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Risk factors associated with Helicobacter pylori infection treatment failure in a high prevalence area.

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Risk factors associated with *Helicobacter pylori* infection treatment failure in a high prevalence area

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SUMMARY

Triple therapy is commonly used for the treatment of *Helicobacter pylori* infection. We determined risk factors associated with its failure in compliant patients focusing on *H. pylori* density, virulence marker and 23S ribosomal RNA (rRNA) point mutations associated with clarithromycin resistance. *H. pylori* infection was diagnosed by ^{14}C urea breath test (^{14}C UBT) and rapid urease test or histology. Triple therapy with esomeprazole 20 mg b.i.d., amoxicillin 1 g b.i.d. and clarithromycin 500 mg b.i.d. was prescribed for 10 days. ^{14}C UBT was repeated 4 weeks after treatment. In total, 111 patients [69 (62%) males] with a mean age of 46 ± 16 years were enrolled. The mean age of treatment failure was 39 ± 14 years compared to 48 ± 16 years with eradication ($P = 0{\cdot}002$). Treatment failure was associated with younger mean age, point mutations in the *23S rRNA* gene of *H. pylori* and *vacA s1a* and *m1* when associated with *cagA* negativity.

Key words: *cagA*, *H. pylori*, histology, point mutations, *vacA*.

INTRODUCTION

The Gram-negative bacterium, *Helicobacter pylori*, colonizes the human stomach, with prevalence rates from 25% in Western countries to over 90% in developing countries [1, 2]. *H. pylori* plays a significant role in the pathogenesis of gastritis and peptic ulcer disease, low-grade mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma (GC) [3, 4]. Its eradication dramatically affects the natural history of both peptic ulcer and gastric lymphoma [5]. European and U.S. guidelines advised the use of triple therapies [proton-pump inhibitor (PPI), clarithromycin + amoxicillin, or metronidazole] for 7–14 days to cure this infection [6]. However, *H. pylori* resistance against clarithromycin is increasing worldwide, reducing the success rate of standard triple therapies to mean values as low as 18–44% in some part of the world [7, 8]. In a previous multicentre study in patients with active duodenal ulcer, 1-week triple therapy with PPI, amoxicillin + clarithromycin eradicated *H. pylori* infection with per protocol (PP) rates of 72% and 62%, respectively, with intention to treat (ITT) analysis in patients recruited from Pakistan [8]. Low cure rate and a higher resistance to clarithromycin are observed in *H. pylori*-positive patients with functional dyspepsia than for peptic ulcer disease [9–11]. Clarithromycin resistance, the major cause of *H. pylori* treatment failure, is associated with point mutations within the peptidyltransferase-encoding region in domain V of the *23S rRNA* gene [12]. These mutations include A2142G or C, A2143G or C, A2115G, G2141A, and A2142T [12–14]. These common mutations can be detected using polymerase chain reaction (PCR)–DNA enzyme immunoassay, PCR line probe assay, and real-time PCR [15–17]. The

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method of detecting *H. pylori* by PCR directly on DNA derived from gastric biopsy specimens, and detection of macrolide resistance with PCR–restriction fragment length polymorphism (PCR–RFLP) directly on biopsy specimens, have been evaluated previously [18–20]. Peptic ulcer disease is commonly associated with cytotoxin-associated gene (*cagA*)-positive and vacuolating cytotoxin gene (*vacA*) *s1*-type strains [21, 22]. Therefore, the *cagA* and *vacA* subtypes and bacterium-related histopathological lesions may play an important role in *H. pylori* eradication. The aim of this study was to determine the factors associated with treatment failure and prevalence of point mutations directly from gastric biopsy and to investigate the effect of these mutations and *H. pylori* genotypes on eradication therapy using PPI, clarithromycin + amoxicillin for 10 days.

