Original article

Detection of clarithromycin resistance and 23SrRNA point mutations in clinical isolates of Helicobacter pylori isolates: Phenotypic and molecular methods

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Abstract

Background and objectives: Peptic ulcer disease, chronic gastritis, and stomach cancer are all caused by H. pylori. The most notable drug for the treatment is the antibiotic clarithromycin, which is currently the drug of choice. H. pylori clarithromycin resistance has been associated with point mutations in 23srRNA, the most prominent of which are A2143 and A2144G. In H. pylori bacteria, methylase synthesis, macrolide-inactivating enzyme activity, and active efflux have all been found to be resistance mechanisms. The goal of the study is to determine how resistant H. pylori is to clarithromycin and what the minimum inhibitory concentration is for various antimicrobials. Furthermore, gastro-endoscopy will be performed on Iraqi patients to detect the presence of A2143G and A2144G point mutations in Helicobacter pylori infections, as diagnosed from the pyloric region and other anatomical regions.

Methods: One hundred fifteen samples were collected from patients strongly suspected of H. pylori infection presented for upper gastrointestinal endoscopy at Ramadi Teaching Hospitals and Private Clinics for the period from January 2020 until February 2021. Specimens were cultured on brain heart infusion agar containing various antibiotics and were incubated at 37°C under microaerophilic conditions. For identification of H. pylori, isolates of the biochemical tests and RT-PCR assay were applied. The Epsilometer test was used in the antibiotic susceptibility testing as dependent on the CLSI standard. The Restriction Fragment Length Polymorphism technique was used to determine point mutations.

Results: In total, 55 (47.8%) Helicobacter pylori isolates were cultured from the 115 biopsy specimens, among which 16 (29.1%), 38 (69.1%), 20 (36.4%), and 40 (72.7%) revealed some degree of resistance to levofloxacin, clarithromycin, ciprofloxacin, and metronidazole, respectively. The frequency of A2144G and A2143 point mutations were 23 (60.5%) and 19 (50%), respectively.

Conclusions: According to our results, Helicobacter pylori showed high resistance to clarithromycin. Our results demonstrate the requirement for antibiotic susceptibility testing and molecular methods in selecting drug regimens.

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1. Introduction

The bacterium Helicobacter pylori (H. pylori) is microaerophilic, a curved, Gram-negative bacilli isolated in 1983 from gastric biopsy in patients with gastritis (Charitos et al., 2021). Also, it causes persistent gastric infection by colonization of the human gastric mucosa (Ansari and Yamakoa, 2019). H. pylori can cause peptic ulcer disease and chronic gastritis (Vazirzadeh et al., 2020). Furthermore mucosa-associated lymphoid tissue and gastric cancer are associated with this microorganism (Arévalo-Jaimés et al., 2019). For these reasons, the WHO has announced H. pylori to be a class I carcinogen. The colonization of H. pylori must be removed...
in patients with peptic ulcers as the prevention of long-term ulcers is a major aim in addition to the acceleration of the healing of such (Reddy and Marsicano, 2018).

The spread of H. pylori infection differs across the world depending on geographic area, race, sanitary conditions, and age (Mehta et al., 2021). H. pylori infection is mainly acquired in one’s early years. It can persist for the host’s lifetime unless specifically treated (Seia et al., 2012). It is well known that more than half of a percent of the world’s population harbour H. pylori in the upper part of their GIT, making it the most highly distributed infection worldwide (Khoder et al., 2019). Antibiotic resistance in H. pylori is on the increase, which is concerning because it is one of the leading causes of therapy failure. H. pylori resistance is substantially higher in underdeveloped countries than in industrialized countries (Savoldi et al., 2018). Antibiotic resistance in H. pylori is usually acquired via chromosomal mutations rather than plasmid acquisition. Other resistance mechanisms have been discovered in bacteria, including methylase production, macrolide-inactivating enzyme activities, and active efflux (Agudo et al., 2010). Vertically transmitted point mutations in the DNA are the main cause of H. pylori resistance (Zhang et al., 2020).

