Emerging trends of antimicrobial resistance in Helicobacter pylori isolates obtained from Pakistani patients: The need for consideration of amoxicillin and clarithromycin

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Abstract

Objective: To present antimicrobial susceptibility profile of helicobacter pylori isolated from gastric biopsies of dyspeptic patients.

Methods: The cross-sectional study was conducted from March 2008 to May 2013 at the Jinnah Postgraduate Medical Centre (JPMC), Karachi, and comprised patients with suspected peptic ulcer disease who underwent upper gastrointestinal endoscopy. Multiple gastric antral biopsies were taken. One sample was imbedded in the Campylobacter-like organism test, while other biopsies were processed for culture and confirmed on polymerase chain reaction. Isolates were subjected to antimicrobial susceptibility testing and minimum inhibitory concentrations of antimicrobial agents were estimated by Epsilometer test. SPSS 21 was used for statistical analysis.

Results: Of the 889 samples available, 254(28.6%) were positive after campylo-like organism test, of them, 92(36.2%) isolates were cultured. Susceptibility test showed 90(97.8%) resistance to metronidazole, 25(30.1%) ofloxacin, 6(16.2%) levofloxacin, 14(15.2%) furazolidone, 5(5.4%) clarithromycin, 4(4.3%) tetracycline and 2(2.2%) isolates showed resistance to amoxicillin. Resistance to more than one drug was observed in 37(40.2%) isolates.

Conclusion: Low clarithromycin resistance was surprising since Pakistan is considered a hot bed of drug-resistant bacteria.

Keywords: Antimicrobial susceptibility, Amoxicillin, Clarithromycin, Furazolidone, Helicobacter pylori. (JPMA 66: 710; 2016)

Introduction

Helicobacter pylori (H. pylori) is a gram-negative, microaerophilic and acidophilic bacterium that has tendency to reside in human stomach and duodenal epithelia for years either asymptomatically or develop a variety of chronic ulcer diseases such as peptic and gastric ulcer. Previous studies suggest that the eradication of H. pylori infection helps in the treatment of gastric ulcer and abolishes the intensity of inflamed gastric mucosa.[1] H. pylori is also considered the biggest risk factor for the development of non-cardiac gastric cancer and its removal is recommended as a part of first-line therapy to reduce cancer prognosis.[2] In addition, the eradication of H. pylori is undertaken as a long-term prophylactic when given to targeted population at high risk of developing gastric cancer. Success of this approach in patients with baseline intestinal metaplasia or atrophic gastritis is reported worldwide.[3] However, the time and length of treatment required to eliminate H. pylori induced cancer cases from population is yet to be determined. Significant improvement was also observed in other H. pylori-associated gastric and non-gastric diseases following anti-H. pylori regime.[4]

A combination of clarithromycin (CLR) with amoxicillin (AML) or metronidazole (MTZ) and a proton pump inhibitor (PPI) have been used as treatment of H. pylori infection since many years.[5] This regime is either given simultaneously for two weeks or in the form of sequential treatment constituting PPI-AML for one week and PPICLR-MTZ/AML for another week. In certain countries, bismuth-containing quadruple therapy is also practised due to low toxicity and better treatment efficiency.[6] However, recent data reports poor patient compliance with an eradication rate of 70% to 85%,[7] mainly due to MTZ and CLR resistance. On that basis in 2012, Maastricht consensus report recommended the use of bismuth or levofloxacin (LEV) containing quadruple therapy as second-line option[6] whereas tetracycline (TE) and furazolidone (Fefficacy of treatment, baseline data on antimicrobial drug susceptibility should be generated so it can be used to modify treatment regimens. Nonetheless the consensus is generated on this issue; antimicrobial susceptibility testing (AST) cannot be performed in every case due to the involvement of invasive procedure to obtain clinical sample.[8] However, in developing countries, AST is still not a part of routine laboratory investigations for those patients who undergo endoscopic examination.

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Therefore insufficient evidence about the frequency of antimicrobial resistance is available.

Pakistan is one of the H. pylori endemic countries where scanty data is available about patient compliance with first-line therapy.[9] A local study[10] reported that 61% physicians preferred CLR-based triple therapy and 23% opted for MTZ-based triple therapy. This current study was planned to evaluate the antimicrobial susceptibility pattern of H. pylori against first- and second-line antimicrobial agents from a cohort of samples.

