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Antibiotic susceptibility patterns of *Helicobacter pylori* and triple therapy in a high-prevalence area

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Introduction

Helicobacter pylori is a Gram-negative spiral bacterium. Its association with gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma is well established,¹ and has a worldwide distribution. Seroprevalence of *H. pylori* in Karachi, Pakistan, in healthy children aged 11–15 years is reported as 53.5%.²

Successful eradication of *H. pylori* infection requires the use of an antibiotic regimen to which the infecting strain is susceptible.³ The Maastricht consensus report on *H. pylori* advised that the first-line treatment should be a drug regimen that includes a proton pump inhibitor (PPI) and two antimicrobial agents (clarithromycin plus amoxicillin or clarithromycin plus metronidazole).⁴ The triple therapy usually results in an eradication rate >80%.

In recent years, the emergence of antibiotic-resistant *H. pylori* strains has been reported, posing a significant threat to successful treatment.^{3,5–6} In a previous multicentre study in patients with active duodenal ulcer, one week of triple therapy with PPI, amoxicillin and clarithromycin eradicated *H. pylori* infection.⁷ Low cure rate and a higher resistance to clarithromycin have been observed among *H. pylori*-positive patients with functional dyspepsia.^{8–10}

In practice, *H. pylori* infection currently is being treated in most centres in Pakistan without knowledge concerning the incidence of resistant strains. For most bacterial infections, antibiotic susceptibility testing is routinely carried out but the specialised growth requirements of *H. pylori* preclude such tests in most diagnostic medical laboratories. There is limited local published information that includes data on the antibiotic susceptibility of this pathogen.^{11–12} In a study conducted in the north of Pakistan, the antimicrobial susceptibility of *H. pylori* isolates from 43 specimens determined by the Kirby Bauer technique against amoxicillin, clarithromycin and metronidazole demonstrated that 67% were resistant to metronidazole, while all the isolates were sensitive to amoxicillin, clarithromycin and tetracycline.¹²

The aim of this study is to determine the effect of primary *H. pylori* resistance to antibiotics used for triple therapy that included clarithromycin (CLR, 500 mg), amoxicillin (AMX, 1 g) and omeprazole (40 mg) twice a day for two weeks, and

ABSTRACT

This study aims to determine primary *Helicobacter pylori* resistance and its effect on eradication of the organism. Ninety-two patients with dyspeptic symptoms were enrolled. *H. pylori* was cultured and antibiotic sensitivity was determined by the Epsilon test (Etest) for clarithromycin (CLR), amoxicillin (AMX) and metronidazole (MTR). 23S ribosomal RNA (rRNA) point mutations associated with clarithromycin resistance were also detected. Patients were treated with omeprazole (40 mg daily), CLR (500 mg) and AMX (1g twice a day) for 14 days. A ¹⁴C-urea breath test (¹⁴C-UBT) was repeated four weeks after completion of treatment to confirm eradication. Triple therapy failure was seen in 30(33%) patients. The resistance rates were: CLR 33% (30/92), MTR 48% (44/92) and AMX 2% (2/92). Clarithromycin resistance (CLR-R) was present in the 16–39 age group in 21 (47%) ($P=0.007$) compared to nine (19%) in the 40–79 age group. CLR resistance was seen in 30 *H. pylori* isolates, 20 (67%) from patients with non-ulcer dyspepsia (NUD), six (20%) with gastric ulcer (GU) and four (13%) with duodenal ulcer (DU). Triple therapy failure was associated with CLR-R in 28 (93%) ($P<0.001$). CLR-R mutations were present in 30 (33%) and were associated with treatment failure in 27 (90%; $P<0.001$). They were present in 20 (44%) isolates obtained from patients in the 16–39 age group ($P=0.018$). Treatment failure was associated with A2142G mutation in 20 (67%; $P<0.001$), A2143G mutation in 12 (40%; $P<0.001$) and A2142C mutation in five (17%; $P=0.003$). In conclusion, triple therapy failure was associated with CLR-R. Metronidazole resistance exceeded that of CLR, hence it cannot be substituted for CLR in a triple therapy.

