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Molecular characterization and antibiotic susceptibility profiles of *Helicobacter pylori* isolated from patients with Gastrodeudenal diseases in Jordan

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Abstract

Introduction: *Helicobacter pylori* is a major cause of more than 80% of chronic active gastritis and other gastrodeudonal diseases worldwide. Successful treatment of *H. pylori* routinely requires the use of multiple agents with different mechanisms including compounds inhibiting acid secretion in conjunction with antibiotics. However, recent data showed the emergence of resistant clinical strains particularly against metronidazole and clarithromycin. The aim of this study is to determine the prevalence of and the susceptibility of *H. pylori* isolates recovered from patients with gastrodeudonal diseases to several antimicrobial agents.

Materials and Methods: A prospective study has been conducting on Jordanian patients attended the gastrointestinal unit of the Jordan university hospital starting from 2014-2015 with gastroduodenal diseases. Antral and corpus mucosal biopsies from the stomach of each patient were used for the isolation of *H. pylori* on selective culture media. Presumptive *H. pylori* colonies were subsequently confirmed by biochemical tests and standard 16S rDNA PCR assay. The antimicrobial susceptibility testing was performed by standard agar diffusion methods according to CLSI. Subsequently, MICs were determined by E test and standard agar dilution method. Molecular typing of the clinical strains was performed using multiplex PCR for the detection of *vacA* and *cagA* genotypes. Metronidazole resistance was characterized by molecular methods for the detection of *rdxA* gene mutations.

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Results: Among 72 symptomatic patients, 13(23%) patients showed positive *H. pylori* infection by both rapid urease test and culture. The antibiotic susceptibility profile showed that all of the isolates were sensitive to amoxicillin. Resistance to, clarithromycin, ciprofloxacin and levofloxacin were observed in 15%, 23% and 8% of the isolates respectively while 92% of the strains were resistant to metronidazole (MIC ≥ 32ug/ml). Metronidazole resistance due to mutations in *rdxA* gene was only observed in one strain (8%) suggesting other resistance mechanisms. Correlation between antibiotic resistance and virulence factors was statistically not significant ($p > 0.05$).

Conclusion: The present study showed that the prevalence of metronidazole resistance among clinical isolates of *H. pylori* is very high. Lower resistance to other antibiotics are reported. Concern should be taken into consideration when triple therapy is used for the treatment of *H. pylori* in our region.

Keywords: *Helicobacter pylori,* Jordanian patients, Antibiotic resistance, Metronidazole

Introduction

Recently, the medical community has been increasingly interested in *Helicobacter pylori* (*H pylori*) related diseases as several studies have been reporting a major role of *H. pylori* in chronic gastritis and peptic ulcer worldwide [1, 2]. Colonization of the gastric mucosa with *H. pylori* constitutes a major risk factor in the pathogenesis of gastric cancer and gastric mucosa-associated lymphoid tissue (MALToma) [3-5].

H. pylori is equipped with an armamentarium of biological factors that enable this microaerophilic bacterium to survive in the harsh conditions present in human stomach. For instance, all strains of *H. pylori* possess a unique stable urease enzyme which is used to neutralize the stomach acidity and provide a suitable pH for the growth of this bacterium. In addition, the presence of numerous binding proteins and adhesion molecules ensure optimal colonization of *H. pylori* in the gastric mucosa [6]. Among the *H. pylori* virulence factors, cytotoxin A (CagA) and vacuolating cytotoxin A (VacA) are the most studied since the production of these toxins increases the risk for gastric atrophy, intestinal metaplasia and gastric carcinoma [7, 8]. However, genetic variation within the genes encoding these virulence factors contributes to differences in the pathogenicity of strains [9]. It has been reported that a round 70% of the *H. pylori* strains isolated in the western countries possess the *cagA* gene while higher prevalence of *cagA*-positive strains is

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reported in East Asian countries [10, 11]. On the other hand, *vacA* is present in approximately all *H. pylori* strains however, allelic variation in three main genetic regions encoding and regulating this protein has been observed [12, 13]. Depending on the type and combinations of *vacA* different alleles, distinct abilities of *vacA* to induce vacuolation in epithelial cells can occur [14]. Associations between infection with strains containing certain *vacA* alleles and increased risk for progression of gastric precancerous lesions and for gastric carcinoma were also reported [15, 16].