Materials and Methods

The cross-sectional study was conducted from March 2008 to May 2013 at the Gastroenterology and Hepatology Unit of Pakistan Medical Research Council (PMRC), Jinnah Postgraduate Medical Centre (JPMC), Karachi, and comprised patients with suspected peptic ulcer disease who underwent upper gastrointestinal (GI) endoscopy due to functional dyspepsia. PMRC Research Centre is a public resource centre providing care for gastroenterology and hepatology illness for patients residing in Karachi and other cities of Sindh. It is a referral unit for gastroenterology and hepatology, providing outpatients primary care, including diagnostic and therapeutic procedures. The patients were selected using non-probable purposive sampling.

To determine the risk factors, potential participants were interviewed for their hygienic habits, such as overcrowding, hand-washing, consumption of treated water (either by boiling or filtration), socio-economic status and abdominal complaints in the preceding one year. More than three persons living per room was considered overcrowding, while the residential area of the participant was used to estimate the socioeconomic status. Written informed consent was obtained from all study participants.

All patients with dyspeptic symptoms whether (duodenal/gastric) ulcer or non-ulcer dyspepsia, with 15 years or above were included. Patients with a history of PPIs or antibiotic intake in the preceding 4 weeks, or those with liver disease or malignancies, or those who were on immunosuppressive drugs were excluded.

Multiple gastric antral biopsies were taken from each patient. One sample was embedded in the Campylobacter-like organism (CLO) gel to check rapid urease activity, while the second sample was transferred immediately to the adjacent Microbiology Laboratory of PMRC, JPMC, in a sterile 1.5ml Eppendorf tube. Biopsy was chopped and inoculated on Wilkins-Chalgren agar (Oxoid, Basingstoke, United Kingdom) enriched with 10% blood and Dent supplement (Oxoid, Basingstoke, United Kingdom) and blood agar. Inoculated plates were incubated for three days in kit-generated microaerobic atmosphere (Campygen, Oxoid). Suspected colonies of H. pylori were confirmed by the presence of gram staining, rapid urease, catalase, and oxidase activities. Strains were stored at -40°C in Brain Heart Infusion (BHI) broth with 20% glycerol.

Strains identified as H. pylori by biochemical testing were cultured in brain BHI broth and total genomic deoxyribonucleic acid (DNA) was extracted using Wizard genomic DNA purification kit (Promega Inc.), according to the manufacturer's described protocol. Polymerase chain reaction (PCR) detection of H. pylori was carried out using specific primers targeting 16S ribosomal ribonucleic acid (rRNA) gene. For PCR amplification, 800ng of DNA samples were added to a PCR mixture containing 0.5mM forward (CTGGAGAGACTAAGCCCTCC) and reverse (ATTACTGACGCTGATTGTGC) (16S rRNA product size 109bp) primers, 1.5 mM MgCl2, 1U of thermus aquaticus (Taq) polymerase (Invitrogen, Italy), 2.5µl PCR buffer (Qiagen, Germany), and 200?M of deoxynucleotide (dNTPs). PCR was held for 5 min at 95°C, followed by 35 cycles of 30 sec each at 95°C, 60°C and 72°C with an extension by 5 min at 72°C.[11]

Strains were freshly cultured in BHI broth. Bacterial suspensions comparable with McFarland's standard No. 4 were prepared in 0.85% saline, as recommended by European Helicobacter pylori Study Group.[12] Minimum Inhibitory concentrations (MIC) of commonly prescribed antimicrobial agents, such as MTZ, AML and CLR, were tested by Epsilometer (E) test. For CLR, AB Biomerieux E-strips were used and for MTZ and AML, Oxide MIC Evaluator E-strips were used. For CLR, MICs breakpoint considered for susceptible strains was 0.25µg/ml, for resistance strains >0.5µg/ml and for intermediate 0.5µg/ml. For AML >0.5µg/ml was considered resistance, and H. pylori strains were considered resistant when the MIC was ≥8µg/ml for MTZ. In case of ofloxacin, levofloxacin, TE and furazolidone, susceptibility was performed by disc diffusion method.

Statistical analysis was done on IBM Statistic 21 and Microsoft Excel. Age was expressed as mean ± standard deviation (SD). Age range were transformed from respondent age and the association between categorical variables like socioeconomic status, overcrowding, hand-washing habit, consumption of treated water, recurrent heart burn (RHB) and abdominal pain in the preceding year was checked with CLO results, while culture positivity was checked with Chi square test. Chi square test were also used to check the

possible correlation between susceptibility pattern and age/gender/recurrent abdominal pain (RABP)/RHB. For any correlation p<0.05 was considered significant.

