1} (range 0.016 to 0.094 μg ml^{-1})	N/A
Amoxicillin	**Disc**	*n*=4	*n*=26	N/A
	E-Test	*n*=4 mean MIC 0.46 μg ml^{-1} (range 0.19 to 0.75 μg ml^{-1})	*n*=26 mean MIC 0.036 μg ml^{-1} (range 0.016 to 0.125 μg ml^{-1})	N/A
Levofloxacin	**Disc**	*n*=6	*n*=24	N/A
	E-Test	*n*=6 mean MIC >256 μg ml^{-1} (all >256 μg ml^{-1})	*n*=24 mean MIC 0.207 μg ml^{-1} (range 0.064 to 0.75 μg ml^{-1})	N/A
Metronidazole	**Disc**	*n*=36	*n*=6	*n*=4
	E-Test	*n*=25 mean MIC 198.56 μg ml^{-1} (range 12 to >256 μg ml^{-1})	*n*=21 mean MIC 0.292 μg ml^{-1} (range 0.016 to 0.5 μg ml^{-1})	N/A

N/A=not applicable.

visible zones were smaller than the breakpoint of 21 mm. In the four isolates with intermediate resistance from disc tests (diameters of 17 to 20 mm), these were all found to be sensitive by E-Tests (mean MIC 0.29 μg ml^{-1}, range 0.016 to 0.5 μg ml^{-1}). The disc tests were also less accurate in determining the susceptibility to metronidazole, when the zone diameters were 5–15 mm (14 resistant by disc diffusion; 3 resistant and 11 sensitive by E-Test). This meant that the disc diffusion test results were unreliable and the isolates with zones in this range had to be re-tested using E-Tests.

The remaining isolates were then tested using the disc diffusion method, or E-Tests as appropriate, to generate a full dataset on the total of 241 (details are provided in Table S1).

Antimicrobial resistance profiles of 241 *H. pylori* isolates to five commonly used antibiotics

Of the whole collection of isolates, 31.9% were not resistant to any of the five antibiotics. 61.8% were resistant to metronidazole, 27.8% were resistant to clarithromycin and 2.5% were resistant to amoxicillin (Table 5). These are the commonly used antibiotics in first-line therapies in the UK. If the first round of treatment fails, then other drugs may be used in second- and third-line therapies including levofloxacin and tetracycline. No isolates were found to be resistant to tetracycline, but 4.1% were resistant to levofloxacin. In these tests using individual antibiotics, 40.7% were resistant to one, 24.1% were resistant to two (the vast majority of these being metronidazole and clarithromycin), and 3.3% were resistant to three antibiotics (clarithromycin, amoxicillin and metronidazole, or clarithromycin, levofloxacin and metronidazole, or clarithromycin, amoxicillin and levofloxacin). None of the isolates were resistant to more than three antibiotics tested.

The eight isolates resistant to three antibiotics originated from five patients. Four had previously received eradication therapy, including one who had undergone five failed rounds of therapy (their paired antrum and corpus isolates were both resistant to clarithromycin, amoxicillin and metronidazole).

Resistance profiles of the paired isolates

Of the 162 infected patients who donated gastric biopsies to the study, isolates were successfully cultured from both the antrum and the corpus tissues in 79 cases. Forty-six isolates were cultured only from the antral biopsies, and 37 isolates were cultured only from the corpus.

Of the 79 pairs of isolates, the antibiotic resistance profiles were identical in all but six. Two of these pairs differed in metronidazole resistance. In one instance, the antral isolate was resistant with an MIC of 64 μg ml^{-1}, and the corpus isolate was sensitive (MIC 0.19 μg ml^{-1}). In another case the antral isolate was resistant to metronidazole (MIC 192 μg ml^{-1}) and clarithromycin (MIC >256 μg ml^{-1}), whilst the corpus isolate was sensitive to both antibiotics (MICs 0.047 and 0.5 μg ml^{-1}, respectively). Three additional pairs differed in clarithromycin resistance, also with large differences in MICs (>256 and 0.016 μg ml^{-1}; 0.016 and 1.5 μg ml^{-1}; 0.38 and 0.125 μg ml^{-1} for antral and corpus isolates, respectively). One pair differed in levofloxacin resistance (MICs of 0.25 and 12 μg ml^{-1}). Interestingly in one case, the isolate from the corpus biopsy was resistant to metronidazole (MIC >256 μg ml^{-1}),