The eradication of *H. pylori* and attenuating its associated virulence factors can contribute to improve clinical conditions of the patients infected with this bacterium including accelerating peptic ulcer healing and minimizing the recurrence of gastric cancer. Current approaches for effective eradication of *H. pylori* include the use of at least two antibiotics and a proton pump inhibitor (triple and more recently quadruple therapy [17, 18]. Triple therapy, including two antibiotics, amoxicillin and clarithromycin, and a proton pump inhibitor given for a week has been recommended as the treatment of choice [19]. Other treatments have also been proposed, including metronidazole, as well as tetracycline, fluoroquinolones, and rifamycins. However, due to the emergence of antibiotic resistance among *H. pylori* clinical strains particularly against clarithromycin, and to a lesser extent metronidazole, higher rates of treatment failure of triple therapy were reported [21, 22]. Global studies in Europe reported a prevalence of *H. pylori* resistance to clarithromycin among adults and children of approximately 20%. In contrast, higher rates of resistance against metronidazole were reported in Europe and USA [23, 24].

Only few data regarding the resistance of *H. pylori* clinical strains to the conventional antimicrobial agents used in the clinical practice were reported which might be due to the technical difficulties to isolate and perform the standard methods for antimicrobial susceptibility testing on *H. pylori*. Our aim is to study the prevalence of *H. pylori* resistance to these various antibiotics especially in light of the resistance mechanism using both microbiological and molecular methods in addition to characterize the virulence factors and their possible association with antibiotic resistance among *H. pylori* strains.

Materials and methods

Patients and specimens. Antral and corpus mucosal gastric biopsies from stomach of 72 Jordanian patients attending the gastrointestinal unit of the Jordan University Hospital presented with any of the following clinical manifestations active gastritis, peptic ulcer or gastric cancer were obtained. Informed written consent was obtained from all patients participating in this study after ethical approval has been provided. Biopsies were transferred into 4 ml aliquots containing 1% Proteose Peptone (Oxoid, UK) for transport purposes.

Isolation and identification of *H. pylori*. For optimal recovery of clinical strains of *H. pylori*, all biopsy tissues were homogenized using a tissue homogenizer (IKA, Germany) and aliquots of 100 µl of the homogenate of each sample were inoculated into Columbia blood agar base (Oxoid, UK) containing 7 % laked horse blood and *Helicobacter pylori* selective supplement (Dent, Oxoid, UK) [25]. All of the plates were incubated at 37°C under microaerophilic conditions using CampyGen atmosphere generating system (Oxoid, UK) in anaerobic Jars for 5-7 days. Growth of *H. pylori* was confirmed according to colony morphology, Gram staining, microaerophilic growth at 37°C, oxidase, catalase and urease and subsequently by standard PCR of 16S rDNA test [26]. *H. pylori* cultures were stored at -70°C in Trypticase soy broth (Oxoid, UK) containing 10% v/v fetal calf serum (PAA, Austria) and 15% glycerol.

Antimicrobial susceptibility testing and MIC measurements. Bacterial suspensions were prepared to the 2 McFarland's standard and subse-

quently uniformly spread on a solid growth medium in a Petri dish. Antibiotic discs including amoxicillin (25 µg), metronidazole (5 µg), clarithromycin (15 µg), ciprofloxacin (5 µg), and levofloxacin (5 µg) (Mast, USA) were used. Plates were incubated for 5-7 days under appropriate cultivation conditions. Antibacterial activity was determined by the production of an inhibition zone around the disc. The minimal inhibitory concentrations (MICs) of each antimicrobial agent were determined by both E- test (Mast, **USA**) and by the two fold agar dilution method according to CLSI recommendations [27]. Antimicrobial agents were provided as powder from local pharmaceutical manufacturers and dissolved in distilled water for stock solutions. Next, two fold serial dilutions of each antimicrobial agent incorporated to molten Columbia blood agar plates supplemented with 7% (v/v) horse blood were prepared. Spots of *H. pylori* (1x 10^5 CFU) were applied on the surface of each plate and the growth of visible colonies was determined after 5-7 days of incubation at 37°C under microaerophilic conditions. MIC was recorded as the lowest concentration that inhibited visible growth of *H. pylori* in the agar dilution method and by measuring the ellipse edge intersects the strip in E-test. Triplicates of each antimicrobial agent were performed and the average of the results was taken. The resistance breakpoints of antibiotics were defined for amoxicillin ≥ 0.5 µg/ml, metronidazole > 8.0 µg/ml, ciprofloxacin and levofloxacin > 1.0 µg/ml and clarithromycin ≥ 1.0 µg/ml.