Results

Out of 889 patients, 424(47.6%) were males and 465(52.3%) were females. Overall mean age was 35.6±14.32 years. Besides, 754(84.8%) patients belonged

to low socioeconomics group, and 474(53%) resided in overcrowded settings. Significant association was found between poor hand hygiene 208(23%), consumption of untreated water 345(39%), and anti-H. pylori treatment 468(53%) with positive CLO results (p=0.000; p=0.000; p=0.019) (Table-1).

Out of 889 patients, 254(28.6%) biopsies were CLO-
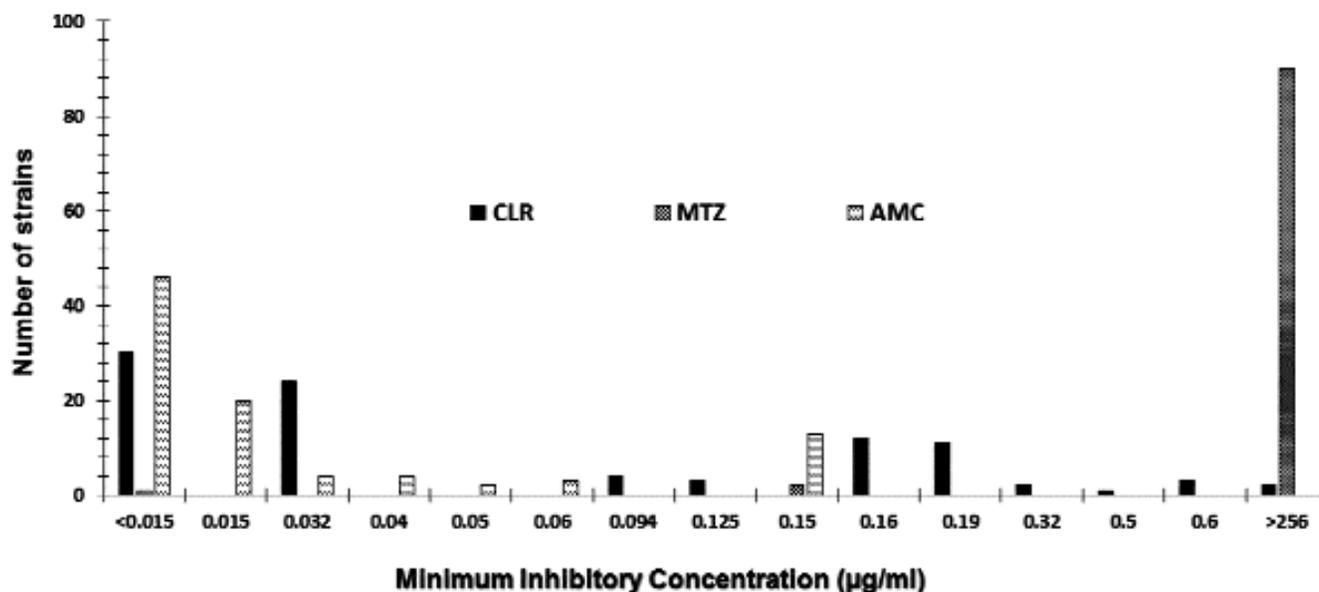
Figure-1: Distribution of minimum inhibitory concentration (MIC) of CLR: clarithromycin, AML: Amoxicillin, MTZ: Metronidazole against Helicobacter pylori isolates.

Table-1: Association of CLO test results with demography and other parameters.

| Parameter		No. of CLO test samples (n =889)		P-value
		Yes (n=254)	No (n=635)	
Age range	15-20	34	87	0.389
	21-40	149	340	
	>41	71	204	
Gender	Male	118	306	0.639
	Female	136	329	
Overcrowding*	Yes	128	346	0.269
	N o	126	289	
Poor hand washing practices*	Yes	137	71	0.000*
	No	117	564	
Consumption of untreated drinking water	Yes	198	147	0.000*
	No	56	488	
Recurrent abdominal pain in last 1 year	Yes	173	463	0.151
	N o	81	172	
Recurrent Heart Burn: In last 1 Year	Yes	169	432	0.662
	No	85	203	
anti- H. pylori treatment in last 1 year	Yes	118	350	0.019*
	No	136	285	

CLO: Campylo-like organism test
H. Pylori: Helicobacter Pylori.