KEY WORDS: Clarithromycin.
Drug resistance, microbial.
Helicobacter pylori.
Metronidazole.

its effect on eradication of *H. pylori* in patients with non-ulcer dyspepsia (NUD) and peptic ulcer disease (PUD). Eradication was documented by performing a ¹⁴C-urea breath test (¹⁴C-UBT) four weeks after completion of treatment. Also determined is the prevalence of 23S ribosomal RNA (rRNA) point mutations associated with clarithromycin resistance in *H. pylori* isolates.

Materials and methods

H. pylori strains

Over the 24 months of the study period (February 2007 to January 2009), a total of 92 *H. pylori* isolates were obtained from patients referred for endoscopy at the Aga Khan

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University (Table 1). Inclusion criteria comprised a history of upper abdominal pain requiring histological definition, symptoms suggestive of peptic ulcer, and where gastric biopsy was indicated by endoscopy finding. Ethical approval for the collection of extra biopsies was granted by the Aga Khan University Ethics Review Committee.

Upper endoscopy was performed and gastric biopsy samples were obtained in triplicate from within 5 cm of the pylorus along the greater curvature for culture and histopathology. *H. pylori* infection was defined by i) positive culture, ii) positive histopathology, and iii) positive rapid urease test.

Culture and identification of *H. pylori*

The specimens were transported immediately in sterile normal saline to isolate *H. pylori*. Thus, within three hours of collection each specimen was homogenised in sterile Eppendorf tubes with an electric homogeniser. The resulting suspension was inoculated onto Columbia blood agar (Oxoid) medium and Dents supplement (containing vancomycin, trimethoprim and polymyxin) and incubated at 37°C under microaerophilic conditions for 4-6 days. Plates were then examined for bacterial growth and typical colonies were selected for identification. The identity of *H. pylori* was confirmed by Gram stain and by the production of urease and catalase. One half of the homogenate was used for culture and the other half was kept at -80°C. *H. pylori* isolates were defined as Gram-negative spiral-shaped bacilli that were catalase-positive and rapidly (less than 1 h) urease-positive. *H. pylori* NCTC

11637 (type strain) was used as a positive control for the culture conditions and identification tests.

Antibiotic susceptibility testing

Antibiotic susceptibility was determined on Mueller Hinton agar (Oxoid, UK) containing 10% defibrinated sheep blood and a cell suspension calibrated at three McFarland units by Epsilon meter test (Etest) using metronidazole, clarithromycin and amoxicillin Etest strips (AB Biodisk, Solna, Sweden). Plates were read after three days at 37°C. The tests were carried out according to the manufacturer's instructions. For Etests, minimum inhibitory concentration (MIC) breakpoints of ≥ 2 mg/L and ≥ 8 mg/L were used for metronidazole and clarithromycin, respectively. Intermediate susceptibility (MIC 2-8 mg/L) was also recorded for metronidazole. High-level resistance to both antibiotics was defined as MIC > 256 mg/L. The breakpoint MIC used for amoxicillin was ≥ 2 mg/L.¹³ *H. pylori* NCTC 11637 was used as a sensitive control.

Rapid urease test

Urea agar base enriched with 40% urea solution (eUAB, Oxoid) was used as a rapid urease test.¹⁴ It shows 97% sensitivity, 86% specificity, 84% positive predictive value (PPV), 97% negative predictive value (NPV) and 91% accuracy to detect urease activity of *H. pylori* in gastric biopsies.

Histological analysis

Formalin-fixed, paraffin wax-embedded gastric biopsy specimens were routinely processed. Gastritis activity was graded on a four-point scale of none (grade 0), mild (grade 1), moderate (grade 2), and severe (grade 3) according to the guidelines of the Sydney system.¹⁵ Presence of *H. pylori* was assessed on modified Giemsa-stained sections.