MATERIALS AND METHODS

Patients

A total of 111 patients undergoing diagnostic oesophagogastroduodenoscopy (OGD) for various symptoms in the endoscopy suite of the Department of Gastroenterology were enrolled from January 2007 to December 2008. There were 69 male patients (male : female ratio 1·65 : 1). The mean age of patients was 46 ± 16 years (range18–82 years). Informed consent was obtained for the study and for OGD with biopsies from antrum and corpus of the stomach. The study was approved by the institutional ethics review committee of The Aga Khan University, Karachi. At the time of enrolment, clinical symptoms, physical findings and previous drug treatment, dosage and duration were noted. The patients enrolled had not received previous treatment with antibiotics, PPIs, histamine 2-receptor blockers (H2-RB) and bismuth compounds. Seventy-six (69%) patients were diagnosed as non-ulcer dyspepsia with gastritis, nine (8%) as gastric ulcer, 16 (14%) as GC, and 10 (9%) as duodenal ulcer. Sydney classification of endoscopic gastritis according to localization was used to classify it as pangastritis, gastritis of the body and antral gastritis [21]. Gastric biopsy specimens were obtained from an erythematosus area of the antrum and corpus. In the case of a lesion, ulcer or suspected malignancy, biopsy was taken from both the involved and uninvolved mucosa. Biopsy specimens, two each were obtained for rapid urease test, histology and DNA extraction for PCR. Patients with *H. pylori* infection were diagnosed on the basis of rapid urease test and histology. They were treated with triple therapy comprising of PPI, esomeprazole 40 mg b.i.d., clarithromycin 500 mg b.i.d. and amoxicillin 1 g b.i.d. for 10 days. Eradication of *H. pylori* infection was documented by ^{14}C urea breath test (UBT) performed 4 weeks after completion of treatment. Patients were advised not to take PPIs and to use an antacid if symptomatic before the UBT. At the end of treatment, 44 (40%) patients were positive, and 53 (47%) were negative by ^{14}C UBT, and for 14 (13%) with GC the test could not be done.

Rapid urease test

Pronto Dry (Medical Instrument Corp., Switzerland) results were read in 30 min after sampling according to the manufacturer's instructions. The colour change from yellow to pink was considered positive [22].

Histology

Gastric biopsy specimens for histopathology were stained with haematoxylin and eosin (H&E) stain for the detection of *H. pylori* and degree of gastritis. In doubtful cases Giemsa staining was performed to ascertain the presence of *H. pylori*. The degree of gastritis as determined on H&E stain was scored in accordance with the Sydney system [23]. Histological features of gastritis were graded according to the updated Sydney System by an experienced pathologist. Mononuclear cell infiltration, glandular atrophy, and intestinal metaplasia were recorded on a four-point scale (0, normal; 1, mild; 2, moderate; 3, marked). Polymorphonuclear cell infiltration was coded as present (1) or absent (0) at biopsy sites. Acute or chronic inflammation was defined as presence of features of both acute and chronic inflammation. The criteria for diagnosis of GC were the presence of acini lined by atypical cells, abnormal mitoses, sheets of signet ring cells with intracellular mucin. Special stain for acid mucin (PAS Alcine Blue) was performed in difficult cases. In undifferentiated cases, immunohistochemical epithelial markers, i.e. cytokeratins highlighted the tumour cells [24].

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system [17]. The presence of *H. pylori* was determined by the positive rapid urease test and histology. All biopsy specimens for histological examination were fixed in 10% formalin, embedded in paraffin wax on the oriented edge, and cut into 5-μm-thick sequential sections. All tissue sections were stained with H&E for histological examination. The degree of acute and chronic inflammation, as well as the *H. pylori* density was scored according to the updated Sydney system. Bacterial density was graded from 0 to 3 (0, absent; 1–3, from few and isolated bacteria to colonies). The infiltration of gastric mucosa by mononuclear cells and polymorphonuclear leucocytes, atrophy, and intestinal metaplasia were graded on a four-point scale (0, none; 1, mild; 2, moderate; 3, marked). Chronic inflammation was defined according to an increase in lymphocytes and plasma cells in the lamina propria graded into mild, moderate or marked increase in density. Chronic active gastritis indicated chronic inflammation with neutrophilic polymorph infiltration of the lamina propria, pits or surface epithelium graded as: 0 = nil; mild, <33% of pits and surface infiltrated; moderate, 33–66%; and marked >66%. Antrum and corpus gastritis were scored by total sum of grade of gastritis (1, mild; 2, moderate; 3, marked, infiltration with lymphocytes and plasma cells) and activity of gastritis (1, mild; 2, moderate; 3, marked, infiltration with neutrophilic granulocytes) either in the antrum or in the corpus, a maximum sum of 6 points for each individual patient. Atrophy was defined as the loss of inherent glandular tissue, with or without replacement by intestinal-type epithelium. For optimal histological evaluation, all gastric biopsy specimens included surface epithelium and muscularis mucosae. Lymphoid aggregates were defined as accumulations of lymphocytes and plasma cells without a germinal centre.

Tissue DNA extraction

DNA was extracted from gastric tissue as previously described [25]. Briefly, gastric tissue was homogenized to uniformity in 500 μl sterile water and centrifuged at 12000 ***g*** for 3 min. Next 500 μl lysis buffer [100 mM NaCl, 10 mM Tris–HCl (pH 8·0), 25 mM EDTA, 0·5% sodium dodecyl sulfate], and 10 μl Proteinase K (10 mg/ml) were added. Incubation was performed at 50 °C for 20 h; this was followed by phenol–chloroform extraction and ethanol precipitation. The resulting pellet was allowed to dissolve in 40 μl TE buffer [10 mM Tris–HCl (pH 7·4) and 0·1 mM EDTA (pH 8·0)] for 20 h at 37 °C. Samples were stored at −20 °C before PCR amplification was performed. DNA content and purity was determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer (Beckman DU-600, USA).

