Comparative Study of *Helicobacter Pylori* Resistance to Clarithromycin and Metronidazole and Its Association with Epidemiological Factors in A Moroccan Population

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Abstract

**Objective:** Knowledge of local antibiotic resistance is crucial to the adaption of the effective empirical first-line treatment for Helicobacter Pylori (*H. pylori*) infection. This study aimed to evaluate the prevalence of *H. pylori* resistance to clarithromycin and compare it with that of metronidazole, and highlight the impact of epidemiological factors and gastric lesions severity on *H. pylori* resistance. **Methods:** The susceptibility to clarithromycin of 96 isolates was determined by PCR-RFLP and the susceptibility to metronidazole of 185 isolates was determined by classic PCR. **Result:** Our results showed that the prevalence of *H. pylori* resistance to clarithromycin (14.6%) was low compared to that recorded with metronidazole (62.7%). Moreover, we remarked that 7.3% of isolates were co-resistant to both antibiotics. The assessment of epidemiological factors’ impact on the resistance to studied antibiotics has revealed no association. Besides, our results had demonstrated that the metronidazole and clarithromycin resistance was not related to the severity of gastric lesions. **Conclusion:** In our population, clarithromycin seems to be an effective antibiotic as long as the resistance rate of *H. pylori* is low. In contrast to metronidazole, it appears that this antibiotic will lose its efficacy, due to the high rate of resistance among our population. Therefore, each population must conduct their epidemiologic studies separately to survey the resistance profile of strains and choose the appropriate antibiotic, in order to avoid the failure of *H. pylori* eradication and the development of severe gastric diseases.

**Keywords:** *Helicobacter pylori*- Resistance- Metronidazole- Clarithromycin- Eradication

*Asian Pac J Cancer Prev, 23 (8), 2755-2761*

Introduction

Helicobacter Pylori (*H. pylori*) is a Gram-negative, microaerophilic, spiral bacterium that colonizes the stomach of approximately half the world’s population. In Morocco, the prevalence of *H. pylori* infection exceeds 70% (Bounder et al., 2017). This bacterium represents the main risk factor for chronic gastritis, peptic ulcer disease, gastric carcinoma, and mucosa-associated lymphoid tissue (MALT) (Cancer, s. d.). The International Agency for Research on Cancer has classified *H. pylori* as a group 1 carcinogen pathogen (Cancer, s. d.). The eradication of *H. pylori* is an important component of treatments for peptic ulcer disease and other gastrointestinal disorders (Malfertheiner et al., 2005; Kabir, 2009).

Several studies had indicated that the eradication of *H. pylori* could reduce the incidence of gastric cancer in patients without precancerous lesions (Malfertheiner et al., 2005; Kabir, 2009).

Unfortunately, the resistance of *H. pylori* to antibiotics has been increased in the last decades in most parts of the world (Mohammadi et al., 2005; Ben Mansour et al., 2010; Bachir et al., 2018). It is well known that the increased resistance to antibiotics plays an important role in the failure of eradication treatment (Koletzko et al., 2006). The most effective treatment regimens for *H. pylori* combine a proton pump inhibitor (PPI), and two antimicrobial agents selected from amoxicillin, a 5-nitroimidazole (usually metronidazole), or clarithromycin.

However, the efficiency of standard triple therapy containing a PPI, clarithromycin, with amoxicillin or metronidazole has been declining in many countries...
because of rising antibiotic resistance. Although, there are several factors influencing efficiency of an anti-\emph{H. pylori} therapy, \emph{H. pylori}-resistance to antibiotics remains the most relevant. According to World Health Organization, \emph{H. pylori} ranked among the 12 most resistant bacteria in the world. While different antibiotics have been used for the treatment of \emph{H. pylori} infection, this bacterium has evolved several mechanisms to protect itself against antimicrobial activities, in particular for clarithromycin and metronidazole (Lu et al., 1999).