A point mutation is a form of genetic mutation in which one of the DNA sequence’s base pairs is changed, either by insertion or deletion. A point mutation that occurs as a result of mutagens can be physical, such as X-rays and UV radiation, or due to chemical species, which can alter base pairs and DNA structure (Mobley et al., 2001). A2144G, A2143G, and A2142C are the most prevalent mutations. A2115G, C2141A, C2147G, T2190C, C2195T, A2223G, and C2694A mutations have also been discovered, but their significance in clarithromycin resistance is unclear (Zhang et al., 2020). The most common mutations in 23SrRNA gene are A2143G or A2144G in resistant H. pylori strains (Matta et al., 2018).

In spite of the sensitivity of H. pylori to various drugs in vitro, its elimination is difficult in vivo. This is believed to be due to the deactivation of antibiotics in the acidic pH of the stomach (Vala et al., 2016). Generally, triple therapy consists of a proton pump inhibitor together with double antibiotics such metronidazole and amoxicillin or clarithromycin, and is used for treatment of H. pylori infection (Ong et al., 2019). Due to an increase in resistance of H. pylori to clarithromycin, the efficacy of this regimen has dramatically decreased (Huang et al., 2017).

Clarithromycin interacts with the peptidyl transferase region of domain V of the 23S rRNA subunit, decreasing bacterial protein synthesis and suppressing bacterial ribosome activity, hence 23S rRNA was chosen for amplification and point mutation detection (Marques et al., 2020). Point mutations in the 23S rRNA gene and post-transcriptional methylation of the 23S rRNA region have been found to cause a change in ribosome structure, decreasing clarithromycin affinity and resulting in bacterial resistance to the antibiotic (Zhang et al., 2020). This antibiotic has an option for elimination of H. pylori as compared to other macrolides due to its stability in acid pH and its good absorption in the stomach (Marques et al., 2020). Clarithromycin resistance is responsible for clarithromycin treatment failure in over 50% of such cases (Yoon et al., 2014a, 2014b). High outpatient consumption of clarithromycin, particularly in the treatment of respiratory tract disorders, is one of the key contributors to H. pylori strains’ clarithromycin resistance (Klesiewicz et al., 2014).

As a result, in any region, determining antibiotic sensitivity before using clarithromycin to treat H. pylori infection is critical (Vala et al., 2016). Approved antibiotic susceptibility testing (AST) methods against bacteria, such as agar dilution, E-test, and disk diffusion, make this possible. The gold standard approach is agar dilution, however it is time consuming and costly. As a result, E-test or disk diffusion procedures could be used in place of agar dilution. The E-test is a simple and straightforward method for measuring the minimal inhibitory concentration (MIC) of a range of antibiotics, however it is more costly (Vala et al., 2016). The underlying aim of the study is to detect the resistance of H. pylori to clarithromycin and the minimal inhibitory concentration for selected antimicrobials and, in addition, to detect the occurrence of A2143G and A2144G point mutations in 23S rRNA of clarithromycin-resistant H. pylori isolates from a sample in Anbar, Iraq.

2. Patients study and techniques

2.1. Specimen collection

In total, 115 patients who submitted for routine upper gastrointestinal endoscopy at Ramadi Teaching Hospitals during the period from January 2020 until February 2021 constituted the sample. The patients included 80 (69.6%) males and 35 (30.4%) females with ages ranging from 17 to 69 years. The clinical diagnoses based on endoscopy included antral gastritis (n = 53), combined gastritis and duodenitis (n = 2), duodenitis (n = 7), gastric tumour, and adenocarcinoma (n = 1), hiatus hernia (n = 4), combined gastric and duodenal ulcers (n = 19), esophagitis (n = 1), and patients with dyspepsia (n = 28) (Table 1). The exclusion criteria were applied to patients who had received H2 receptor blockers, antimicrobial therapy, PPI, and/or non-steroid anti-inflammatory drugs one month pre-endoscopy. Subjects with the following clinical conditions were also excluded from the study: cirrhosis, nephropathy in critical stages, and pregnancy. Information about demographic and socioeconomic factors, and the personal treatment histories of the included patients was already reported in a questionnaire. The gastric biopsy samples obtained from the antrum and corpus of the stomach during routine endoscopy by an expert clinician (gastroenterologist) were placed in sterile tubes containing brain heart infusion broth medium and 5% of foetal bovine serum for transportation. Further, the results of an invasive rapid urease test (RUT) were found using a urea agar slant tube, RT-PCR amplification of 16SrRNA using the thermal cycler (Sacace - Italy) with a RT-PCR kit for qualitative detection of H. pylori ((Sacace- Italy), UBT using a HUBT-20p H. pylori detector (HEADWAY, China) using14C-urea, 99-atom%14C-labelled urea capsule. SAT was performed using the H. pylori Ag Rapid Test CE (CTK - Biotech, USA), whilst cagA-IgG was performed using a commercial Human H. Pylori Cytotoxin-Associated Gene A Protein IgG (HP-CagA-IgG) ELISA Kit (CUSABIO, USA) by ELISA system (Human, Germany) (Hussein et al., 2021).

