Novel Research in Microbiology Journal (2024), 8(4): 2542-2554

(Print) (ISSN 2537-0286)
(Online) (ISSN 2537-0294)
https://nrmj.journals.ekb.eg/

Research Article

DOI: 10.21608/NRMJ.2024.305189.1652

Development of polymerase chain reaction coupled with a high-resolution melting technique for detection of *Helicobacter pylori* clarithromycin resistance

Souad Oirdi Zahir[1,2]; Mounia El Khadir[2,3]; Dafr-Allah Benajah[1,4]; Sidi Adil Ibrahimi[1,4]; Laila Chbani[5]; Mohamed El Abkari[1,4]; Bahia Bennani[1,2*]

[1]URL CNRST N°15 Laboratory of Human Pathology, Biomedicine and Environment, Faculty of Medicine, Pharmacy and dental Medicine of Fez (FMPDF), Sidi Mohammed Ben Abdellah University (USMBA), Fez, Morocco; [2]Laboratory of Microbiology and Molecular Biology, FMPDF, USMBA, Fez, Morocco; [3]The Higher Institute of Nursing Professions and Health Techniques (ISPITS), Taza, Morocco; [4]Department of Hepato-gastroenterology Hassan II University Hospital Center, Fez, Morocco; [5]Department of Pathological Anatomy Hassan II University Hospital Center, Fez, Morocco

[*]Corresponding author E-mail: bahia.bennani@usmba.ac.ma

Received: 5 July, 2024; Accepted: 14 August, 2024; Published online: 15 August, 2024

Abstract

Helicobacter pylori, a bacterial pathogen that causes severe gastric diseases, has developed antibiotics resistance; mainly against clarithromycin. Consequently, the latest international recommendations have advised clarithromycin susceptibility testing before prescribing the first line of therapy. This resistance is predominantly related to the *A2143G* point mutation of 23S rRNA. The aim of this study was to develop a highly sensitive and cheap molecular technique to detect *H. pylori* clarithromycin resistant strains. For that, plasmids of *H. pylori* 23S rRNA region harboring *A2143G* point mutation were constructed and used to develop polymerase chain reaction coupled with a high-resolution melting technique (PCR-HRM). Then, this method was applied on 233 gastric *H. pylori* positive samples. The obtained results showed that the developed PCR-HRM technique allowed specific identification of clarithromycin resistant and heteroresistant *H. pylori* samples, even if the ratio of resistant/ sensitive samples was low. In this series, the detected rate of *H. pylori* clarithromycin resistance was 7.6 %, which concord with the resistance rate determined using molecular sequencing. So, the developed PCR-HRM represents a good tool for detecting resistant and sensitive *H. pylori* samples and can be used in routine clinical practices. This will help in managing *H. pylori* infection, increase its eradication rate, and avoid the use of therapeutic protocols that have serious side effects.

Keywords: *H. pylori*, PCR-HRM, 23S rRNA, Clarithromycin resistance, Hetero-resistance