Clarithromycin is a bacteriostatic antibiotic, belonging to the macrolide group and connecting to the peptidyl transferase region of the 23S ribosomal RNA (rRNA) molecule V domain. Three-point mutations (A2143G, A2142G, A2142C) in the V domain of 23S rRNA is responsible for more than 90% of adult cases for clarithromycin resistance (Mégraud, 2004).

Metronidazole is belonging to the nitroimidazole class, and it is frequently used to treat gastrointestinal infections. Metronidazole has been adopted as an antibiotic for several decades, with added antiparasitic properties that set it apart from many other antibacterial drugs, allowing it to treat a wide variety of infections (Dingsdag and Hunter, 2018; Hernández Ceruelos et al., 2019). The molecular mechanism of metronidazole resistance is mainly due to the inactivation of an oxygen-insensitive NADPH nitroreductase (rdxA) as a result of a deletion in the rdxA encoding gene, and may also be boosted by mutations in the frxA gene that encodes a NAD (P) H-flavin oxidoreductase (Goodwin et al., 1998; Debets-Ossenkopp et al., 1999).

This study aimed to evaluate the prevalence of \emph{H. pylori} resistance to metronidazole and clarithromycin and to highlight its relationship with epidemiological factors (gender, age, living area, alcohol-tobacco consumption) and gastric lesions characteristic of carcinogenesis.

Materials and Methods

\textbf{Study population and Data collection}

The present study was carried out on patients with gastric diseases consulting at Ibn Rochd University Hospital Center, Casablanca (Gastroenterology and Oncology departments), aged from 18 to 86 years. Three biopsies (1 from antrum, 1 from fundus, and 1 from lesser curvature) were taken from each enrolled patient by endoscopy, from 2017 to 2020.

Clinical information about demographic characteristics of participants including age, sex, living area, smoking, and alcohol habits was collected using a structured survey. All participants were informed about their inclusion in the study and agreed to it in writing form. The study protocol has been performed in accordance with the ethical standards of Helsinki and was approved by the committee of the Pasteur Institute of Morocco.

\textbf{Histopathology Test}

The biopsy samples were transported in 10% formalin and embedded in paraffin. Multiple histological sections were obtained from each biopsy. Biopsy sections were then obtained and stained with hematoxylin-eosin for the detection of gastric lesions. The blades were read by a pathologist.

\textbf{DNA extraction}

Whole genomic DNA was extracted from gastric biopsies using a genomic DNA extraction kit (Isolate Genomic DNA Kit, BioLine) according to the manufacturer’s instructions. Then it was stored at −20 °C until use.

\textbf{\emph{H. pylori} detection by PCR}

The ure C gene (296 bp) was amplified to detect \emph{H. pylori} infection, using the primers described by Lu et al. PCR reaction mixture was prepared with 0.5 mM dNTPs, 1.5 mM MgC2, 0.5 μM of each primer, 1 U of DNA Polymerase (MyTaq DNA Polymerase, BioLine), and 300 ng of DNA in a final volume of 20 μL. PCR thermocycling conditions were: 1 cycle at 95°C for 1 min, 35 cycles at 95°C for 15 s, 55°C for 30 s, 72°C for 30 s and a final extension cycle at 72°C for 7 min (Lu et al., 1999).

\textbf{Clarithromycin Susceptibility Detection}

PCR-RFLP was performed for genotyping two mutations of the 23S rRNA gene at the position A2142 C and A2143 G.

The fragment of the gene containing these mutations was amplified using specific primers (Table 1). The PCR reaction is carried out in a final volume of 25 μl included: 300 ng of genomic DNA, 0.8 μM of primers, 0.5 mM dNTPs, 1.5 mM MgC2, and 1U of Taq polymerase (MyTaq DNA Polymerase, BioLine). The amplification conditions were as follow: initial denaturation at 95°C for 1 minute, followed by 35 cycles of 95°C for 15 seconds, 60°C for 30 sec, 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. The electrophoresis on a 1% agarose gel was performed to achieve amplicons of 768 bp.