2.2. Ethics’s committee

All study techniques that involved patients were approved by the Ethical Approval Committee, University of Anbar, Ramadi, Iraq

Table 1

Guideline characters for the main topics regarding the study patients and their diseases.

| Items | Findings |
|-------|---------|
| Number of patients (study isolates) | 115 (55) |
| Age | 17–69 years |
| Men: women (men: women %) | 80: 35 (69.6%: 30.4%) |
| Disease | |
| Antral gastritis: Duodenitis: Esophagitis: Hiatus- Hernia | 53:7:1:4 |
| Combined gastritis/Duodenitis: GDU | 2:19 |
| Gastric tumour | 1 |
| Patients with dyspepsia | 28 |

*GDU, combined gastric and duodenal ulcer.
Samples were cultivated on brain heart infusion agar (Oxoid, UK) consisting of 5% foetal bovine serum (Capricorn, South America), 7% horse blood, and antibiotics including nystatin, nalidixic acid, and vancomycin. The cultures were then incubated overnight (with specific conditions regarding microaerophilic conditions and saturated humidity: < 0.1 oxygen within 2.5 h and 7–15% CO2 within 24 hr. for 5–7 days (Anaerocult A; Darmstadt, Germany)) (Amin et al., 2019). Colonies of H. pylori within 24 hr. for 5–7 days (Anaerocult A; Darmstadt, Germany)) were isolated and identified bacteriologically. In addition, H. pylori isolates were confirmed by reverse transcription polymerase chain reaction amplification. H. pylori preservation were stored in brain heart infusion broth containing 10% foetal bovine serum and 15% glycerol at −20 °C. These stock solutions were thawed and sub-cultured for the study experiments (Amin et al., 2019).

2.4. Determination of antibiotic MICs using the E-Test

The MIC values of clarithromycin, levofloxacin, ciprofloxacin, and metronidazole against H. pylori isolates were confirmed with epsilometer strips (E-test, Ezy MIC™ Strips, HiMedia Laboratories, India). This test is performed and standardized based on criteria laid down by the appropriate Clinical Laboratory Standard Institute protocol. H. pylori isolates were sub-cultured on brain heart infusion agar (Oxoid, England) supplemented with 7% of horse blood, 5% foetal bovine serum, and dent supplement (vancomycin, nystatin, and nalidixic acid) for 72 h. A suspension of the bacteria was prepared equivalent to the McFarland turbidity standard (9x10⁸ CFU/mL; turbidity, 3 McFarland) and inoculated to Mueller-Hinton agar (Oxoid, England) containing 7% horse blood. The E-test strips were placed on the Mueller-Hinton agar after drying the surface of the medium. They were incubated under microaerophilic conditions at 37 °C for 72 h. Resistant breakpoints of MICs for clarithromycin, levofloxacin, ciprofloxacin, and metronidazole, were defined as ≥ 8, ≥ 8, ≥ 4, and ≥ 32 µg/mL, respectively, whereas the susceptible strains had MICs for clarithromycin, levofloxacin, ciprofloxacin, and metronidazole, ≤ 2, ≤ 2, ≤ 1, and ≤ 8 µg/mL, respectively (Amin et al., 2019; Al-Qaysi et al., 2020).