Table 5. Antimicrobial resistance data for all 241 isolates analysed in the study

Antibiotic	No. of resistant isolates (from *n*=241), percentage
Clarithromycin	67, 27.8%
Metronidazole	149, 61.8%
Levofloxacin	10, 4.1%
Amoxicillin	6, 2.5%
Tetracycline	0, 0%
Resistant to none of the antibiotics	77, 31.9%
Resistant to one antibiotic	98, 40.7%
Resistant to two antibiotics	58, 24.1%
Resistant to three antibiotics	8, 3.3%
Resistant to >three antibiotics	0, 0%
Clarithromycin and metronidazole only	58, 24.1%
Levofloxacin and metronidazole only	5, 2.1%
Clarithromycin, amoxicillin and levofloxacin	2, 0.8%
Clarithromycin, levofloxacin and metronidazole	2, 0.8%
Clarithromycin, amoxicillin and metronidazole	4, 1.7%

All tests were done with single antibiotics. Only the detected resistance profiles to more than one antibiotic are listed.

clarithromycin (MIC 1.5 μg ml^{-1}) and amoxicillin (MIC 0.75 μg ml^{-1}), whilst the antral isolate was only resistant to metronidazole (MIC >256 μg ml^{-1}). Of these six pairs of isolates, five had identical *vacA* i genotypes and matching presence or absence of *cagA*. One pair differed in *cagA* status, but were of the same *vacA* i1 type.

Apart from the mismatches in antimicrobial resistance between pairs of isolates from the same patient, different virulence factor genotypes were noted in a further five pairs. One pair had completely different *vacA* and *cagA* types (*vacA* i2, *cagA*+ in the antral isolate and *vacA* i1, *cagA*- in the corpus), three had the same *vacA* type but differed in *cagA* type, and one pair of isolates were *cagA* positive but had different *vacA* types. None of these had differences in their antimicrobial sensitivity profiles, however.

The frequencies of drug-resistant isolates from patients with and without previous eradication therapy

To avoid bias from double counting the 79 pairs of isolates in the dataset, only one isolate per patient was considered further, and all data from the six discordant pairs were excluded. It was then determined for each individual patient (from the remaining total of 156) whether they harboured a drug-resistant isolate. We compared the proportions of patients with resistant isolates between two groups: 39 with a previously failed eradication therapy, and 117 for which there was no record of previous therapy to eradicate *H. pylori* (Fig. 1).

In total, 23 of the 39 patients (59.0%) who previously received therapy were colonized with a clarithromycin-resistant isolate, compared with 19 of the 117 patients who had not (16.2%) (Fisher's exact test, $P<0.0001$). Altogether, 33 of the 39 patients (84.6%) who previously received therapy harboured a metronidazole-resistant isolate, compared with 71 of the 117 patients who had not (60.7%) ($P=0.006$). Only three patients in total had amoxicillin-resistant strains, all having had previous therapy. Seven patients had levofloxacin-resistant isolates, three of whom had previously received therapy (3/39) and four who had not received therapy (4/117). There was no significant difference.

Antibiotic resistance of *H. pylori* isolates over time

Trends in the incidence of antibiotic resistance were then examined over the 2001–2018 period of isolate collection, again based on whether the 156 patients harboured a resistant strain (Fig. 2). The data were also broken down according to whether the patients had previously received *H. pylori* eradication therapy or not.