The A2142G substitution was assessed by restriction digestion with Mbo II restriction endonuclease that recognizes the GAAGA sequence and cleaves the mutated gene to 418 and 350 bp. The A2143G substitution was assessed by Eco 31I restriction endonuclease that recognizes the AATTC sequence and cleaves the mutated gene to 350, 310, and 108 bp. The cleaved product was resolved on 2% agarose gel electrophoresis (Suzuki et al., 2013).

\textbf{Metronidazole Susceptibility Detection}

Deletion of a 200-bp fragment from the rdxA gene is one of the molecular mechanisms of resistance to metronidazole. To determine the gene deletion, the PCR method using specific primers was performed. In the case of susceptible strains (non-mutated \emph{H. pylori}), the amplification of this gene by PCR leads to the production of an 850-bp fragment. In the case of the defective gene (resistant-(\emph{H. pylori})), the PCR leads to the production of a 650-bp fragment.

The rdxA gene was amplified by PCR in a total volume of 20 μL of reaction mix included: 300 ng of genomic DNA, 0.8 μM of primers, 0.5 mM dNTPs, 1.5 mM MgC2, and 1U of Taq polymerase (MyTaq DNA Polymerase, BioLine). Thermocycling conditions of PCR were as follow: initial denaturation at 95°C for 1 minute, followed
by 35 cycles of 95°C for 15 seconds, 58°C for 30 sec, 72°C for 30 seconds, and a final extension at 72°C for 7 minutes. The PCR products were analyzed by 1.5% agarose gel electrophoresis (Debets-Ossenkopp et al., 1999).

Statistical analysis
Data entry and analysis were performed using RStudio software. The descriptive data are presented as frequencies and means ± standard deviations (SD). The differences between the groups were analyzed with Wilcoxon test for continuous variables, and Chi-square test or Fisher’s exact test for categorical variables. The differences were considered significant at p < 0.05.

Results

Description of the population studied
A total of 195 gastric biopsies were obtained from patients with histopathological diagnosis. Of all patients, 59.5% (116/195) had active chronic gastritis, and 40.5% (79/195) had precancerous lesions [26.2% (51/195) had atrophic gastritis and (14.4%) (28/195) had intestinal metaplasia]. The mean age of patients was 49.9 ± 16.4 (range 18–86 years). The rate of female and male was, respectively 54.4% (106/195) and 45.6% (89/195). Of 195 patients, H. pylori infection was detected in 185 individuals (94.9%) based on the detection of ure C gene by PCR technique.

The prevalence of clarithromycin resistance compared to metronidazole resistance
Regarding clarithromycin resistance, our study showed that 14.6% (14/96) of patients are infected with H. pylori strains resistant to clarithromycin, indicating a relatively low level of clarithromycin resistance, while 85.4% (82/96) are infected with clarithromycin susceptible strains. Moreover, we remarked that the A2143G mutation was the commonest among our isolates while the A2142G mutation was not found.

Concerning metronidazole resistance our results showed that 62.7% (116/185) of patients are infected with H. pylori strains resistant to metronidazole, indicating a high level of metronidazole resistance, compared to clarithromycin resistance (14.6%). Seven strains (7.3%) were resistant to both antibiotics, clarithromycin, and metronidazole.

Epidemiological factors and H pylori resistance variation by gender
Analysis of H. pylori antibiotic resistance by gender using the data available (Table 1 and 2) showed that males and females were infected in similar proportions by clarithromycin-resistant isolates, with a rate of 14% for men versus 15.4% for women. Likewise, analysis of metronidazole resistance showed similar proportions in men (62.7%) and women (63.3%).