2.5. Molecular technique:- (Sambrook et al., 1989)

a) Extraction of DNA:
DNA was extracted using SaMag Tissue DNA extraction kits (Sacace, Italy) using the SaMag-12 automatic nucleic acid extraction system for the extraction of genomic DNA (Samaga, Cepheid, Italy). For the RFLP technique, the SaMag Bacterial DNA extraction kit (Sacace, Italy) was used to extract the nucleic acid from 38 (69.1%) H. pylori isolates which were resistant to clarithromycin. The extracted DNA was stored at a temperature of −20 °C (Vaziri et al., 2013). A QuantusTM Fluorometer (Promega, USA) was used to determine the concentration of extracted DNA to detect the goodness of the sample for further applications (Khalaf and Al-Ouqaili, 2018; Al-Ouqali et al., 2020).

b) Quantitative real-time PCR (qRT-PCR):
This was achieved using a thermal cycler (Sacace - Italy) with a Real-Time PCR kit for the qualitative detection of H. pylori ((Sacace, Italy). PCR conditions consisted of 1 cycle of 15 min at 95 °C. The cycling programme consists of 45 denaturation cycles for 10 s at 95 °C followed by annealing for 30 s at 60 °C, and an extension of 10 s at 72 °C.

c) 23SrRNA amplification and PCR-RFLP
DNA was extracted from H. pylori isolates using SaMag Bacterial DNA extraction kits (Sacace, Italy) which act as a template for amplifying a 425 bp fragment of 23SrRNA gene peptidyl transferase. Oligonucleotide primers (sense, 5'-CCACAGCGATGTGGTCTC-3'; antisense, 5'-CTCCATAAGGGCACAAGCCC-3') were used to investigate the mutations in the 23S rRNA gene that emerged in clarithromycin resistance. The polymerase chain reaction followed a program of initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 55 °C, an extension of 1 min at 72 °C and a final extension for 7 min at 72 °C to amplify the 23SrRNA. InTRON is the Maxime PCR PreMix Kit (Intron, Korea), which includes 5 U/µl i-Taq DNA polymerase, 2.5 mM DNTPs, 1X reaction buffer (10X), and 1X gel loading buffer. The PCR reaction was achieved using a final volume of 25 µl containing 5 µl Taq PCR PreMix, 10 picomols/µl (1 µl) forward and reverse primer, 1.5 µl genomic DNA as a template, and 16.5 µl distilled water. Red Safe Nucelic Acid Staining (Intron, Korea) was selected to stain the PCR product (425 bp) through electrophoresis using 1.5% agarose (Amin et al., 2019).

d) PCR-RFLP and detection of point mutations
The PCR-RFLP assay was performed to identify point mutations in clarithromycin-resistant strains of H. pylori. Amplicons (425 bp each) of the 23S rRNA gene were digested with either Bsal enzymes (BioLabs, USA) for 30 min at 37 °C or BbsI (BioLabs, USA) for 30 min at 37 °C to detect the A2143G and A2144G mutations, respectively, as shown in Table 2. Digested fragments were separated on a 2.5% agarose gel and viewed under UV light (Vilber Lourmat, France). A2144G and A2143G mutations in clarithromycin resistance strains were identified according to fragment numbers and size (Yoon et al., 2014a, 2014b).

3. Results

In total, 55 (47.8%) H. pylori cultures were obtained from 115 biopsies. A total of 41 (51.3%) and 14 (40%) of the isolates were collected and diagnosed from both males and females. The males and females were within the age ranges of 18–69 and 17–66, respectively. The patients had a mean age of 36.53 ± 14.544. The macroscopic cultural characteristics of H. pylori on the culture plate were confirmed by using all requested biochemical and molecular diagnostic tools, which include urease, oxidase, catalase, and the reverse transcription polymerase chain reaction, where the latter was performed to amplify and detect 16S rRNA (Figs. 1, 2). The endoscopy-based clinical diagnoses included antral gastritis, combined gastritis and duodenitis, duodenitis, gastric tumour or adenocarcinoma, hiatus hernia, combined gastric and duodenal ulcers, esophagitis, and patients with dyspepsia.