In the whole dataset, from 156 patients (Fig. 2a), there was a significant increase in the frequency of patients with clarithromycin-resistant isolates from 15.6% (7/45) in 2001–2005 to 40.0% (16/40) in 2011–2018 (Chi-square test for trend, $P=0.011$). There was also a marked increase in metronidazole-resistant isolates, from 57.8% (26/45) in 2001–2005 to 77.5%

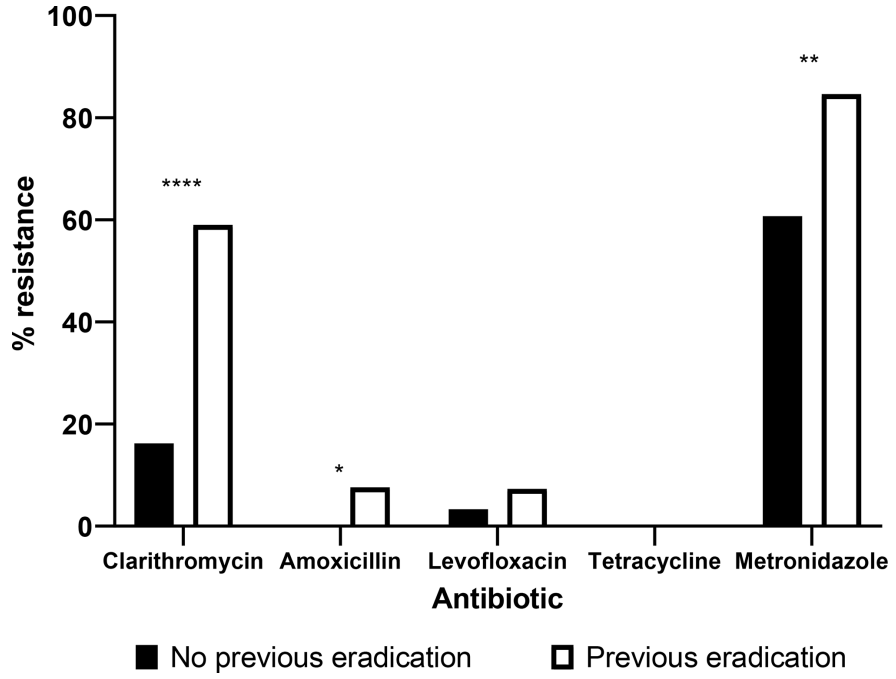

Fig. 1. The frequencies of patients with resistant isolates, who had (*n*=39) or had not (*n*=117) previously undertaken *H. pylori* eradication therapy. The frequency of clarithromycin-resistant isolates was significantly higher amongst the patients with previous therapy (23/39), compared to those who had not previously been treated (19/117; *P*<0.0001****). The frequency of amoxicillin-resistant isolates was significantly higher amongst the patients with previous therapy (3/39) compared to those who had not previously been treated (0/117; *P*<0.05*). The frequency of metronidazole-resistant isolates was significantly higher amongst the patients with previous therapy (33/39), compared to those who had not previously been treated (71/117; *P*<0.01**). There were no significant differences in the frequencies of levofloxacin-resistant isolates amongst patients who had (3/39) or had not (4/117) previously been treated. None of the patients had tetracycline-resistant isolates.

in 2011–2018 (31/40) (trend *P*=0.05). When examining the data on patients who had previously received *H. pylori* eradication therapy (Fig. 2b), there was also an increase in the frequency of clarithromycin-resistant isolates from 40% (2/5) in 2001–2005 to 76.9% (10/13) in 2011–2018 but this did not achieve statistical significance. The frequency of patients with metronidazole-resistant isolates was 80.0% (4/5) in 2001–2005 and 92.3% (12/13) in 2011–2018, which was also not statistically significant. The frequencies of clarithromycin-resistant and metronidazole-resistant isolates from patients who had not previously received eradication therapy also followed the same trends (Fig. 2c), and increased during the period of the study. The differences were not statistically significant, however. In 2001–2005, the frequencies of clarithromycin-, metronidazole-, amoxicillin-, levofloxacin- and tetracycline-resistant isolates from this sub-group were 12.5% (5/40), 55.0% (22/40), 0% (0/40), 2.4% (1/40), and 0% (0/40). In 2011–2018, the corresponding frequencies were 25.9% (7/27), 70.4% (19/27), 0% (0/27), 3.7% (1/27) and 0%, respectively.

The frequencies of antibiotic-resistant isolates with differing virulence factor type

Since there is an increased risk of disease arising from infection with a more virulent strain of *H. pylori*, it was investigated whether there were any differences in the frequencies of antibiotic-resistant isolates amongst the *cagA* positive and negative groups. Isolates from the 162 patients were all PCR genotyped for presence and absence of the *cagA* gene, and this result was confirmed by testing for anti-CagA antibodies in samples of their serum (details in Table S1).