PCR

Amplification of *cag A* and *vac A* alleles by PCR was performed in a volume of 50 μl containing 10 mmol/l Tris–HCl (pH 8·3), 50 mmol KCl, 1·5–2·5 mmol/l $MgCl_2$, 200 μmol/l deoxynucleoside triphosphates, 2 U *Taq* DNA polymerase (Promega, USA) and 25 pmol of both forward and reverse primers (synthesized by an MWG Automatic synthesizer) (Table 1) used previously [26]. PCR was performed in a PerkinElmer 9700 thermal cycler. Positive and negative reagent control reactions were performed with each batch of amplifications. DNA from *H. pylori* strains ATCC 43504 (*vacA s1am1*, *cagA* positive), ATCC 51932 (*vacA s2m2*, *cagA* negative) and ATCC 43526 (*vacA s1bm1, cagA* positive) were used to define the accuracy of the *cagA*. The amplified PCR products were resolved in 2% agarose gels containing Tris/acetate/EDTA, stained with ethidium bromide, and visualized under a short wavelength ultraviolet light source.

A 267-bp fragment of the *23S rRNA* gene of *H. pylori* (GenBank accession no. U27270) was amplified by PCR using extracted DNA using primers as previously described corresponding to nucleotides 1931 to 1952 and 2197 to 2175 (Table 1) [27, 28]. PCR amplification was performed in a total volume of 50 μl containing 1 × PCR reaction buffer, 1·5 mM $MgCl_2$, 200 μM each of the four dNTPs, 0·2 μM of primers (synthesized by MWG), 2 U *Taq* DNA polymerase (Promega) and 2 μl of extracted DNA. Negative reagent control reactions were performed with each batch of amplifications, consisting of tubes containing DNA isolated from biopsies of *H. pylori*-negative patients. After amplification PCR products were ethanol precipitated, the pellets were washed with 70% ethanol and resuspended in 25 μl sterile distilled water. Then, 3 μl PCR product was electrophoresed on a 2% agarose gel to ensure homogeneity and yield. PCR amplification resulted in a homogeneous DNA fragment of the expected size.

PCR–RFLP

The amplified products obtained by PCR were subjected to restriction endonuclease digestion for 2 h at

Table 1. *Oligonucleotide primers used in typing of* H. pylori cagA *and* vacA *alleles*

Region amplified	Primer designation	Primer sequence (5′ to 3′)	Size of PCR product	PCR cycles
cagA				
C-5′	D008	GGTCAAAATGCGGTCATGG	297 bp[19]	1 cycle of 94 °C for 5 min,
	R008	TTAGAATAATCAACAAACA-TCACGCCAT		35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 90 s, 1 cycle of 72 °C for 5 min
vac A alleles				
S1a	SS1-F	GTCAGCATCACACCGCAAC	190 bp[19]	1 cycle of 95 °C for 5 min;
	VA1-R	CTGCTTGAATGCGCCAAAC		35 cycles of 95 °C for 1 min,
S1b	SS3-F	AGCGCCATACCGCAAGAG	187 bp[19]	52 °C for 1 min and 72 °C
	VA1-R	CTGCTTGAATGCGCCAAAC		for 1 min; 1 cycle of 72 °C
S2	SS2-F	GCTAACACGCCAAATGATCC	199 bp[19]	for 5 min
	VA1-R	CTGCTTGAATGCGCCAAAC		
m1	VA3-F	GGTCAAAATGCGGTCATGG	290 bp[19]	
		CCATTGGTACCTGTAGAAAC		
m2	VA3-R			
	VA4-F	GGAGCCCCAGGAAACATTG	352 bp[19]	
	VA4-R	CATAACTAGCGCCTTGCAC		
23S rRNA	HPY-S	AGGTTAAGAGGATGCGTCAGTC	279 bp[29]	1 cycle of 94 °C for 5 min,
	HPY-A	CGCATGATATTCCCATTAGCAGT		35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and 1 cycle of 72 °C for 5 min

37 °C in 20 μl volume, according to recommended procedures [29]. The digested samples were analysed by agarose gel (3%, w/v) electrophoresis. Restriction enzyme *Bbs*I (5 U), *Bsa*I (5 U) and *Bce*AI (0·5 U) (New England Biolabs, USA) were used on the basis of sequence data available for this amplified product. RFLP allowed the identification of mutations A2142G and A2143G using the *Bbs*I and *Bsa*I restriction enzymes, respectively, as previously described [17, 18]. The enzyme *Bce*AI recognized two sites on the 267-bp amplified product: 5′-ACGGC(N)122N-3′ and 5′-N2(N)12GCCGT-3′ yielding two restriction fragments from the wild type and three from A2142G and A2143G mutants 195, 48, 24, while *Bce*AI yielded four from A2142C mutation recognizing an additional site, 5′-ACGGC(N)122N-3′ (Fig. 1). The amplified PCR products were resolved in 3% agarose gels containing Tris/acetate/EDTA, stained with ethidium bromide, and visualized under a short wavelength ultraviolet light source.