Urea breath test

A ¹⁴C-UBT procedure was undertaken using methodology described previously.¹⁶ In a previous study, the accuracy of ¹⁴C-UBT was compared to histopathology and rapid urease test. Accuracy was 93% compared to histopathology, while PPV and NPV were 97% and 84%, respectively.¹⁷ Comparison of ¹⁴C-UBT with rapid urease test gives an accuracy of 96%, with PPV and NPV of 95% and 97%, respectively. These results were highly reproducible ($P < 0.001$).¹⁷

Extraction of genomic DNA

Bacterial cells grown on chocolate agar were washed (x2) with phosphate-buffered saline (PBS) then centrifuged at 3000 xg for 20 min. *H. pylori* DNA was extracted by a phenol/chloroform method as described previously.¹⁸ Briefly, the bacterial pellet was resuspended in Tris-HCl buffer containing ethylenediaminetetraacetate (TE, pH 8.0) and lysozyme (30 mg/mL) and then incubated at 37°C for 30 min. The suspension was treated with sodium dodecyl sulphate (SDS, 10%), proteinase K (2.5 mg/mL) and RNase A (40 mg/mL). DNA was extracted with phenol/chloroform/isoamyl alcohol, precipitated by sodium acetate and ice-cold absolute alcohol, and the washed with ice-cold alcohol (70%). The DNA pellet was resuspended in TE buffer. The DNA content and purity were determined by measuring absorbance at

Table 1. Characteristics of the study patients from which *H. pylori* isolates were obtained.

Age	Mean \pm SD	43 \pm 15
	Range	16-79
	Age group 16-39 years	43(47)
	Age group 40-79 years	49(53)
Gender	Male	62(67)
	Female	30(33)
Symptoms	Abdominal pain	46(83)
	Heartburn	5(5)
	Diarrhoea	8(9)
	Haematemesis	3(3)
Diagnosis	Non-ulcer dyspepsia	57(62)
	Gastric ulcer	15(16)
	Duodenal ulcer	20(22)
¹⁴ C Urea breath test	Positive	30(33)
	Negative	62(67)
Clarithromycin	Sensitive	62(67)
	Resistant	30(33)
Metronidazole	Sensitive	48(52)
	Resistant	44(48)
Amoxicillin	Sensitive	90(98)
	Resistant	2(2)
Data shown as patient numbers (%).		
Results presented as mean \pm standard deviation for quantitative variables.		

Table 2. Distribution of antibiotic sensitivities.

		Clarithromycin			Metronidazole			Amoxicillin		
		Sensitive (n=62)	Resistant (n=30)	P value	Sensitive (n=48)	Resistant (n=44)	P value	Sensitive (n=90)	Resistant (n=2)	P value
Age groups	16–39 years	24(53)	21(47)	0.005	22(49)	23(51)	0.537	44(98)	1(2)	0.975
	40–79 years	38(81)	9 (19)		26(55)	21(45)		46(98)	1(2)	
Diagnosis	Mean age									
Non-ulcer dyspepsia	40±15	37(65)	20(35)		30(53)	27(47)		56(98)	1(2)	
Gastric ulcer	46±15	9(60)	6(40)	0.725	4(27)	11(73)	0.088	14(93)	1(7)	0.376
Duodenal ulcer	45±13	16(80)	4(20)	0.269	14(70)	6(30)	0.177	20(100)	0(0)	
¹⁴C Urea breath test	Positive	2(7)	28(93)	<0.001	16(53)	14(47)	0.877	29(97)	1(3)	0.548
	Negative	60(97)	2(3)		32(52)	30(48)		61(98)	1(2)	

Data shown as patient numbers (%).

$P < 0.05$ was considered statistically significant.

260 nm and 280 nm using a spectrophotometer (Beckman DU-600, USA).