Table-2: Culture positivity according to different parameters.

Parameter		No. of CLO positive samples (n =254)		P-value
		Yes (n=93)	No (n=161)	
Overcrowding*	Yes	45	92	0.177
	N o	48	69	
Poor hand washing practices*	Yes	45	68	0.342
	No	48	93	
Consumption of untreated drinking water	Yes	78	64	0.000*
	No	15	97	
Recurrent abdominal pain in last 1 year	Yes	58	72	0.006*
	N o	35	89	
Recurrent Heart Burn: In last 1 Year	Yes	65	85	0.007*
	No	28	76	
anti- H. pylori treatment in last 1 year	Yes	36	95	0.001*
	No	57	66	

CLO: Campylo-like organism test
H. Pylori: Helicobacter Pylori.
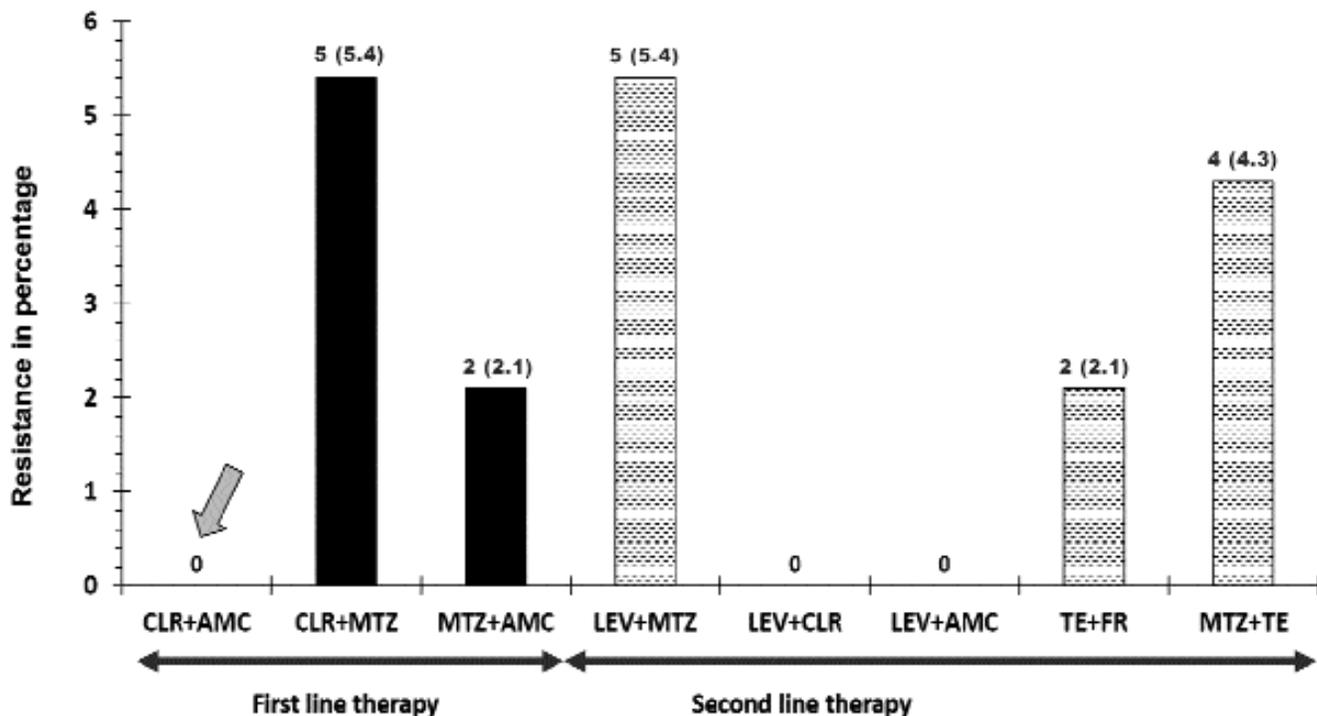
Figure-2: Resistance profile of H.Pylori isolates to drug combinations.
CLR: Clarithromycin, AMC: Amoxicillin, MTZ: Metronidazole, LEV: Levofloxacin, TE: Tetracycline, FR: Furazolidone.

positive, and, of them, 118(46.4%) were males and 136(53.5%) were females. Mean age of these patients was 35.1±14.02 years with 144(57.6%) in the 21-40 year age bracker. H. pylori was isolated from 93(36.6%) samples. Consumption of untreated drinking water 142(56%), RHB 150(59%) and RABP 130(51%) showed significant culture-

positive cases (p=0.000; p=0.008; p=0.004) respectively. No association was found with other symptoms such as, fever, diarrhoea and vomiting (p>0.05). Interestingly, H. pylori was not isolated from patients with history of taking anti-H. pylori treatment in the preceding one year (p=0.002) (Table-2).