Statistical assessment

SPSS software was used for data analysis (version 11.5, SPSS Inc., USA). The descriptive analysis was done for demographic and clinical features. Results are presented as mean ± standard deviation for quantitative variables and number (percentage) for qualitative variables. Differences in proportion were assessed using Pearson's χ^2, Fisher's exact or likelihood ratio tests where appropriate. P value $<0{\cdot}05$ was considered as statistically significant, all P values were two-sided.

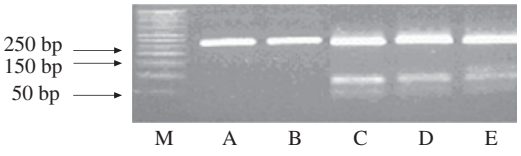

Fig. 1. Representative polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) pattern of *23S rRNA* genes from patients with clarithromycin resistance. Lane M, DNA size marker; PCR–RFLP pattern obtained with *Bbs*I (lane A); *Bsa*I (lane B); *Bce*AI (lane C).

RESULTS

All patients completed the therapy. After triple therapy, repeat ^{14}C UBT was negative in 51 (46%) patients, positive in 44 (40%) while 16 (14%) did not

receive a repeat ^{14}C UBT to document eradication of infection. Those with a negative ^{14}C UBT had a mean age of 48 ± 16 years. There were 44 (40%) patients with treatment failures with a positive ^{14}C UBT [29 (54%) males, 15 (37%) females], with a mean age of 39 ± 14 years (range 23–53 years). The most frequent side-effects were nausea experienced by 30 (34%) and diarrhoea by 18 (20%) patients. Failure to eradicate *H. pylori* infection was seen in 40 (53%) of gastritis patients ($P = 0{\cdot}02$) (Table 2*a*).

Distribution of histopathological changes

The density of *H. pylori* was of grade 1 in 37 (39%) and grade 2 in 58 (61%) patients. *H. pylori* density of grades 1 and 2 were seen with moderate inflammation in 37 (42%) and 56 (58%) patients compared to nine (37%) and 15 (63%) with mild inflammation, respectively ($P = 0{\cdot}658$). In treatment failure, moderate inflammation was present in 38 (49%) compared to mild inflammation in six (35%) patients ($P = 0{\cdot}315$) (Table 2). The features of lymphoid aggregates, intestinal metaplasia and gastric atrophy were not significantly associated with *H. pylori* eradication (Table 2). One (1%) patient with gastric atrophy and three (3%) with intestinal metaplasia were diagnosed as GC. No histopathological changes were associated with *H. pylori* treatment failures (Table 2*a*).

Distribution of virulence markers

The *H. pylori* virulence marker *cagA* was positive in 55 (49%), *vacA s1a* in 68 (61%), *vacA s1b* in 38 (34%), *vacA m1* in 59 (53%) and *vacA m2* in 63 (57%) of patients. *CagA*-positive strains ($n = 19$, 45%) were not more readily eradicated compared to *cagA*-negative strains ($n = 23$, 55%) ($P = 0{\cdot}85$) (Table 2*a*). *VacA s1a* was positive in 31 (56%, $P = 0{\cdot}02$), *vacA s1b* in eight (30%, $P = 0{\cdot}04$), *vacA m1* in 28 (58%, $P = 0{\cdot}02$) and *vacA m2* in 19 (33%, $P = 0{\cdot}002$) patients, respectively, with treatment failure (Table 2*a*). When *cagA* was negative *VacA s1a* ($n = 18$, 75%, $P < 0{\cdot}001$), *m1* ($n = 16$, 89%, $P < 0{\cdot}001$) and *s1am1* ($n = 15$, 79%, $P = 0{\cdot}001$) were associated with treatment failure (Table 2*b*).

Distribution of mutations

Overall 55 mutations were present in 44 (40%) patients. The commonest mutation was 2142G present in 26 (27%) patients while 12 (12%) had multiple mutations (Fig. 1) (Table 2*a*). The mutations concomitantly present were 2142G and 2143G seen in nine (9%) and 2142G, 2143G and 2142C seen in three (3%) patients (Table 2). The distribution of mutations were not associated with age and gender ($P = 0{\cdot}07$ and $P = 0{\cdot}795$, respectively).