Detection of cagA and Vac A alleles. Detection of *CagA* gene, and *VacA* allele variation was performed as described by Atherone et al., 1995 and Zheng et al., 2000 with slight modifications [28, 29] **(Table 1).** In brief, multiplex PCR was performed with the primers of concentrations of 0.2 µM each and 1X ready to use master mix (New England biolab, UK) with adjusted concentration of $MgCl_2$ of 2.5 µM in a final reaction volume of 50 µl. The amplification was performed with the following parameters; 95°C for 10 min followed by 40 cycles of 30s at 95°C, 45s at 58°C and 45s at 62°C. Positive and negative controls were used in all experiments and the PCR amplified products were analyzed by 2% agarose gel electrophoresis.

Detection of *rdx A*gene deletion in Metronidazole resistant *H. pylori*. Detection of NADPH nitroreductase was performed by conventional mutagenic-based PCR with the following conditions and parameter; reactions were carried out in 25 µl mixtures containing 12.5 µl PCR master mix (New

Table 1. Oligonucleotide sequences used in this study

Primer name	Sequence 5`-3`	Target size (bp)	Annealing temp	References
HP1	GCAATCAGCGTCAGTAATGTTC	16S rRNA 521	55	26
HP2	GCTAAGAGATCAGCCTATGTCC			
cagA1	TTGACCAACAACCACAAACCGAAG	Cag 183	60	29
cagA2	CTTCCCTTAATTGCGAGATTCC			
vacA m1 F	GGTCAAAATGCGGTCATGG	VacA M1 allele 290	56	28
vacA m1 R	CCATTGGTACCTGTAGAAAC			
vacA m2 F	GGAGCCCCAGGAAACATTG	vacA M2 allele 352	56	
vacA m2 R	CATAACTAGCGCCTTGCAC			
rdxA F	AATTTGAGCATGGGGCAGA	NADPH reductase 850	55	30
rdxA R	GAAACGCTTGAAAACACCCCT			

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England biolab, UK), 9.5 µl sterile deionized water, 1 µl template DNA (100 ng) and 1 µl of each oligonucleotide primers (0.4 µM). Initial denaturation at 94°C for 5 mins followed by 30 cycles of denaturation at 94°C for 45s, annealing for 1min at 55°C, extension at 72°C for 30s. The expected fragment was 800 bp if the gene is normal and 650 bp if the gene is mutated. PCR amplified products were analyzed by 1.5% agarose gel electrophoresis [30].

Statistical analysis

The results were statistically analyzed using the Chi square or Fisher's exact test. The level of significance was set at $p < 0.05$.

Results

Prevalence of *H. pylori* and patients characteristics. A total of 72 patients presented with gastroduedenal diseases were included in this Study. 13 cases (23%) presented positive culture for *H. pylori*, with one of the patients (8%) presenting growth only in the biopsy of the gastric corpus, five patients (38%) in the biopsy of the antrum, while 7 patients (54%) were positive for *H. pylori* growth in both biopsy cultures. In general, statistically significant differences were not reported for the prevalence of *H. pylori* as a function of gender, age groups and endoscopic findings ($p > 0.05$) **(Table 2).** Fur-

Table 2. Clinical and epidemiological characteristics of patients enrolled in this study

Characteristics Culture (+)		Occurrence of *H. pylori*		Total	P value
		Culture (–)			
Age	15-35	5	13	18	>0.05
	36-55	4	26	30	
	56-80	4	20	24	
Sex	Male	3	25	28	>0.05
	Female	10	34	44	
Gastrointestinal complains	Anemia	2	6	8	>0.05
	Dyspepsia	2	3	5	< 0.02
	Dysphagia	1	3	4	>0.05
	Epigastric pain	5	34	39	>0.05
	Heartburn	4	4	8	< 0.02
	Vomiting	2	6	8	>0.05
Endoscopic finding	Mild gastritis	3	19	22	>0.05
	GERD	2	15	17	
	Erosions and Ulceration	3	19	22	
	Duodenitis	3	11	14	
Use of antibiotics	Yes	3	21	24	>0.05
	No	6	42	48	
Use of PPI	Yes	7	32	39	>0.05
	No	2	31	33	
Use of NSAIDs	Yes	2	22	24	>0.05
	No	7	41	48	

thermore, the presence of *H. pylori* in patients who used antibiotics and or NSAID was not statistically significant. Although the rate of *H. pylori* colonization increased with the use of PPI as reported in this work, no statistical significance was reported. In regards to the clinical manifestations, *H. pylori* colonization was significantly higher in patients presented with heartburn and dyspepsia ($p < 0.05$).