3.1. Detection of antibiotics’ minimal inhibitory concentrations (MIC)
Clarithromycin, levofloxacin, ciprofloxacin, and metronidazole resistance was found in 38 (69.1%), 16 (29.1%), 20 (36.4%), and 40 (72.7%) of the 55 H. pylori strains, respectively. (Table 3). Additionally, the antibiotic MICs based on the E-test are represented in Fig. 3. The clarithromycin, levofloxacin, ciprofloxacin, and metronidazole MIC values ranged from 0.25 to 128 µg/mL, 0.064 to 32 µg/mL, 0.094 to 64 µg/mL, and 0.19 to 256 µg/mL, respectively.
3.2. The multidrug resistance study isolates

44 of 55 strains (80%) were resistant to two antimicrobial agents (revealing MDR) (Table 4). Clarithromycin and metronidazole resistance were the most prevalent multidrug-resistant isolates (54.5%). Resistance to more than two drugs were observed in nine (16.4%) and four (7.3%) strains, respectively.

3.3. Amplification of the 23S rRNA in clarithromycin-resistant strains

The PCR technique was performed to amplify a fragment (425 bp) from the PT variable domain of 23srRNA to detect point mutations in clarithromycin-resistant isolates. The BsaI and BbsI endonuclease enzymes influence the PCR product. The PCR product of the strains containing the A2144G mutation produced 304-bp and 101-bp fragments if digested with BsaI. The H. pylori strains containing the A2143G mutation produced 332-bp and 93-bp fragments if digested with BbsI, as per Fig. 4. The A2144G mutation was found in 23 (60.5%) of the 38 clarithromycin-resistant H. pylori strains studied, whereas the A2143G mutation was found in 19 (50%) and the A2144G and A2143G double mutation was found in 9 (23.7%). The amplified products were not digested by BsaI and BbsI (15; 39.5%) (19; 50%), respectively (Table 6), which did not contain any of the above mentioned mutations. (The MICs for the A2144G mutant strains ranged from 8 to 32 μg/mL The MICs for the A2143G mutant strains ranged from 32 to 128. The MICs
for the double mutant strain were 16 μg/mL. The MICs for non-restricted strains were relatively low (MICs of 8 μg/mL) (Table 6).

3.4. Relationship between the type of mutation and the level of clarithromycin resistance

In total, of the 38 H. pylori-resistant strains, 14 (41.2%) A2144G H. pylori mutants had a MIC \(< 32 μg/mL\), six (17.6%) A2143G H. pylori mutants had a MIC \(\leq 32 μg/mL\), nine (26.5%) double mutant H. pylori mutants had a MIC \(\leq 32 μg/mL\) and 5 (14.7%) isolates that lacked either mutation and whose basis for clarithromycin resistance remains undetermined, had a MIC \(< 32 μg/mL\), while only 4 (100%) A2143G H. pylori mutants had a MIC \(> 32 μg/mL\) (Table 5).