1. Introduction

Helicobacter pylori is one of the most common human infectious agents that infects more than half of the world's population (Li *et al*., 2023; Malfertheiner *et al*., 2023). It is responsible for the development of various gastric pathologies notably gastric cancer (Salvatori *et al*., 2023). Management of *H. pylori* infection is generally based on antibiotic therapy within different protocols. Thus, clarithromycin (CLR), metronidazole (MTR), amoxicillin (AMX), levofloxacin (LEV), tetracycline (TET) or rifabutin (RIF) were used in different combinations with proton pump inhibitors (PPI) and with or without bismuth (Katelaris *et al*., 2023; Malfertheiner *et al*., 2023). Unfortunately, despite this diversity in antimicrobial molecules, we faced an increasing treatment failure related to increasing rates of *H. pylori* resistance. Clarithromycin used in triple therapy protocol (CLR-AMX-PPI), which was recognized for more than two decades as a first-line treatment has become obsolete in many world regions, due to high rate and rapid emergence of *H. pylori* resistance (Morehead and Scarbrough, 2018). Thus, the effectiveness of this treatment protocol has fallen from over 90 % to 70 %, which prompted the recommendations for its exclusive use in regions with low CLR resistance rates (less than 15-20 %) (Malfertheiner *et al*., 2022; Katelaris *et al*., 2023). So, determination of *H. pylori* CLR resistance prevalence is a key element in determining the most suitable therapeutic protocol for each region. This resistance is attributed to point mutations in 23S rRNA (Marques *et al*., 2020), notably *A2143G*, *A2142G*, and *A2142C*, which are the most widely described mutations. Various phenotypic and genotypic methods have been suggested to determine *H. pylori* CLR resistance; however each of them has its strengths and limitations notably in terms of cost and time consumption. So, it's of prompt interest to develop a highly sensitive technique to identify *H. pylori* CLR resistance that can be applied in countries with limited resources. In this study, our objective was to develop a new tool using polymerase chain reaction coupled with a high-resolution melting technique (PCR-HRM) to screen *H. pylori* CLR resistant samples, which can be used in routine diagnosis.

2. Materials and methods

2.1. Clinical samples

A total of 233 *H. pylori* positive DNA were extracted from gastric biopsies previously obtained from consenting patients aged between 16 and 99 years old who consulted the gastroenterology Department of Hassan II Hospital University (CHU) of Fez, Morocco, and who had undergone upper gastrointestinal endoscopy for various reasons (*i.e*., pain, discomfort, indigestion…etc.) between 2009 and 2018, were used in this study. These patients constituted of 50.2 % men and 49.8 % women.

2.2. Cloning the gene of interest

DNA extracted from two clinical samples, one harboring *H. pylori* clarithromycin resistant point mutation *A2143G* of 23S rRNA gene (CLR-R) and the other was harboring *H. pylori* clarithromycin sensitive strain (CLR-S) (with no mutation on the region 1952–2173 of 23S rRNA gene), was used to prepare CLR-S and CLR-R plasmid controls, respectively. PCR was used to amplify this region of interest using two primers: *HPY-S* and *HPY-A* (Ménard *et al*., 2002) shown in Table (1). The amplified products of 267 pb were cloned in *Escherichia coli* chemically competent cells using the "Original TA Cloning Kit" (Invitrogen, Life technologies, USA), following the manufacturer's recommendations. The plasmids were then extracted (Mülhardt and Beese, 2007), which were accordingly noted as resistant and sensitive plasmids and stored at −80 °C until use.

2.3. Primer design

Helicobacter pylori 23S rRNA gene sequence (GenBank: U27270.1) was used to design a forward

Table 1: Primers used in this study

Primers	Sequences	Reference
HpHR-S	5' ATTCAGTGAAATTGTAGTGGAGGTG 3'	This study
HPY-S	5′-AGGTTAAGAGGATGCGTCAGTC-3′	(Ménard *et al*., 2002)
HPY-A	5'-CGCATGATATTCCCATTAGCAGT-3′	(Ménard *et al*., 2002)

primer "HpHR-S" to be used in combination with a reverse primer HPY-A, to amplify a small DNA sequence delimiting the A2142/ A2143 region. In this study, the newly designed primer "HpHR-S" was located between 2089 and 2114 nucleotides of *H. pylori* 23S rRNA gene and its sequence was 5' ATTCAGTGAAATTGTAGTGGAGGTG 3' (Table 1).