Polymerase chain reaction

A 267 bp fragment was amplified by PCR using extracted DNA as the template, as previously described, using primers 5'-AGGTTAAGAGGATGCGTCAGTC-3' (HPY-S) and 5'-CGCATGATATCCCATAGCAGT-3' (HPY-A), corresponding to nucleotides 1931–1952 and 2197–2175, respectively, of the 23S rRNA gene of *H. pylori* (GenBank Accession No U27270).¹⁹ Amplification was carried out in a total volume of 50 mL containing 1xPCR reaction buffer, 1.5 mmol/L MgCl₂, 200 μmol/L each of four dNTPs, 0.2 mmol/L primers (synthesised by MWG), 2 unit of *Thermus aquaticus* (*Taq*) DNA polymerase (Promega) and 2 mL extracted DNA. The reaction was carried out in a Perkin Elmer 9700 thermal cycler. Amplification cycle comprised an initial denaturation of target DNA at 94 °C for 5 min and then denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The final cycle included an extension step for 7 min at 72 °C to ensure full extension of the product. The samples were amplified through 35 consecutive cycles. Negative reagent control reactions were performed with each batch of amplifications, consisting of tubes containing DNA isolated from *H. pylori*-negative patients. After amplification, PCR products were precipitated and the pellets washed in 70% ethanol and resuspended in 25 μL sterile distilled water. The PCR products (3 μL) were subjected to electrophoresis in 2% agarose gel to ensure homogeneity and yield. Amplification resulted in a homogeneous DNA fragment of the expected size.

The amplified products obtained were subjected to restriction endonuclease digestion for 2 h at 37 °C in a 20 μL volume.²⁰ The digested samples were analysed by agarose gel (3% [w/v]) electrophoresis. Restriction enzymes *Bbs*I (5 units), *Bsa*I (5 units) and *Bce*AI (0.5 units) (New England Biolabs) were used on the basis of sequence data available for this amplified product. Restriction fragment length polymorphism (RFLP) allowed the identification of the A2142G and A2143G mutations using the *Bbs*I and *Bsa*I restriction enzymes, respectively, as previously described.^{21,22} The enzyme *Bce*AI recognised, on the 267 bp

amplified product, two sites 5'-ACGGC(N)122N-3' and 5'-N2(N)12GCCGT-3', yielding two restriction fragments from the wild-type and three from the A2142G and A2143G mutants (195, 48 and 24), while *Bce*AI yielded four from the A2142C mutation, recognising an additional site, 5'-ACGGC(N)122N-3'. The amplified PCR products were resolved in 3% agarose gel containing Tris/acetate/EDTA, stained with ethidium bromide and visualised under an ultraviolet light source.

Statistical assessment

The Statistical Package for Social Science (SPSS, Release 16, standard version) was used for data analysis. Results were presented as mean ± standard deviation (SD) for quantitative variables and number (%) for qualitative variables. Differences in proportion were assessed using Pearson's χ^2 , Fisher exact or likelihood ratio tests where appropriate. $P < 0.05$ was considered statistically significant.

Results

Triple therapy failure was seen in 30(33%) patients. The primary antibiotic resistance rates are shown in Table 1. Dual resistance to CLR and AMX was present in two (2%) cases, to CLR and MTR in 13 (14%), and one patient showed resistance to all three antibiotics.

Sensitivity and resistance data by age group are shown in Table 2. Dual resistance to CLR and MTR was seen in 11(85%) patients in the 16–39 ago group compared to two (15%) in the 40–79 age group ($P=0.007$). The resistance rate

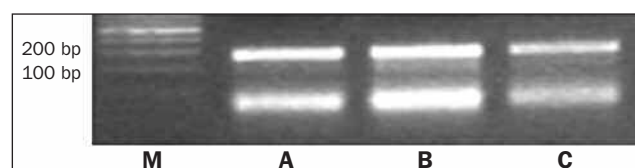


Fig. 1. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) pattern of 23S rRNA genes from patients with clarithromycin resistance. Lane M, DNA size marker; PCR-RFLP pattern obtained with *Bbs*I (lane A), *Bce*AI (lane B) and *Bsa*I (lane C).

to CLR and MTR did not show a gender distribution. Correlation of diagnosis with antibiotic susceptibilities is shown in Table 2, as is association with triple therapy failure, which was associated with resistance to CLR in 28 (93%) cases ($P < 0.001$). A breakdown of treatment failure is shown in Table 3. Details of the correlation between treatment failure and mutation is shown in Figure 1 and Table 3.