4. Discussion

The resistance of H. pylori to antibiotics has been observed worldwide. Iraq is among those countries in which resistance to the drug of choice is spreading. Clarithromycin is used largely in H. pylori treatment. Therefore, the increased resistance has become a major issue in the eradication of study bacterium (Goderska, 2018). H. pylori resistance to clarithromycin is predominantly related to the point mutations in the peptidyl transferase-encoding region of the V domain of the 23S rRNA gene. The most prevalent point mutations responsible for this process are A2143G, A2142G, and A2142C (Klesiewicz et al., 2014). Norway (5.9%) has the lowest levels of clarithromycin resistance in Europe, whereas Spain (32.01%) and Portugal have the highest (42.35%). H. pylori resistance decreased from 36.65% in 2009 to 24.38% in 2014 according to European research conducted at six-year intervals, whilst India (58.8%) and China (58.8%) both saw high levels of clarithromycin resistance in the Asian regions (Ghotaslou, 2015). In Pakistan and Iran, resistance has been estimated to be 47.8% and 31.7%, respectively (Rasheed et al., 2014). Clarithromycin resistance has increased in several countries in recent years as a result of the extensive use of clarithromycin for respiratory infections within the general public, particularly in children, and there is a link between outpatient use of long-acting macrolides and clarithromycin resistance (Ghotaslou, 2015). Epidemiological factors and inadequate therapy leading to misuse of antibiotics may play a vital role in the variation of resistance to clarithromycin across the world (Aslam et al., 2018). Depending on the E-test method used, the isolated H. pylori strains showed resistance to clarithromycin in 38 (69.1%) cases in this study, which is close to the values observed by Abdollahi et al. (2011) in Iran (69.3%). Our results were higher than those of de Franceso et al. (2010) from Italy (51.2%), Amin et al. (2019) from Iran (53.4%), and Arenas et al. (2019) from Chile (26%). However, the results are largely inconsistent with the studies by Lottspeich et al. (2007) from Germany (13.3%), Tani and Ndip (2013) from South Africa (15.4%), Jaka et al. (2018) from Africa (29.2%) and Wang et al. (2019) from China (31.0%).

Klesiewicz et al. (2014) reported that different types of mutations are associated with different MIC values. The relationship between the MICs and the type of mutation for the 38 analysed H. pylori isolates is shown in Table 5. Versalovic and associates’ (1997) observations are inconsistent with those from our study, in which MIC values exceeding 32 mg/L were defined as having high-level resistance to clarithromycin. Our results showed that all of the A2144G H. pylori mutants demonstrated low MICs to clarithromycin (MICs \(< 32 mg/L\)), while in the A2143G mutants we observed strains of both phenotypes, that is, with high- or low-level resistance. However, the results of our study demonstrated that the strains with the A2144G mutation had lower average MICs than strains with the A2143G mutation (8 mg/L and 32 mg/L, respectively). These results are in line with those found by other researchers who concluded that the A2144G point mutation was correlated with lower clarithromycin MICs than A2143G (Klesiewicz et al., 2014). For the first time, Versalovic and associates stated that point mutations in the 23srRNA variable region are related to H. pylori resistance to clarithromycin (Abdollahi et al., 2011). Point mutations, which result in A to G transitions in the 23 s rRNA sequence, can be found at positions 2143 and 2144 and which have been subsequently confirmed by other investigators (Yoon et al., 2014a, 2014b; Amin et al., 2019). In this study, A2144G and A2143G mutations were found in H. pylori clarithromycin-resistant strains, based on the PCR-RFLP assay; among the 38 (69.1%) clarithromycin-resistant H. pylori strains included in our study, 23 (60.5%) of the isolates carried the A2144G mutation, 19 (50%) carried the A2143G mutation, and 9 (23.7%) carried the double mutation (A2144G and A2143G), PCR products were not digested by Bsal and BbsI were (15; 39.5%) (19; 50%), respectively, which did not contain any of the above mentioned mutations. Therefore, our research confirms the results reported by several other authors that the predominant mutations responsible for clarithromycin resistance in H. pylori are A2144G and A2143G (Yoon et al., 2014a, 2014b; Vazirzadeh et al., 2020). The majority of isolates (60.5%) contained the A2144G mutation, whereas the A2143G mutation was found in half (50%), where our results were consistent with Álvarez et al. (2009) from Colombia, Yoon et al., 2014a, 2014b from Korea, Klesiewicz et al. (2014) from Poland, and

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**Table 5**

Association between MICs of clarithromycin (mg/L) and type of mutations in the 23S rRNA of clarithromycin-resistant H. pylori strains.

| Mutation          | Number (%) of strains |
|-------------------|-----------------------|
|                   | MIC \(< 32\) mg/L n = 34 | MIC \(> 32\) mg/L n = 4 | Total n = 38 |
| A2143G            | 6 (17.6%)             | 4                         | 10 (26.3%)   |
| A2144G            | 14 (41.2%)            | 0                         | 14 (36.8%)   |
| Double mutation   | 9 (26.5%)             | 0                         | 9 (23.7%)    |
| (A2144G, A2143G)  | 5 (14.7%)             | 0                         | 5 (13.2%)    |
| Undetermined (NR) |                      |                           |              |
PCR-RFLP method and mutations in 38 clarithromycin-resistant strains.