Of the total 241 isolates in the study, 166 were *cagA* positive and 66 isolates were *cagA* negative. Nine isolates were removed from the analysis, as the PCR and serology results did not match. The proportions of antibiotic-resistant isolates in the *cagA* positive group were very similar to the *cagA* negative group (Table 6). There were no statistically significant differences.

Similar results were found when stratifying the isolates according to *vacA* i1 and i2 types (data not shown), where i1 strains are associated with increased risk of disease [26]. In addition, there were no significant differences in the frequencies of antibiotic-resistant isolates from male or female patients, and there were no differences according to whether or not they smoked cigarettes (not shown).

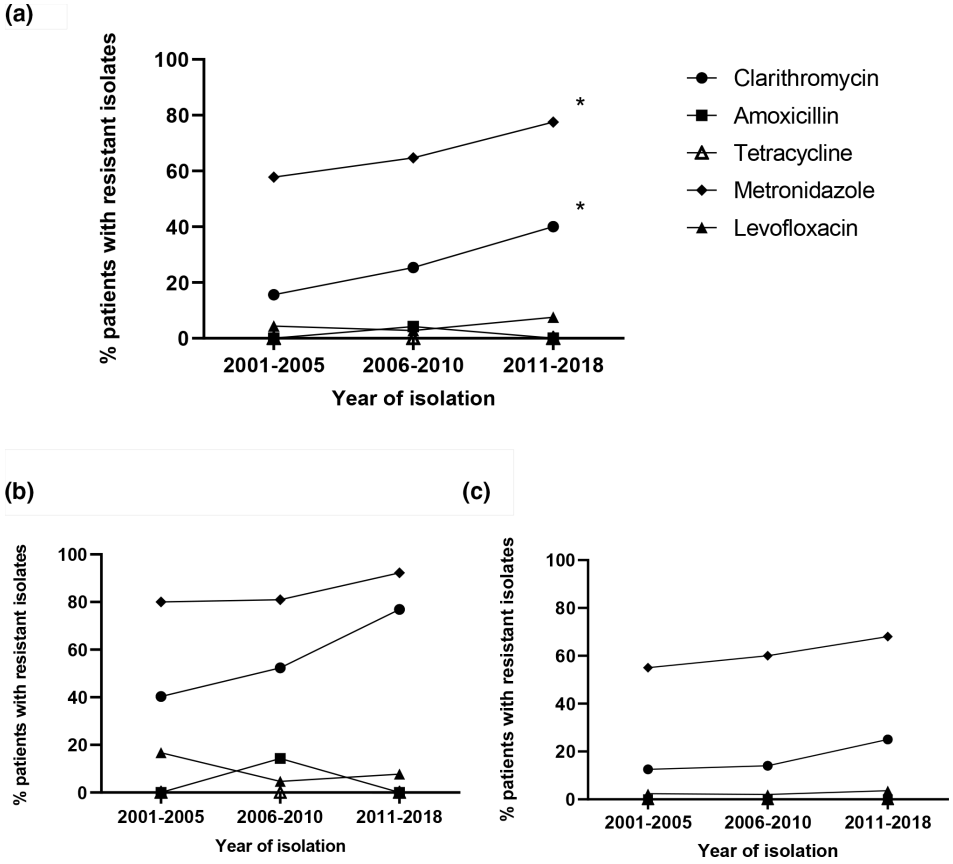

Fig. 2. The frequencies of patients with resistant isolates, who had or had not previously undertaken *H. pylori* eradication therapy over the period of the study. For the whole dataset (a), 2001–2005 *n*=45, 2006–2011 *n*=71 and 2011–2018 *n*=40. For the patients with previous eradication therapy (b), 2001–2005 *n*=5, 2006–2011 *n*=21, and 2011–2018 *n*=13. For the patients without previous eradication therapy (c), 2001–2005 *n*=40, 2006–2011 *n*=50, and 2011–2018 *n*=27. Significant trends in the data were found for clarithromycin-resistant ($P \leq 0.05^*$) and metronidazole-resistant ($P \leq 0.05^*$) isolates, but only in the whole dataset (a). No significant trends were found for the other antibiotics, or within the subgroup analyses (b and c).