Antimicrobial susceptibility profile of *H. pylori*. All of the tested strains were sensitive to amoxicillin with values of MICs range of 0.015-0.03 µg/ml. Resistance to ciprofloxacin and levofloxacin was observed in 23% and 8% respectively **(Figure 1).** MIC values of ciprofloxacin resistance in *H. pylori* were 4-16 µg/ml **(Table 3).** 15% of the strains were resistant to clarithromycin with MIC value of 8 µg/ml. Resistance to metronidazole was dominating with 92% of the strains showed moderate to high levels of resistance with MIC range of 32-128 µg/ml.

Characterization of *cagA* and *vacA* genotypes and their relation to antibiotic resistance. Molecular analysis of different alleles of the *vacA*

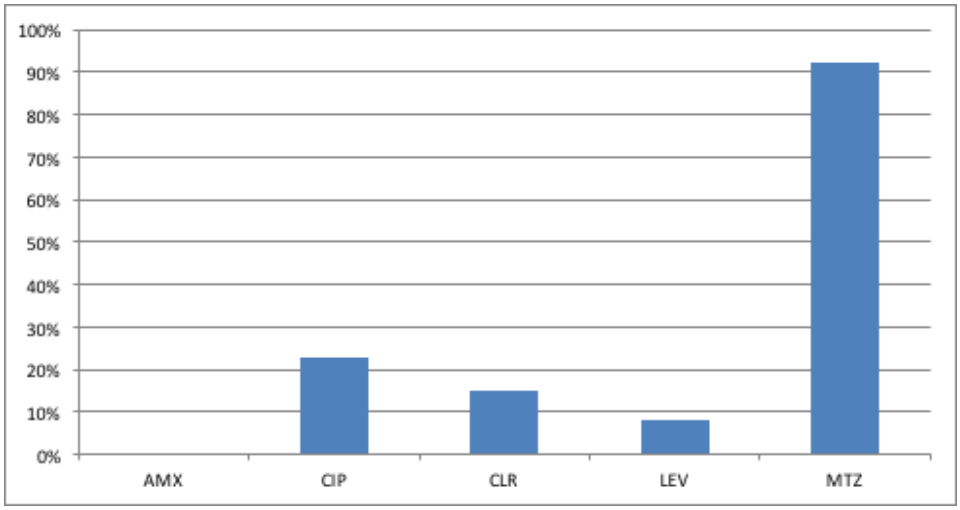

Figure 1. Percentages of *H. pylori* resistant to antimicrobial agents used in this study

Table 3. Antimicrobial susceptibility profile of *H. pylori* clinical strains to conventional antibiotics

Antibiotic	Sensitivity to antibiotics (MIC µg/ml)												
	Strain number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
AMX	S	S	S	S	S	S	S	S	S	S	S	S	S
CLR	S	S	R (8)*	S	S	S	S	S	S	R (8)	S	S	S
CIP	S	S	R (4)	S	S	S	S	S	R (4)	R (16)	S	S	S
LEV	S	S	S	S	S	S	S	S	S	R (8)	S	S	S
MTZ	R (64)	S	R (128)	R (64)	R (64)	R (32)	R (32)	R (64)	R (64)	R (128)	R (64)	R (64)	R (64)

AMX= amoxicillin, CLR= clarithromycin, CIP= ciprofloxacin, LEV= levofloxacin, MTZ= metronidazole. R indicates resistant, S indicates sensitive

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gene showed that one sample (8%) were found to have both *m1* and *m2* alleles with notable moderate multi-drug resistant behavior against the tested antibiotics **(Table 4).** The majority of the tested *H. pylori* strains possess the m2 allele (78%). No statistical significant association between the *vacA* allelic genotype and resistance to antibiotics or the clinical manifestation could be established ($p > 0.05$).

cagA gene was detected in 46% of the tested strains with no correlation to antibiotic resistance and clinical manifestations.