Diagnosis and histological changes associated with mutations

These mutations were seen in 36 (47%, $P = 0{\cdot}009$) gastritis cases with a very low rate of mutations in patients with GC ($n = 1$, 6%). These mutations were associated with moderate degree of inflammation in 37 (43%, $P = 0{\cdot}236$) and inflammatory activity in 19 (38%, $P = 0{\cdot}749$) patients. The predominant mutation A2142G was also associated with endoscopic gastritis in 21 (28%, $P = 0{\cdot}021$) and moderate inflammation in 13 (26%, $P = 0{\cdot}562$) patients (Table 2*a*).

Failure of triple therapy associated with mutations

Triple therapy failure was associated with mutations in 34 (79%, $P < 0{\cdot}001$) patients (Table 2). Mutation A2142G was associated eradication failure in 21 (81%, $P < 0{\cdot}001$), A2143G in 17 (89%, $P < 0{\cdot}001$) and A2142C in seven (78%, $P = 0{\cdot}04$) patients (Table 2*a*).

DISCUSSION

In this study, triple therapy failed to eradicate *H. pylori* infection in a large number of patients who presented at a younger age with gastritis compared to those with successful eradication (Table 2*a*). Triple therapy failure was significantly observed in 53% with gastritis compared to gastric ulcer and duodenal ulcer (Table 2*a*). There was also a high frequency of point mutations 47% in patients with gastritis. However, mutations were significantly negative in our patients with GC compared to those with gastritis. Mutations were not associated with grade or activity of inflammation. The mutations observed in gastritis may have occurred subsequent to previous treatments with antibiotics. It is known that there is a high frequency of mutation in the strains caused by variation in the efficiency of DNA repair functions or alternatively, in the accuracy of DNA polymerase [30]. In the presence of an antibiotic all bacteria in the stomach die and there is a stepwise selection from sensitive to resistant to compensated mutant [30].

Table 2*a*. *Factors associated with eradication failure*

Total no. of patients	(*N*=111)	Eradication		*P* value
		Failure, *n* (%)	Successful, *n* (%)	
Patients with repeat UBT	95	44 (46)	51 (54)	
Age (years)		39±14	48±16	0·002
Gender				
Male	54 (57)	29 (54)	25 (46)	0·09
Female	41 (43)	15 (37)	26 (63)	
Diagnosis				
Gastritis	76 (80)	40 (53)	36 (47)	0·02
Gastric ulcer	9 (9)	1 (11)	8 (89)	
Duodenal ulcer	10 (11)	3 (30)	7 (70)	
Histopathology				
H. pylori *colonization density*				
Grade 0				
Grade 1	37 (39)	15 (41)	22 (59)	0·367
Grade 2	58 (61)	29 (50)	29 (50)	
Grade 3				
Inflammation				
Grade 0				
Grade 1	17 (18)	6 (35)	11 (65)	0·315
Grade 2	78 (82)	38 (49)	40 (51)	
Grade 3				
Activity				
Grade 0				
Grade 1	52 (55)	24 (46)	28 (54)	0·972
Grade 2	43 (45)	20 (47)	23 (53)	
Grade 3				
Lymphoid aggregate				
Positive	44 (46)	21 (48)	23 (52)	0·795
Negative	51 (54)	23 (45)	28 (55)	
Virulence marker				
cagA				
Positive	42 (44)	19 (45)	23 (55)	0·85
Negative	53 (56)	25 (47)	28 (53)	
vacA s1a				
Positive	55 (58)	31 (56)	24 (44)	0·02
Negative	40 (42)	13 (32)	27 (68)	
s1b				
Positive	27 (28)	8 (30)	19 (70)	0·04
Negative	68 (72)	36 (53)	32 (47)	
m1				
Positive	48 (51)	28 (58)	20 (42)	0·02
Negative	47 (49)	16 (24)	31 (76)	
m2				
Positive	57 (60)	19 (33)	38 (67)	0·002
Negative	38 (40)	25 (66)	13 (34)	
Point mutation				
Over all				
Positive	43 (45)	34 (79)	9 (21)	<0·001
Negative	52 (55)	10 (19)	42 (81)	
Individual				
A2142G				
Positive	26 (27)	21 (81)	5 (19)	<0·001
Negative	69 (73)	23 (33)	46 (67)	

Table 2*a* (*cont.*)

Total no. of patients	(*N* = 111)	Eradication		*P* value
		Failure, *n* (%)	Successful, *n* (%)	
A2143G				
Positive	19 (20)	17 (89)	2 (11)	< 0·001
Negative	76 (80)	27 (43)	49 (57)	
A2142C				
Positive	9 (9)	7 (78)	2 (22)	0·04
Negative	86 (91)	37 (43)	49 (57)	
More than one				
2142G + 2143G				
Positive	9 (9)	9 (100)	0	< 0·001
Negative	86 (91)	35 (41)	51 (59)	
2142C + 2142G + 2143G				
Positive	3 (3)	3 (100)	0	0·03
Negative	92 (97)	41 (45)	51 (55)	

The great majority of *vacA s1* strains are known to be associated with a good response to treatment when associated with *cagA* [12]. However, the majority of our patients that were *vacA s1a* had an eradication failure since 47% of them were *cagA* negative. Previously, it has been shown that *cagA*-negative strains *s2/m2* were resistant to therapy compared to the *cagA*-positive *s1am1* or *s1am2* strains [12]. The virulence genotype is an important determinant for the severity of the gastritis and the formation of intestinal metaplasia.