DISCUSSION

The success rate of standard triple therapy for eradication of *H. pylori* is decreasing in many parts of the world, where increasing prevalence of antibiotic resistance has been found. The efficacies of regimens containing clarithromycin, levofloxacin and amoxicillin are seriously impacted by resistance to these antibiotics [28]. In order to ensure that treatment choices are appropriate and likely to be effective, it is important to investigate the antibiotic sensitivities of isolates in the local geographic region. We performed these assays for a collection of UK isolates, and also developed a disc diffusion method that was reliable for clarithromycin, amoxicillin and levofloxacin susceptibility testing, which could provide others with a cheaper way to assess their isolates. This could overcome some cost barriers and facilitate better surveillance. The assay was not reliable for metronidazole tests, but this antibiotic can be an effective part of treatment regimens even when the strain is 'resistant'. Thus the clinical utility of resistance testing for metronidazole is arguable.

Table 6. Frequencies of antibiotic resistance amongst the *cagA* positive and negative isolates (*n*=241)

	No. and percentage of antibiotic-resistant isolates				
	Clarithromycin	**Amoxicillin**	**Levofloxacin**	**Tetracycline**	**Metronidazole**
***cagA*+ (*n*=166)**	43; 25.9%	5; 3.0%	4; 2.4%	0; 0%	100; 60.2%
***cagA*- (*n*=66)**	20; 30.3%	0; 0%	2; 3.0%	0; 0%	43; 65.2%

*isolates with inconclusive *cagA* status were removed from the dataset.

Both types of tests were quick and easy to perform, taking the same amount of time and requiring the same numbers of culture plates. The main advantages of E-Tests are that they provide an MIC value, rather than a binary designation of susceptible or resistant. This provides additional useful data, which can inform on emerging resistance from trends in MIC levels. The main disadvantage of using E-Test strips was their cost, at over 30-fold that of antibiotic discs. Whilst disc diffusion tests were equally effective as E-Tests in determining clarithromycin, amoxicillin and levofloxacin susceptibility, their main disadvantage was the lack of reliability for metronidazole susceptibility testing. This issue has also been reported by others [29].

In our collection of isolates gathered between 2001 and 2018, we found that 32% were sensitive to all the main therapeutic antibiotics tested (clarithromycin, amoxicillin, metronidazole, levofloxacin and tetracycline). In total, 41% of the isolates were resistant to one of these drugs, 24% were resistant to two, and 3% were resistant to three. The prevalence of antibiotic resistance in our study has similarities to that reported by McNulty *et al.* in 2012 [30], based on isolates gathered in 2009–2010 from three UK centres (London, Bangor and Gloucester). In our study we found 28% clarithromycin resistance overall, and 62% metronidazole resistance. For those without known previous *H. pylori* eradication therapy 16% were colonized by a clarithromycin-resistant strain. McNulty *et al.* [30] found higher rates of resistance to these first-line treatment antibiotics, at 68 and 88%, respectively, in their isolates from London. The rates were lower (18 and 43%) in isolates from Bangor, and it was postulated that there was a higher frequency of resistant isolates in London due to more of the patients having been born outside the UK.

It is recommended that sampling is carried out to assess the prevalence of clarithromycin-resistant strains [4], however no such sampling is routinely carried out in the UK at the present time. We detected that the frequency of clarithromycin-resistant isolates from patients without previous eradication therapy increased from 12.5% in 2001–2005, to 14.0% in 2006–2010 and to 25.0% in 2011–2018. This is above the recommended 15% limit for its effective use as a first-line agent [4]. The observed trend is likely to increase further, as increasing global migration is resulting in the spread of *H. pylori* from countries with much higher frequencies of antibiotic resistance [31]. If clarithromycin is inappropriately prescribed for the eradication of *H. pylori*, it greatly reduces the likelihood of treatment success, and unfortunately also increases the acquisition of resistance by other bacteria in the host microbiome [32, 33].