Variation by age
The distribution of antibiotic-resistance patterns according to age is shown in tables 1 and 2. We found that no patient was resistant to clarithromycin in the age group of [61-90]. However, in this age group, most patients were infected with strains resistant to metronidazole with a rate of 74.4%. The age group of [51-60] was the most infected by the resistant strains of clarithromycin (20.8%). However, the difference was not statistically significant between groups.

variation by the living area
Regarding the living area, antibiotics resistance was

Table 1. Prevalence of Clarithromycin Resistance and Social Factors

| Factors (n) | Clarithromycin-resistance (%) | Clarithromycin-susceptibility (%) | P-value |
|------------|------------------------------|----------------------------------|---------|
| Gender     |                              |                                  |         |
| Men (57)   | 8 (14)                       | 49 (86)                          | 0.99    |
| Women (39) | 6 (15.4)                     | 33 (84.6)                        |         |
| Age        |                              |                                  |         |
| 18-40 (34) | 4 (11.8)                     | 30 (88.2)                        |         |
| 41-50 (26) | 5 (19.2)                     | 21 (80.8)                        | 0.94    |
| 51-60 (24) | 5 (20.8)                     | 19 (79.2)                        |         |
| 61-90 (12) | 0 (0%)                       | 12 (100)                         |         |
| The living area |                      |                                  |         |
| Rural (25) | 5 (20)                       | 20 (80)                          | 0.50    |
| Urban (71) | 9 (12.7)                     | 62 (87.3)                        |         |
| Tobacco    |                              |                                  |         |
| Yes (12)   | 2 (14.3)                     | 10 (85.7)                        |         |
| No (84)    | 14 (16.7)                    | 70 (83.3)                        | 0.99    |
| Alcohol    |                              |                                  |         |
| Yes (14)   | 1 (7.1)                      | 13 (92.9)                        | 0.99    |
| No (82)    | 11 (13.4)                    | 71 (86.6)                        |         |
| Total 96   |                             |                                  |         |

Table 2. Prevalence of Metronidazole Resistance and Epidemiological Factors

| Factors (n) | Metronidazole-resistance (%) | Metronidazole-susceptibility (%) | P-value |
|------------|-----------------------------|---------------------------------|---------|
| Gender     |                             |                                 |         |
| Men (87)   | 54 (62.7)                   | 33 (37.3)                       | 0.86    |
| Women (98) | 62 (63.3)                   | 36 (36.7)                       |         |
| Age        |                             |                                 |         |
| 18-40 (54) | 35 (64)                     | 19 (36)                         |         |
| 41-50 (71) | 19 (43.2)                   | 25 (56.8)                       | 0.16    |
| 51-60 (42) | 30 (71.1)                   | 12 (28.9)                       |         |
| 61-90 (43) | 32 (74.4)                   | 11 (25.6)                       |         |
| The living area |                      |                                 |         |
| Rural (59) | 38 (64)                     | 21 (36)                         | 0.86    |
| Urban (126)| 78 (60.1)                   | 48 (39.9)                       |         |
| Tobacco    |                             |                                 |         |
| Yes (26)   | 17 (65.4)                   | 9 (34.6)                        |         |
| No (159)   | 99 (62.2)                   | 60 (37.8)                       | 0.93    |
| Alcohol    |                             |                                 |         |
| Yes (16)   | 10 (62.3)                   | 6 (37.7)                        |         |
| No (169)   | 106 (62.7)                  | 63 (37.3)                       | 0.99    |
| Total 185  |                             |                                 |         |
more detected in the rural area compared to the urban area in the same way for clarithromycin and metronidazole respectively (60.1% and 64% for rural area versus 12.7% and 20% % for urban area) (Tables 1-2). However, the difference was not statistically significant.

**Variation by the consumption of alcohol- tobacco**

The study of the impact of tobacco and the consumption of alcohol on the risk of clarithromycin and metronidazole resistance showed that there was no difference between *H. pylori* resistance and the consumption of tobacco or alcohol. These results were shown in tables 2-3.

**Study of the impact resistance on gastric lesions characteristic of carcinogenesis**

It is well known that *H. pylori* infection is associated with the development of precancerous lesions such as atrophic gastritis, intestinal metaplasia, and gastric cancer. For that, we tried to study the relationship between the resistance of antibiotics (clarithromycin- metronidazole), and the degree of severity of precancerous gastric lesions. Our patients were categorized into two groups according to the severity of gastric lesions related to *H. pylori* infection: chronic gastritis and precancerous lesions (atrophic gastritis and intestinal metaplasia). Clarithromycin -resistance rates in each of these two groups were respectively 13.3% (8/60) and 16.7% (6/36). The corresponding Metronidazole -resistance rates were 62% (67/108) and 63.6% (49/77) respectively. The difference was not statistically significant.