| Isolates | Diagnosis                  | Age/sex | RFLP    | MIC (µg/mL) | Mutation      |
|----------|----------------------------|---------|---------|-------------|---------------|
| 4        | Antral gastritis           | 31/M    | BsaI, BbsI | 8           | NR, NR        |
| 5        | GDU                        | 20/F    | BsaI, BbsI | 8           | NR, NR        |
| 6        | Antral gastritis           | 34/M    | BsaI, BbsI | 16          | A2144G, A2143G|
| 7        | GDU                        | 45/M    | BsaI, BbsI | 16          | A2144G, A2143G|
| 10       | Antral gastritis           | 20/M    | BsaI, BbsI | 16          | A2144G, A2143G|
| 11       | GDU                        | 56/M    | BsaI, BbsI | 16          | A2144G, A2143G|
| 22       | Antral gastritis           | 30/M    | BsaI, BbsI | 32          | NR, A2143G    |
| 24       | Antral gastritis           | 24/M    | BsaI, BbsI | 8           | A2144G, NR    |
| 25       | GDU                        | 25/M    | BsaI, BbsI | 32          | NR, A2143G    |
| 26       | Duodenitis                 | 18/M    | BsaI, BbsI | 8           | NR, NR        |
| 28       | Antral gastritis           | 35/M    | BsaI, BbsI | 16          | A2144G, NR    |
| 29       | GDU                        | 69/M    | BsaI, BbsI | 128         | NR, A2143G    |
| 30       | Duodenitis                 | 31/M    | BsaI, BbsI | 16          | A2144G, A2143G|
| 31       | GDU                        | 32/F    | BsaI, BbsI | 16          | A2144G, A2143G|
| 34       | Antral gastritis           | 38/F    | BsaI, BbsI | 8           | NR, NR        |
| 36       | Duodenitis                 | 30/M    | BsaI, BbsI | 16          | A2144G, NR    |
| 40       | Antral gastritis           | 20/M    | BsaI, BbsI | 16          | A2144G, NR    |
| 41       | Antral gastritis           | 52/M    | BsaI, BbsI | 16          | A2144G, NR    |
| 43       | Antral gastritis           | 25/F    | BsaI, BbsI | 64          | NR, A2143G    |
| 45       | Dyspepsia                  | 23/F    | BsaI, BbsI | 8           | NR, A2143G    |
| 51       | Antral gastritis           | 25/M    | BsaI, BbsI | 32          | NR, A2143G    |
| 59       | GDU                        | 18/M    | BsaI, BbsI | 64          | NR, A2143G    |
| 60       | GDU                        | 26/M    | BsaI, BbsI | 32          | NR, A2143G    |
| 61       | Antral gastritis           | 31/F    | BsaI, BbsI | 8           | A2144G, NR    |
| 62       | Antral gastritis           | 36/M    | BsaI, BbsI | 16          | A2144G, NR    |
| 63       | Antral gastritis           | 26/M    | BsaI, BbsI | 32          | A2144G, NR    |
| 64       | GDU                        | 32/M    | BsaI, BbsI | 16          | A2144G, NR    |
| 65       | GDU                        | 69/M    | BsaI, BbsI | 16          | A2144G, A2143G|
| 66       | Antral gastritis           | 45/F    | BsaI, BbsI | 16          | A2144G, A2143G|
| 67       | Antral gastritis           | 32/M    | BsaI, BbsI | 16          | A2144G, NR    |
| 68       | Antral gastritis           | 50/M    | BsaI, BbsI | 16          | A2144G, NR    |
| 69       | Duodenitis                 | 20/M    | BsaI, BbsI | 16          | A2144G, NR    |
| 70       | Antral gastritis           | 31/M    | BsaI, BbsI | 16          | A2144G, NR    |
| 71       | GDU                        | 19/F    | BsaI, BbsI | 16          | A2144G, NR    |
| 72       | GDU                        | 30/M    | BsaI, BbsI | 32          | NR, A2143G    |
| 73       | Antral gastritis           | 18/M    | BsaI, BbsI | 64          | NR, A2143G    |
| 74       | GDU                        | 20/F    | BsaI, BbsI | 32          | NR, A2143G    |
| 83       | GDU                        | 36/M    | BsaI, BbsI | 16          | A2144G, A2143G|