Metronidazole resistance was also more prevalent in the samples collected in 2011–2018, although the increase from the 2001–2005 sampling period was not as marked. Metronidazole accounts for just 1.8% of total consumption of antibiotics in the UK, and its use has been declining over the past 5 years [34]. This may indicate that the frequencies of resistant isolates are now stabilizing. We detected a metronidazole-resistant isolate in 78% of patients during 2011–2018, and in 92% of patients who had previously received eradication therapy during this time interval. Although these levels are high, resistance to metronidazole does not preclude its use in a therapeutic combination [35]. Testing for metronidazole resistance is therefore of a lower clinical priority.

We found the frequency of amoxicillin resistance to be very low, with just six from a total of 241 isolates (2.5%). In line with our study, McNulty *et al.* [30] also found that amoxicillin resistance was rare at all three of their UK centres (London, Bangor and Gloucester). We were unable to find any tetracycline-resistant isolates, whereas the McNulty study found four isolates (1.7% overall). Other studies have also shown that tetracycline-resistant isolates are very low or absent in the UK and in Europe [36, 37]. We found that the frequency of levofloxacin-resistant isolates was also low (4%), but McNulty *et al.* reported 13% resistance in London, 17% in Bangor and 1% in the isolates from Gloucester. A study in France in 2018 reported 17.6% primary and 22.7% secondary resistance to levofloxacin [38]. A study of *H. pylori* antimicrobial resistance in 18 European countries in 2018 found that the frequency of primary levofloxacin resistance ranged from zero in Denmark and the Netherlands, to 29.2% in Italy [39]. Use of levofloxacin is much less common in the UK than in other countries, and has also been decreasing between 2017 and 2021 [34]. This may explain why we found fewer levofloxacin-resistant isolates than reported for other countries outside the UK.

Our data agreed with many other previous studies showing that a previous course of clarithromycin or metronidazole was associated with an increase in the risk of antibiotic resistance to that drug [30, 40], and we also found significantly increased frequencies of clarithromycin-resistant and metronidazole-resistant isolates over the time of sample collection [40, 41]. We were unable to find any differences in resistance rates between isolates from male and female patients, and unlike some other reports, there were no associations with cigarette smoking [42, 43].

We were surprised to find six isolates (from four patients, three of whom previously had undergone eradication therapy) were resistant to amoxicillin. One isolate had an MIC of 0.19 μg ml^{-1}, close to the breakpoint concentration (0.125 μg ml^{-1}). Three had MICs of 0.5 μg ml^{-1} or greater. A recent study from Vietnam found a frequency of 25.7% amoxicillin-resistant isolates, which had an MIC range of 0.19–1.5 μg ml^{-1}. 44% of their resistant isolates yielded MICs of 0.5–1.5 μg ml^{-1}, which is extremely concerning [44]. Although *H. pylori* does not usually possess a beta lactamase gene, they showed that the resistance phenotype was linked to seven amino acid changes in penicillin-binding protein 1A. We now plan to conduct genome sequence analysis on our amoxicillin-resistant isolates to investigate mutations in their penicillin-binding protein genes.

We found that the vast majority of paired isolates, from the antrum and corpus tissues of the same patient, had identical antimicrobial resistance profiles. Six sets of isolates had different profiles, however, and one of these also differed in *cagA* status. This may indicate that some of these patients were infected with multiple strains. Presence of more than one strain could be a factor

in the failure of eradication therapy, and it has been recommended that this possibility should be considered when carrying out antimicrobial susceptibility testing [45]. Genomic analysis will be carried out on the discordant pairs of isolates to confirm whether there were multiple strain infections in these six patients. In previous studies we performed whole-genome deep sequencing on a small subset of the isolates described here (but not the six described above), and found extensive allelic diversity amongst populations within the antrum and corpus regions of each patient's stomach [46]. Antrum and corpus populations from the same patient grouped together in phylogenetic analyses, indicating that most patients were initially infected with a single strain, which then diversified. Recombination was observed both within and between different regions of the same patient's stomach. These findings were in agreement with Ailloud *et al.* (2021) [47], who found gastric region-specific diversity and recombination with bacteria from different regions. Their data also suggested that antibiotic treatments (including those for extra-gastric conditions) are likely to have a major influence on the population structure of *H. pylori* in the stomach. This is another potential driver of the global trend for increasing prevalence of antibiotic-resistant *H. pylori* [4].