**Discussion**

**Antibiotic susceptibilities**

The high antibiotic resistance increases the risk of *H. pylori* transmission among the population and the persistence of infection that leads to gastric mucosa inflammation and increases the risk of gastric cancer (Cancer, s. d.).

The contributory factors to treatment failure are multidimensional and complex. Epidemiological factors, host genetic factors, *H. pylori* virulent factors, antibiotic-resistant *H. pylori* strains, compliance to therapy, and duration of therapy affect the treatment outcome. However, *H. pylori*-resistance to antibiotics remains the most relevant factor of *H. pylori*.

Clarithromycin is the core antibiotic of the standard triple regimen. Many studies have found that resistance to clarithromycin plays an important role in standard triple treatment failure. Recently, primary clarithromycin resistance to *H. pylori* remains a serious problem in most regions of the world (Nogueira et al., 2001; Datta et al., 2005; Hu et al., 2017; Bachir et al., 2018). Metronidazole is an important antibiotic in quadruple therapy. The antibiotic resistance of metronidazole is known to be as high as 50%-80% in developing or developed countries (Bachir et al., 2018).

In 2014, a Moroccan study had revealed a rate of 28.8% for clarithromycin resistance and 40.1% for metronidazole resistance (Bouhat et al., 2017). Compared to our present study, we remarked a decrease in the rate of clarithromycin resistance (14.6%) and an increase in the rate of metronidazole resistance (62.7%). These reports show that clarithromycin will have a good effect on the eradication of *H. pylori* for our population, due to the low rate of resistance to this antibiotic.

Regarding clarithromycin resistance and compared to other countries, the prevalence of resistance in our population (14.6%) seems to be similar to those found in Tunisia (14.6%) (Ben Mansour et al., 2010) and in Spain (17.1%) (Tamayo et al., 2017), lower to that detected in Iran (17%) (Mohammed et al., 2003), in Turkish (27.6%) (Baglan PH et al., 2006) and in European countries (43.5%) (Soloca et al., 2001), and higher than that found in Congo (1.7%) (Ontsira Ngoyi et al., 2015) and Iceland (9%) (Gunnarsdottir et al., 2017). The difference in the clarithromycin resistance rate between several countries might be due to the prescription of this antibiotic.

In general, and in many countries over the world, and since clarithromycin is widely used as an antimicrobial drug to heal other infections such as respiratory infectious diseases and previous consumption of macrolides, the prevalence of clarithromycin-resistant is continuously increasing. In Europe, a positive correlation was established between long-acting macrolide consumption and *H. pylori* resistance to clarithromycin. However, in our population, the rate of clarithromycin resistance remains low because macrolides are not frequently prescribed in Morocco apart from *H. pylori* eradication. So Morocco is among the countries where it is still possible to use the standard triple therapy, which uses clarithromycin as empiric first-line treatment for this infection (Megraud et al., 2013).

Concerning the point mutations conferring resistance to clarithromycin, we found that A2143G was dominant and the A2142G was not detected in all samples. The A2143G was more prevalent in two Spanish and Tunisian studies (Alarcón et al., 2000; Bachir et al., 2018). De Francesco (2010) (De Francesco et al., 2010) reported that the A2143G was prevalent in their Italian study and concluded that this mutation reduces markedly the eradication therapy efficacy. While Hansomburana (2012) (Hansomburana et al., 2012) documented that the A2142G mutation was more frequent than the A2143G in the