The CLR resistance mutations were present in 30 (33%) *H. pylori* isolates. These mutations were associated with treatment failure in 27(90%) cases ($P < 0.001$). These mutations were present in 20 (44%) *H. pylori* isolates from patients in the 16–39 age group and 10 (21%) in the 40–79 age group ($P = 0.018$), and were present in 19 (33%) patients with NUD, four (20%) with DU ($P = 0.262$) and seven (47%) with GU ($P = 0.339$).

Discussion

This study demonstrated a triple therapy failure rate of 30%. It was found more frequently in those under 40 years of age who had NUD. However, the difference was not significant in those with NUD compared to those with PU. Resistance to AMX was seen in a small number of patients, while MTR is not used locally as a part of triple therapy for *H. pylori* infection, although it is commonly prescribed for infectious diarrhoea of both bacterial and parasitic origin.

The implication of this study is that there is a high degree of MTR resistance in *H. pylori* isolates so it cannot be used instead of CLR in triple therapy regimens. Metronidazole resistance may be associated with previous use of the drug.²³ In contrast to the findings reported here, Mirza *et al.*, from the north of Pakistan, reported 100% sensitivity to CLR and AMX, which shows that resistance to CLR has evolved in the south of the country.¹²

Clarithromycin resistance may be associated with pre-existent resistance to other macrolides (e.g., erythromycin) or infection with *H. pylori* strains with DNA mutations associated with CLR resistance. These mutations are known to be associated with different MIC values *in vitro*. A very low eradication rate (48%) occurred in the A2143G mutated strains, while the rate was higher than 90% in the absence of this mutation.²³ In addition to use in the treatment of *H. pylori* infection, CLR is commonly prescribed for the treatment of acute exacerbation of chronic bronchitis in adult patients in the community. In Pakistan, self-prescription is also common and medications are available for sale without prescriptions.^{24,25}

Erythromycin is among the most frequently used agents.²⁴ High levels of resistance have been found to ampicillin, cotrimoxazole and erythromycin.²⁵ Poultry use has increased with rising inflation, and poultry production largely depends on the use of antibiotics, and erythromycin is commonly used.²⁶ Owing to indiscriminate use and unawareness of adequate withdrawal periods, antibiotic residues are known to be present in the meat. This is unfit for human consumption and known to alter intestinal bacterial flora and encourage the development of resistant bacteria.²⁷ Akhtar *et al.* cultured 58 isolates of *Salmonella enteritidis* from stool samples of patients with diarrhoea, all of which showed resistance to erythromycin.²⁸

South Asia is a low-risk region for gastric cancer but has a high prevalence of *H. pylori*.²⁹ Locally, the incidence of gastric

Table 3. Correlation of treatment with diagnosis.

		Treatment		P value*
		Failure (n=30)	Success (n=62)	
Age groups	16–39 years	19(42)	26(58)	0.054
	40–79 years	11(23)	36(77)	
Diagnosis				
Non-ulcer dyspepsia	n=57	19(33)	38(67)	
Gastric ulcer	n=15	6(40)	9(60)	0.629
Duodenal ulcer	n=20	5(25)	15(75)	0.489
A2142G	Positive	20(91)	2(9)	<0.001
	Negative	10(14)	60(86)	
A2143G	Positive	12(92)	1(8)	<0.001
	Negative	18(23)	61(77)	
A2142C	Positive	5(100)	0(0)	0.003
	Negative	25(29)	62(71)	

Data shown as patient numbers (%).
 $P < 0.05$ was considered statistically significant.

carcinoma has been increasing, markedly in males over 40 years of age.²⁹ A recent study concluded that primary care physicians lacked knowledge regarding the management of *H. pylori* infection.³⁰ Only 57% would confirm *H. pylori* eradication after treatment in selected patients, and 47% physicians preferred serological testing for follow-up.

In conclusion, this study has shown that MTR resistance exceeds CLR resistance in *H. pylori* isolates from patients with upper gastrointestinal symptoms. In view of this finding, MTR cannot be used in place of CLR in patients who demonstrate CLR-based triple therapy failure. □

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