Table-3: Susceptibility pattern & its possible correlation with different parameters.

		AMC (n=92)			CLR (n=92)			FR (n=92)			TE (n=92)			MTZ (n=93)			LEV (n=37)			OFX (n=83)		
		S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I
Age range (years)	15-20	14	1	0	14	2	0	8	6	1	13	2	0	0	15	0	4	1	0	8	2	4
	21-40	48	1	3	50	2	0	42	4	5	48	2	1	3	49	0	17	2	2	34	9	5
	>41	21	0	1	21	1	0	17	4	0	22	0	0	0	22	0	7	3	1	10	4	4
P-value			0.795			0.000*			0.011*			0.763			0.485			0.695			0.126	
Gender	M	40	0	1	38	1	1	33	1	6	38	2	0	2	39	0	9	3	2	23	5	7
	F	47	2	3	48	4	0	36	13	2	49	2	1	1	51	0	19	3	1	29	10	8
P-value			0.318			0.294			0.010*			0.548			0.423			0.402			0.831	
RABP	Yes	70	1	3	68	4	1	57	11	5	70	3	1	2	72	0	27	4	2	45	10	11
	No	16	1	1	18	1	0	13	3	3	17	1	0	1	18	0	1	2	1	7	5	5
P-value			0.385			0.665			0.432			0.459			0.399			0.044*			0.169	
RHB	Yes	68	1	3	67	4	1	58	9	6	68	3	1	3	70	0	25	4	2	45	12	10
	No	18	1	1	19	1	0	12	5	2	19	1	0	0	20	0	3	2	1	7	3	6
P-value			0.140			0.200			0.045*			0.199			0.063			0.277			0.015*	

RABP: Recurrent abdominal pain. RHB: Recurrent heart burn. M: Male. F: Female. AMC: Amoxicillin. CLR: Clarithromycin. FR: Furazolidone. TE: Tetracycline. MTZ: Metronidazole. LEV: Levofloxacin. OFX: Ofloxacin.

Resistance against MTZ was in 90(97.8%) isolates having growth inhibition at ≥8µg/ml. In case of AML, 90(97.8%) isolates exhibited MICs ranging from 0.015 to 0.15 µg/ml, whereas only 2(2.2%) strains showed MIC ≥1 µg/ml, indicating resistance to AML. For CLR, 86(93.4%) strains showed inhibition at less than 0.5µg/ml, whereas 5(5.4%) showed MIC >0.5µg/ml, only 1(1.1%) strain showed intermediate inhibition with MIC 0.5 µg/ml (Figure-1).

Four second-line antimicrobial drugs were tested by disc diffusion method. A total of 25(30.1%) strains showed resistance to ofloxacin, 6(16.2%) to levofloxacin, 14(15.2%) to furazolidone, and 4(4.3%) to TE. Resistance to more than one drug was observed in 37(40.2%) isolates; of them resistance to two drugs was 25(27.1%), while triple and quadruple drug resistance was 9(9.7%) and 3(3.2%). No isolate was found to be resistant with a combination of AML and CLR. Resistance rates were higher against combination of MTZ with any of second-line drugs, including levofloxacin 5(5.4%) and Tetracycline 4(4.3%). MTZ showed 5(5.4%) resistance with first line drug CLR and 2(2.1%) with AMC. (Figure-2).

Susceptibility pattern of H. pylori showed significant association with patients' age (Table-3). Resistance against CLR (p=0.000) and furazolidone (p=0.002) was more frequent in younger age group (15-40 years) compared to those 41 years and above. Frequency of resistance against furazolidone was significantly high in females compared to males (p=0.010). When resistance patterns were compared with the patient's symptoms, it was found that patients with RHB had more resistant isolates against furazolidone and ofloxacin (p=0.045; p=0.015).