| Table 3. The Impact of Clarithromycin and Metronidazole Resistance on the Evolution of Gastric Lesions |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Histologic lesions** | **Clarithromycin-resistance (%)** | **Clarithromycin-susceptibility (%)** | **Metronidazole-resistance (%)** | **Metronidazole-susceptibility (%)** |
| Chronic gastritis | 8 (13.3) | 52 (86.7) | 67 (62) | 41 (38) |
| Precancerous lesions | 6 (16.7) | 30 (83.3) | 49 (63.6) | 28 (36.4) |
| P-value | 0.76 | | 0.87 | |
Regarding metronidazole resistance, our rate (62.7%) was similar to the rate found in Egypt (62.9%) (Ramzy et al., 2016), in Tunisia (56.8%) (Ben Mansour et al., 2010), in Algeria (67.5%) (Bachir et al., 2018) and in China (63.8%) (Hu et al., 2017), but was higher than that found in Europe (33.1%) (Lerang et al., 1997; Boyanova, 2009) and Iceland (1%) (Gunnarsdottir et al., 2017) and India (41.9%) (Bhatia et al., 2004), and lower than that found in Colombia (82%) (Alvarez et al., 2009). In a multicenter African study, despite the high heterogeneity of the studies, the overall metronidazole resistance was 75.8% which is significantly higher than that observed in Europe and South America (Jaka et al., 2018). The use of metronidazole in the treatment of other endemic diseases such as diarrheal and protozoa diseases could explain the significantly high rate of metronidazole resistance in Africa ( Głupczynski et al., 2001; Frencik and Clemens, 2003). Due to the increase of H. pylori resistance to metronidazole in our population, it appears that this antibiotic will lose its effectiveness, and it will become a significant limitation for the eradication of H. pylori.

Epidemiological factors and clarithromycin resistance
In this study, we found that the resistance rate to clarithromycin for males was like females. On the opposite, other studies found males are more predisposed to clarithromycin resistance than females (Boyanova et al., 2012; Wang et al., 2019).

According to age, even if our result was not significant, the absence of clarithromycin resistance in elderly patients [60-90] can be explained by the non-use of macrolides in their childhood. It was noted that the prevalence of clarithromycin resistance was more frequent in patients whose age is less than 60 years. This is most likely due to the wide use of macrolides in the treatment of upper respiratory tract infections in Morocco at a young age in the last decades. Serrano (2017) found infected pediatric patients, with a high prevalence of clarithromycin resistance which is indicative of an increased prevalence of this resistance among H. pylori early in childhood. (Serrano et al., 2017).

The study of the impact of tobacco and the consumption of alcohol on clarithromycin resistance showed that there was no association statistically significant.

As we have already noted, H. pylori infection is an important etiological factor for gastric cancer, and its eradication reduces the risk of cancer development (Kabir, 2005; Malfertheiner et al., 2005; Courillon-Mallet A, 2014).

The observed link between antibiotic resistance and disease severity is not well known until now. We have not found a significant difference in the frequency of clarithromycin resistance according to the severity of precancerous gastric lesions (p-value>0.05). However, Farzi (2009) found a significant difference in the frequency of clarithromycin resistance in patients with severe gastritis compared to those with chronic gastritis (Farzi et al., 2019). Exacerbation of gastric disease after ineffective treatment occurs in most of the patients, which is accompanied by the dominance of resistant strains in the stomach. In this view, the resistant strains may act like innocent bystanders, and their persistence in the gastric tissues, due to its resistance, has probably an impact on the evolution of lesions towards gastric cancer.
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Author Contribution Statement

All authors contributed equally in this study.

Acknowledgments

We acknowledge the contributions of our patients’ partners, and we ask God for the recovery and good health of the patients. We also acknowledge the team of the HistoCytopathology Laboratory, Institut Pasteur du Maroc, Casablanca, Morocco; Dr. Meriem Khyati from the Laboratory of Onco-Virology, Institut Pasteur du Maroc.

Funding Statement

This work was supported by the Pasteur Institute of Morocco.

Approval

This work is part of an approved thesis.

Ethical Declaration

The study protocol has been performed in accordance with the ethical standards of Helsinki and was approved by the committee of the Pasteur Institute of Morocco.

Data Availability

The data used to support the findings of this study are included in the article.

Conflict of Interest

The authors report no conflict of interest.

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