Limitations of the study include that the isolates were derived from cohorts of patients attending an endoscopy clinic for investigation of symptoms. These patients are therefore not representative of the general population, and are more likely to include patients with *H. pylori* that has been difficult to treat in primary care. Additionally, as treatment for this infection requires multiple antibiotics in triple or quadruple therapy, the study could have been improved by testing for cross-resistance or synergistic effects using combinations of the drugs. We only tested the sensitivity of the isolates to single antibiotics. Additionally, it would have been more informative to have isolated single colonies of *H. pylori* from the biopsies, rather than combine them together. These single colonies may have varied genetically and in the phenotype of antimicrobial susceptibility. We are planning to investigate this in a future study.

Our data are important because there is currently very little antibiotic sensitivity testing of *H. pylori* isolates in the UK. This is despite guidance that *H. pylori* sensitivity testing should be carried out when patients have a restricted choice of antibiotic due to hypersensitivity, have previously received two unsuccessful courses of antibiotic treatment, or when known local resistance rates are high [11]. The main barriers to testing in the UK include the requirement for endoscopy to obtain isolates and the associated costs. Data on local resistance rates are unavailable for the UK and, considering our findings, this puts patients at high risk of being given ineffective first-line therapies. Multiple further rounds of therapy may be needed in order for the infection to be eradicated successfully, which could have detrimental effects on the patient including exposure to more antibiotic side effects, and the continuation of symptoms. It has recently been recommended that patients are tested for clarithromycin-resistant *H. pylori* prior to prescribing this drug [4], however no such tests are routinely carried out in the UK at the present time. We agree that testing of *H. pylori* isolates should be improved in the UK, especially when considering clarithromycin as a therapeutic option. Stool-based PCR tests for clarithromycin resistance have been shown to be extremely accurate [48, 49]. In a recent study in France, comparing the Amplidiag *H. pylori* +ClariR stool PCR test for detecting resistance to clarithromycin with data from E-Tests, the sensitivity was 98.4% and the specificity was 100% [48]. Such non-invasive testing has the potential to dramatically enhance successful therapeutic choices.

In conclusion, although there was a trend of high and increasing resistance to clarithromycin and metronidazole, there was no resistance to tetracycline and the frequencies of amoxicillin and levofloxacin resistance were very low. The incidence of antibiotic resistance will probably have increased further since the 2018 isolate collection in our study, as observed in many other parts of the world [2, 50]. We recommend the expansion of *H. pylori* sensitivity testing in the UK, including increased availability of stool-based PCR testing for clarithromycin resistance, to prevent unnecessary treatment failures and avoid exacerbating antimicrobial resistance in the gut microbiota.

Funding information
At the University of Nottingham, Elizabeth Garvey was supported by a PhD studentship from the Wellcome Trust Doctoral Training Centre in Antimicrobials and Antimicrobial Resistance, and Suffi Suffian was supported by a PhD studentship from the Malaysian Government. At Nottingham Trent University, Daniel Whiley was supported by a Vice-Chancellor's PhD Scholarship and the School of Science and Technology at Nottingham Trent University, and Farah Mahmood is supported by a PhD studentship from the Biotechnology and Biological Sciences Doctoral Training Programme. Karen Robinson is supported by the Medical Research Council (MR/R026343/1), and the National Institute for Health Research (NIHR) through the Biomedical Research Centre at Nottingham University Hospitals NHS Trust and The University of Nottingham (NIHR203310). The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Acknowledgements
We thank Ms Melanie Lingaya, who also isolated *H. pylori* from human gastric biopsies.

Author contributions
Conceptualization and supervision: K.R., J.A.W. Investigation: J.R., E.G., S.S., F.M., N.B., D.W., D.L., J.W. Data analysis: E.G., J.R., S.S. Writing: K.R., E.G., D.W., F.M., D.L., J.A., J.W., J.A.W.

Conflicts of interest
The author(s) declare that there are no conflicts of interest.

Ethical statement

H. pylori strains were isolated from gastric biopsies donated by patients attending Queens Medical Centre Hospital, Nottingham, UK with written informed consent and ethical approval from the Nottingham Research Ethics Committee 2(08/H0408/195)

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