Table 4. cagA and vacA genotypes of clinical strains of *H. pylori* and their relation to the *rdxA* genetic mutations in metronidazole resistance

Strain	Genotype
1	*cagA+, vacA m1, rdxA 650*
2	*cagA-, vacA m2, rdxA 800*
3	*cagA+, vacA m1,m2, rdxA800*
4	*cagA+, vacA m2, rdxA800*
5	*cagA-, vacA m2, rdxA 800*
6	*cagA-, vacA m2, rdxA 800*
7	*cagA+, vacA m2, rdxA 800*
8	*cagA+, vacA m1, rdxA 800*
9	*cagA-, vacAm2, rdxA800*
10	*cagA-, vacAm2, rdxA800*
11	*cagA-, vacAm2, rdxA 800*
12	*cagA+, vacA m2, rdxA800*
13	*cagA-, vacA m2, rdxA800*

rdxA 650: deletion mutation, *rdxA 800:* normal length gene

Characterization of *rdxA* mutations in metronidazole resistance in *H. pylori*. By mutagenesis based PCR method, deletion mutations of *rdxA* gene in *H. pylori* clinical strains were analyzed. The assay provided two genotypes; Normal and mutant genotypes of *rdxA* gene with PCR products of 800 bp and 650 bp in size respectively. Only one sample (8%) of the metronidazole resistant *H. pylori* strains exhibited a mutation in *rdxA* gene which the rest of the tested strains exhibited a normal *rdxA* gene (Table 4)

Discussion

H. pylori has been increasingly considered as one of the most common bacterial infections affecting all ages worldwide. In Jordan, there is a limited number of epidemiological reports with a remarkable lack of information regarding the susceptibility of the clinical strains to the conventional antimicrobial agents used for the treatment of *H. pylori* infections [31]. One reason for this shortage of information is the technical difficulties of isolating this microaerophilic bacterium and the sensitivity of the methods used for this purpose [32].

This study has been conducted to characterize the susceptibility of the clinical strains of *H. pylori* to the conventional antimicrobial agents used in the region with particular concern for metronidazole and clarithromycin since there is an increasing number of recent reports about the emergence of multi-drug resistant *H. pylori* strains in the developing countries [33, 34].

Our study reported a very high rate of metronidazole resistance among the clinical isolates of *H. pylori* in Jordan compared to countries from Europe and Asia [35-37]. It could be suggested that the misuse of metronidazole, which is one of the common prescribed drugs by the general practitioners particularly in adults, would be a major factor for this high rate although the differences in gender and age were not statistically significant. In addition, the chemical nature of this antimicrobial agent plays a significant role in selecting the resistant strains. One interesting finding of our work is the low occurrence in deletion mutations in *rdxA* gene among *H. pylori* metronidazole resistant strains which is similar to data from Japan and supports the studies which questioned the mutational inactivation of

rdxA gene as a key mechanism of metronidazole resistance in *H. pylori* [38].

Resistance to clarithromycin was reported in 15% of the *H. pylori* clinical isolates tested in our study. This is in agreement with most of the local and international studies which reported rates that are similar to our results However, the reported MIC values of clarithromycin resistant *H. pylori* in our study were higher. [38, 39]. In a study conducted by PCR for the detection of point mutations associated with clarithromycin resistance, similar rates were reported in Jordan [31]. Although neither the history of treatment failure nor virulence factors of the strains could not be statistically established as factors for this result, there is mounting evidence favoring the testing for the genetic determinants with antimicrobial susceptibility testing in cases of clarithromycin.

Although testing of fluoroquinolones susceptibility is not primary in the screening for antibiotic resistance among *H. pylori* strains, our study showed increase rates of resistance to ciprofloxacin and levofloxacin (23% and 8% respectively). Ciprofloxacin is widely used in the treatment of many infections in our region compared to levofloxacin which is used with concern and only prescribed for specific types of infections. Our results are in agreement with data from other countries which reported higher rates for fluoroquinolones resistance among *H. pylori* clinical strains [40, 41]. Further testing for genetic determinants should be performed to characterize the type of resistance among *H. pylori* strains to ciprofloxacin.

Lastly, the in vitro testing showed that amoxicillin has the least resistance rate where no strains of *H. pylori* exhibited resistance to this antibiotics. In addition, the MIC values where less than 0.125 µg/ml indicating that amoxicillin is a good choice of antimicrobial agents that should be included in the treatment regimens for *H. pylori*. In Brazil, resistance to amoxicillin has been detected in 29% of strains recovered from post-treatment [39]. However, many studies from other countries have shown that all strains examined are susceptible to amoxicillin [42, 43]. The clinical significance of amoxicillin susceptibility testing has not been yet documented.

In conclusion, metronidazole resistance among clinical isolates of *H. pylori* is very high. Care should be taken into consideration when applying the standard regimen for the treatment of infections caused by this bacterium with emphasis of the importance of antimicrobial susceptibility testing in vitro. In addition, metronidazole use in the triple therapy for eradication of *H. pylori* should be revised.

The authors declare that there is conflict of interests

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