The implications of our study are that *H. pylori* infection with point mutations signifies a greater probability of treatment failure. These mutations were seen with *H. pylori* infections that were *cagA* positive in only 33% of cases. It is known that the *cagA* protein produced by certain *H. pylori* strains plays a crucial role in gastric carcinogenesis [31]. Moreover, *vacA s1* and *vacA m1* strains that are known to be associated with a higher degree of inflammation and epithelial damage in the gastric mucosa were positive in only 24–42% of *H. pylori* strains with mutations [32, 33]. Patients with GC are known to have much more severe gastritis that leads to a decrease in acid production and subsequent development of GC. This reduction in acid production results in the suppression of a defence mechanism against dedifferentiated epithelium that leads to persistence, and progression to atypical cells [34, 35]. In our study the mutations were associated with only mild to moderate inflammation and inflammatory activity ($P = 0{\cdot}236$ and 0·749, respectively). However, these were not significant associations.

The presence of either mutation, especially the A2142G and 2143G may prompt a high-dose, non-macrolide-based treatment regimen. Recent data from Western Europe indicate that >60% of treatment failures with clarithromycin-based protocols are associated with the presence of clarithromycin-resistant *H. pylori* isolates after therapy [36]. Our results are similar to a study from Korea that revealed the A2142G mutation was observed in 59% of cases [37], and different from those of Japan and China where the major type of mutation was reported as A2143G [38, 39]. In studies from USA, the prevalence of clarithromycin-resistant *H. pylori* mutant strains with the A2142G mutation varies from 48% to 53%, A2143G from 39% to 45% and A2142C from 0% to 7% [16, 37, 38]. In Europe, the prevalence of the A2142G mutation was reported as 23–33%, the A2143G mutation as 44–67%, and the A2142C mutation as 2–10% [18]. In Japan, >90% of the mutant strains had the A2143G mutation [38]. In a Chinese study, the A2143G mutation was present in 100% of clarithromycin-resistant *H. pylori* strains [39]. Clarithromycin resistance was present in 17% of Iranian *H. pylori* strains, with 74% having the A2143G mutation, 21% A2142C and 5% A2142G mutations [40]. These mutations have a genetic stability and growth advantage compared to the wild-type strain [41]. Moreover, the A2143G mutation is known to give a high level of resistance to clarithromycin [41].

Table 2*b*. *Association of* cagA *and* vacA *with treatment*

	cagA					
	Positive ($n=42$)			Negative ($n=53$)		
vacA	Treatment failure *n* (%)*	Treatment successful *n* (%)	*P* value	Treatment failure *n* (%)	Treatment successful *n* (%)	*P* value
s1a						
Positive	13 (42)	18 (52)	0·504	18 (75)	6 (25)	<0·001
Negative	6 (55)	5 (45)		7 (24)	22 (76)	
s1b						
Positive	3 (37)	5 (63)	0·709	5 (26)	14 (74)	0·043
Negative	16 (47)	18 (53)		20 (59)	14 (41)	
m1						
Positive	12 (40)	18 (60)	0·323	16 (89)	2 (11)	<0·001
Negative	7 (58)	5 (42)		9 (26)	26 (74)	
m2						
Positive	7 (37)	12 (63)	0·366	12 (32)	26 (68)	0·001
Negative	12 (52)	11 (48)		13 (87)	2 (13)	
s1am1						
Positive	11 (44)	14 (56)	0·845	15 (79)	4 (21)	0·001
Negative	8 (47)	9 (53)		10 (29)	24 (71)	
s1bm1						
Positive	2 (33)	4 (67)	0·673	2 (67)	1 (33)	0·597
Negative	17 (47)	19 (53)		25 (46)	27 (54)	
s1am2						
Positive	4 (33)	8 (67)	0·495	5 (56)	4 (44)	0·719
Negative	15 (50)	15 (50)		20 (45)	24 (55)	
s1bm2						
Positive	1 (20)	4 (80)	0·356	4 (25)	12 (75)	0·041
Negative	18 (49)	19 (51)		21 (57)	16 (43)	

Differences in proportion were assessed by using Pearson's χ^2, Fisher's exact or likelihood ratio tests where appropriate. *P* value <0·05 was considered as statistically significant, all *P* values were two-sided.