* GDU, combined gastric and duodenal ulcer; MIC, minimal inhibitory concentration; NR, non-restriction; RFLP, restriction fragment length polymorphism.

Vazirzadeh et al. (2020) from Iran. Therefore, there are geographical variations in clarithromycin resistance, which highlights the significance of identifying regional patterns of resistance in H. pylori for the selection of proper treatment. Furthermore, the clarithromycin resistance is attributed to the efflux system in H. pylori, which forces the bacteria to extrude macrolides. Our PCR-RFLP analysis also showed the occurrence of five (14.7%) isolates without any digestion with BsaI and BbsI. Therefore, resistance of these isolates to clarithromycin might be associated with other less common mutations or with the efflux mechanism (Klesiewicz et al., 2014).

In our study, 55H. pylori strains were tested; 3.7% were susceptible to all, and 96.3% were resistant to at least one of the tested antibiotics. Multi-resistant strains explained as many as 80% of cases and included those resistant to two (metronidazole and clarithromycin, or clarithromycin and ciprofloxacin, or clarithromycin and levofloxacin, or levofloxacin and ciprofloxacin, or ciprofloxacin and metronidazole, or three (clarithromycin, levofloxacin, and metronidazole, or clarithromycin, ciprofloxacin and metronidazole, or levofloxacin, ciprofloxacin and metronidazole) or four (clarithromycin, levofloxacin, ciprofloxacin and metronidazole). This rate was higher than that (16.5%) for H. pylori isolated from Korean patients (Yoon et al., 2014a, 2014b) and from Polish patients (29%) (Bińkowska et al., 2018). Our study showed a high resistance to metronidazole that was in line with a study in Eastern and Central European countries, reaching almost 50%, whilst resistance to clarithromycin is as high as 30% and still increasing, contributing to the failure of first-line therapy in approximately 70% of patients (Mégraud, 2017). Such a high resistance to clarithromycin as that observed in Poland results mainly from excessive consumption of antibiotics, especially from the group of macrolides, and their widespread use to treat respiratory tract infections. With regard to levofloxacin, our results showed resistance to such was very low (29.1%) which is very close the rates observed by Karczewska et al. (2014) and Korona-Clowniak et al., (2019).

5. Conclusions

According to our results, H. pylori showed high resistance to clarithromycin. Our results demonstrated the necessity for antibiotic susceptibility testing and molecular methods when selecting drug regimens. The correlation between the resistance of H. pylori to clarithromycin and 23srRNA point mutations is clearly high. In addition, H. pylori resistance to clarithromycin is both widespread and considerable, resulting in the reduced potencies seen in studies of antimicrobial agents. However, new antimicrobial resistance studies should be handled periodically and regionally in Iraq to provide information that may help to monitor effective eradication programmes. The PCR-RFLP method reduces the time required for resistance determination of clarithromycin by about four days compared to phenotypic methods of susceptibility testing. Non-restricted isolates were also discovered during our PCR-RFLP study. Resistance to clarithromycin in these isolates could be associated with other, less prevalent mutations or the efflux mechanism. As a result, more research in this area is clearly required. These mechanisms could be determined at some future point via PCR-RFLP, RT-
PCR, or sequencing. Our observations revealed that patients showed considerable clarithromycin resistance and that *H. pylori* can have dual resistance. This shows that existing *H. pylori* infection management in this part of Iraq should be adjusted to account for the high prevalence of *H. pylori* resistance to clarithromycin. Clarithromycin-based triple therapy should not be utilized in the attempt to eradicate *H. pylori*.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Further reading
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