Discussion

We observed high prevalence of resistance against MTZ (97.8%), ofloxacin (30.1%), levofloxacin (16.2%) and furazolidone (15.2%). The resistance rates were exceptionally low (5.4% and 2.2%) for first-line drugs CLR and AML. Consumption of untreated water and poor hand hygiene with CLO positivity showed a significant association, which suggests that improvement in household hygiene practices prevents H. pylori infection. In addition, we observed that H. pylori infection was not present in cases that received anti-H. pylori therapy in the last one year (p=0.001), indicating success of eradication, but these patients still underwent endoscopy due to severe functional dyspepsia or other gastric complaints. It was difficult to obtain the history of particular regime which was recommended to these patients previously, therefore the rate of patient compliance with different antibiotics could not be determined.

CLR is one of the most commonly used antimicrobial agents that determine the fate of first-line therapy. Acquisition of CLR resistance in H. pylori could result in treatment failure and persistent infection.[13] A serious dichotomy in the prevalence of CLR resistance is observed in different regions of the world. High-level resistance with frequency rates 20%-45% is observed in different countries from Europe,[14] South East Asia.[15] Addressing

the issue, separate treatment regimens are advised in current Maastricht IV consensus report and instead of CLR containing triple regime, bismuth containing quadruple therapy is recommended in high CLR resistance regions. In our study, it is very surprising to see the low prevalence of CLR and AML resistance in this cohort which was in sharp contrast with the previous studies undertaken[16,17] on Pakistani patients. Molecular assessment of CLR resistance need to be done for further scrutiny. Though present data is comparable with other studies reported from South Asian region that describe resistance rates between 2% to 6%.[18,19] Similar results were also observed in case of AML. Our findings show that in CLR resistance group, age range has an exceptional trend (p=0.000). CLR resistance is significantly seen in younger age ranges from 5-20 and 21-40 years. As in our population, the use of CLR for respiratory tract infections (RTIs) in younger age group has increased over recent decades due to which resistance may be common in this age group. Our data reflects that intense differences are present in our population presenting different anti-biogram and a multi-centre study based on a large group of patients is necessary to obtain population-based statistics in order to avoid serious repercussions related to CLR therapy.

In this study, MIC distribution showed that 97.8% isolates were resistant to MTZ which is in agreement with our previous observations on Pakistani patients[15] and other developing countries, such as India.[20] In 2012, Maastricht consensus report recommends MTZ can be considered first-line option with CLR when resistance rate is <40% to MTZ and 15-25% to CLR.[6]

H. pylori resistance against second-line drugs was also high in the present study since 30% of isolates show resistance to ofloxacin and 16.2% to levofloxacin which are closer to other local studies.[16,21] Dual resistance with MTZ and levofloxacin, the commonly prescribed second-line combination, was observed in 5.4% cases. Resistance rate to levofloxacin varied up to 50% in different parts of the world.[22] Although fluroquinolone-based therapies are highly effective in countries with less than 12% resistance,[23] but it is still unequivocally used as drug of choice for second-line treatment. The resistance to levofloxacin represents the resistance to all fluoroquinolones, including ciprofloxacin and moxifloxacin, which are commonly used for other bacterial infections.

TE and furazolidone, which are considered low-cost alternative second-line options for the treatment of H. pylori showed 2.7% and 13.5% resistance respectively. Only 2.1% of the isolates in our study were found to be resistant with this combination. These drugs specifically furazolidone have been successfully used in the areas with high MTZ resistance and in patients with multiple treatment failure.[24] In our study, resistance against furazolidone was significantly high in female gender (p=0.010), the possible reason of this trend is the indiscriminate use of furazolidone in female population. Furazolidone has many side-effects, including depression of spermatogenesis, that is why it might not be excessively prescribed in male patients. Because of its low cost, World Gastroenterology Organisation, recommend the use of furazolidone in developing countries where MTZ resistance is high.[25] But due to potential toxicity, the furazolidone containing regime is still not specified. Global data is lacking on prevalence of its resistance since it is not part of AST profile in developed and developing countries.

The study provides up to date information about antimicrobial susceptibility pattern of H. pylori strains isolated from Pakistan. Variation in resistance pattern, particularly for CLR and AML, with respect to previous studies clearly indicates the presence of different patterns of antimicrobial susceptibility niche and necessitate introducing AST profile as a part of routine laboratory investigations for those patients who undergo endoscopic examination.

Conclusion

Low CLR resistance was surprising since Pakistan is considered a hot bed of drug-resistant bacteria. The study indicates the presence of heterogeneous population of H. pylori and its emerging trend of antibiogram in Pakistan, and emphasises reconsideration of the treatment according to the native AST.

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Conflict of Interest: None.

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