* *n* (%) = number and percentage.

This suggests that GC is associated with wild *H. pylori* strains without 23rRNA mutations in our patients. The prevalence of GC in our population is lower than in Japan and China where the prevalence of GC is higher and clarithromycin-resistant mutations are less prevalent, i.e. 9% and 5%, respectively [38, 39]. Moreover, a high frequency of clarithromycin-resistant mutation is described in *H. pylori* isolates from Tehran, Iran which is an area without a high incidence of GC [39]. This suggests that GC in the Japanese and Chinese populations is related to wild-type *H. pylori* strains without clarithromycin-resistant mutations. *H. pylori* strains become resistant by mutations in the chromosomal genes, by acquisition of exogenous DNA by plasmids, transposons or integrons, or by transformation. In conclusion, 23S rRNA point mutations were prevalent in *H. pylori* infection associated with non-ulcer dyspepsia. The eradication failure of *H. pylori* infection was associated with younger mean age, point mutations in the *23S rRNA* gene of *H. pylori* and *vacA s1a* and *m1* when *cagA* was negative.

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DECLARATION OF INTEREST

None.

REFERENCES

1. **Solerman A, *et al.*** Assessment of *Helicobacter pylori* clarithromycin resistance mutations in archival gastric biopsy samples. *Swiss Medical Weekly* 2005; **135**: 327–332.
2. **International Agency for Research on Cancer.** Schistosomes, liver flukes and *Helicobacter pylori*. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* 1994; **61**: 1–241.
3. **Blaser MJ, *et al.*** Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Research* 1995; **55**: 2111–2115.
4. **Bayerdorffer E, *et al.*** Regression of primary gastric lymphoma of mucosa-associated lymphoid tissue type after cure of *Helicobacter pylori* infection. MALT Lymphoma Study Group. *Lancet* 1995; **345**: 1591–1594.
5. **Graham DY, *et al.*** Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer. A randomized controlled study. *Annals of Internal Medicine* 1992; **116**: 705–708.
6. **Bytzer P, O'Morain C.** Treatment of *Helicobacter pylori*. *Helicobacter* 2005; **10**: 40–46.
7. **Megraud F.** *H. pylori* antibiotic resistance: prevalence, importance, and advances in testing. *Gut* 2004; **53**: 1374–1384.
8. **Wong BC, *et al.*** Triple therapy with clarithromycin, omeprazole, and amoxicillin for eradication of *Helicobacter pylori* in duodenal ulcer patients in Asia and Africa. *Alimentary Pharmacology & Therapeutics* 2000; **14**: 1529–1535.
9. **Broutet N, *et al.*** Risk factors for failure of *Helicobacter pylori* therapy results of an individual data analysis of 2751 patients. *Alimentary Pharmacolagy & Therapeutics* 2003; **17**: 99–109.
10. **Graham DY.** Antibiotic resistance in *Helicobacter pylori*: implications for therapy. *Gastroenterology* 1998; **115**: 1272–1277.
11. **Houben MH, *et al.*** A systematic review of *Helicobacter pylori* eradication therapy: the impact of antimicrobial resistance on eradication rates. *Alimentary Pharmacolagy & Therapeutics* 1999; **13**: 1047–1055.
12. **van Doorn LJ, *et al.*** Importance of *Helicobacter pylori* cagA and vacA status for the efficacy of antibiotic treatment. *Gut* 2000; **46**: 321–326.
13. **Occhialini AM, *et al.*** Macrolide resistance in *Helicobacter pylori*: rapid detection of point mutations and assays of macrolide binding to ribosomes. *Antimicrobial Agents and Chemotherapy* 1997; **41**: 2724–2728.
14. **Versalovic JD, *et al.*** Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. *Antimicrobial Agents and Chemotherapy* 1996; **40**: 477–480.
15. **Hulten K, Gibreel A, Engstrand L.** Macrolide resistance in *Helicobacter pylori*: mechanism and stability in strains from clarithromycin treated patients. *Antimicrobial Agents and Chemotherapy* 1997; **41**: 2550–2553.
16. **Taylor DE, *et al.*** Cloning and sequence analysis of two copies of a 23S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23S rRNA mutations. *Antimicrobial Agents and Chemotherapy* 1997; **41**: 2621–2628.
17. **Marais A, *et al.*** Direct detection of *Helicobacter pylori* resistance to macrolides by a polymerase chain reaction DNA enzyme immunoassay in gastric biopsy specimens. *Gut* 1999; **44**: 463–467.
18. **Van Doorn LJ, *et al.*** Accurate prediction of macrolide resistance in *Helicobacter pylori* by a PCR line probe assay for detection of mutations in the 23S rRNA gene: multicenter validation study. *Antimicrobial Agents and Chemotherapy* 2001; **45**: 1500–1504.
19. **Van Doorn LJ, *et al.*** Rapid detection, by PCR and reverse hybridization, of mutations in the *Helicobacter pylori* 23S rRNA gene, associated with macrolide resistance. *Antimicrobial Agents and Chemotherapy* 1999; **43**: 1779–1782.
20. **Matsumura M, *et al.*** Rapid detection of mutations in the 23S rRNA gene of *Helicobacter pylori* that confers resistance to clarithromycin treatment to the bacterium. *Journal of Clinical Microbiology* 2001; **39**: 691–695.
21. **Tee W, Lambert JR, Dwyer B.** Cytotoxin production by *H. pylori* from patients with upper gastrointestinal tract disease. *Journal of Clinical Microbiology* 1995; **33**: 1203–1205.
22. **van Doorn LJ, *et al.*** Expanding allelic diversity of *Helicobacter pylori vacA*. *Journal of Clinical Microbiology* 1998; **36**: 2597–2603.
23. **Tytgat GNJ.** The Sydney system: endoscopic division, endoscopic appearance in gastritis/duodenitis. *Journal of Gastroenterology and Hepatology* 1991; **6**: 223–234.
24. **Said RM, *et al.*** Evaluation of a new biopsy urease test: Pronto Dry, for the diagnosis of *Helicobacter pylori* infection. *European Journal of Gastroenterology and Hepatology* 2004; **16**: 195–199.
25. **Price AB.** The Sydney System: histological division. *Journal of Gastroenterology and Hepatology* 1991; **6**: 209–222.
26. **Rosai J.** *Rosai and Ackerman's Surgical Pathology*, vol. 1, 9th edn. New York: Elsevier, 2004.
27. **Van Zwet AA, *et al.*** Sensitivity of Culture compared with that of Polymerase chain reaction for detection of *Helicobacter pylori* from antral biopsy samples. *Journal of Clinical Microbiology* 1993; **31**: 1918–1920.
28. **Ménard A, *et al.*** PCR-restriction fragment length polymorphism can also detect point mutation A2142C in the 23S rRNA gene, associated with *Helicobacter pylori* resistance to clarithromycin. *Antimicrobial Agents and Chemotherapy* 2002; **46**: 1156–1157.
29. **Sambrook J, Fritisch EF, Maniatis T.** *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring

Harbor: Cold Spring Harbor Laboratory Press, 1989, pp. 1847–1855.
30. **Björkholm B, *et al.*** Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*. *Proceedings of the National Academy of Sciences USA* 2001; **98**: 14607–14612.
31. **Parsonnet J, *et al.*** Risk of gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. *Gut* 1997; **40**: 297–301.
32. **Nogueira C, *et al.*** *Helicobacter pylori* genotypes may determine gastric histopathology. *American Journal of Pathology* 2001; **158**: 647–654.
33. **Atherton JC, *et al.*** Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of *Helicobacter pylori*. *Gastroenterology* 1997; **112**: 92–9.
34. **Meining A, *et al.*** Gastric carcinoma risk index in patients infected with *Helicobacter pylori*. *Virchows Archiv* 1998; **432**: 311–314.
35. **Axon ATR, Lynch DAF.** *Helicobacter pylori*, gastric physiology and cancer. *European Journal of Gastroenterology and Hepatology* 1993; **5**: 109–113.
36. **Xia HX, Buckley M, Hyde D.** Effects of antibiotic resistance on clarithromycin – combined triple therapy for *Helicobacter pylori*. *Gut* 1995; **37** (Suppl. 1): A55.
37. **Kim KS, *et al.*** Mutations in the 23S rRNA gene of *Helicobacter pylori* associated with clarithromycin resistance. *Journal of Korean Medical Science* 2002; **17**: 599–603.
38. **Maeda S, *et al.*** Detection of clarithromycin resistant *Helicobacter pylori* strains by a preferential homoduplex formation assay. *Journal of Clinical Microbiology* 2000; **38**: 210–214.
39. **Pan ZJ, *et al.*** Assessment of clarithromycin resistant *Helicobacter pylori* among patients in Shanghai and Guangzhou, China, by primer-mismatch PCR. *Journal of Clinical Microbiology* 2002; **40**: 259–261.
40. **Mohammadi M, *et al.*** *Helicobacter pylori* antibiotic resistance in Iran. *World Journal of Gastroenterology* 2005; **11**: 6009–6013.
41. **Versalovic J, *et al.*** Point mutations in the 23S rRNA gene of *Helicobacter pylori* associated with different levels of clarithromycin resistance. *Journal of Antimicrobial Chemotherapy* 1997; **40**: 283–286.