2.4. Polymerase chain reaction and high-resolution melt development (PCR-HRM)

In this study, the prepared *H. pylori* CLR-R and CLR-S plasmids were used in different ratios to prepare CLR hetero-resistant plasmids (CLR-H) (CLR-H 25 % R/75 % S noted CLR-H 25 %R, CLR-H 50 %S/50 %R noted CLR-H 50 %R and CLR-H 25 %S/75 %R noted CLR-H 75 %R). All these plasmids were used to develop the PCR-HRM technique that was executed in the QuantStudio5 thermocycler (Applied Biosystems, Thermo Fisher Scientific). So, PCR-HRM experiments were conducted in a final volume of 20 µl including 5 µM of each primer (HpHR-S and HPY-A), 20 ng/ µl of DNA samples, 10 µl of 2X MeltDoctor HRM Master Mix (Applied Biosystems, Thermo Fisher Scientific), according to manufacturer's recommendations (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0014394_HighResMeltExperiment_GSG.pdf). The amplification conditions were 95 °C for 10 min., and 40 cycles of 15 s at 95 ºC and 1 min. at 60 °C. The melt curve phase consisted of 10 s denaturation at 95 °C, 1 min. annealing at 60 °C, high resolution melting curve at 95 °C for 15 s, and finally annealing at 60 °C during 15 s with ramping of 0.025 °C/ s. Melting curve of all DNA samples was analyzed using High Resolution Melting software version 3.1 (Applied Biosystems, Thermo Fisher Scientific). Plasmid controls were used to identify the resistance profiles in our DNA samples by comparing the melting curve profiles with those plasmid controls.

2.5. Application of developed PCR-HRM on DNA of clinical samples

The DNA extracted from 233 gastric samples were analyzed to determine the clarithromycin resistance profile of *H. pylori* using both PCR-HRM technique developed in this study and conventional PCR, exploiting a primer pair HPY-S/ HPY-A (Ménard *et al*., 2002) followed by sequencing. Sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, Lithuania) according the manufacturer's instructions. For PCR-HRM technique, CLR-S, CLR-R, and CLR-H plasmids (including 25 %R, 50 %R, 75 %R) were used as controls, while water was used as a negative control in each reaction.

2.6. Polymerase chain reaction of 23S rRNA and sequencing

A conventional PCR reaction was performed on the 233 purified DNA clinical samples using the primer pair HPY-S and HPY-A (Ménard *et al*., 2002). The reaction was conducted in a total volume of 25 µl

containing 10x Dream green taq buffer (Thermoscientific, Lithuania) including 25 mM $MgCl_2$, 200 µM each dNTP, 10 µM each primer, 2U of dream taq DNA polymerase, and finally 3 µl of purified DNA. The PCR conditions were: 94 °C for 5 min. and then 40 cycles of 94 °C for 1 min., 58 °C for 45 s, and 72 °C for 45 s, and a final extension at 72 °C for 7 min. All the obtained PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, Lithuania) following the manufacturer's instructions.

2.7. Limit of detection (LOD)

The limit of detection (LOD), which corresponded to the lowest concentration of the target bacterial species that can be detected by PCR-HRM technique in at least 95 % of samples, was evaluated using DNA serial dilutions in reference to Broeders *et al*., (2014).

2.8. Statistical analysis

To determine the specificity and sensitivity (Monaghan *et al*., 2021) of PCR-HRM developed in this study, a contingency table (cross table 2×2) was drawn up using SPSS version 20 (Statistical product and services solutions, version 20, SPSS Inc. Chicago, Illinois, USA) software. Results obtained by sequencing were considered as references [*i.e.* sensitive (no-mutation *A2142* or *2143*) and resistant (presenting at least one of these mutations)]. For sequencing and PCR-HRM technique, samples showing hetero-resistant profiles were considered as resistant. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and Kappa coefficient concordance (K) were determined (Monaghan *et al*., 2021).

3. Results

The forward primer HpHR-S was designed in this study to allow an amplification of a 108 bp DNA fragment when used in combination with the reverse primer HPY-A (Table 1). Amplification using HPY-S/HPY-A and HpHR-S/HPY-A primer pairs was performed on prepared plasmids (*i.e*., CLA-S and CLR-R). Agarose gel migration of the resulting PCR products confirmed amplification of the expected 267 bp and 108 bp products, respectively; in addition to absence of a non-specific amplification. No amplification was observed in the negative control.

3.1. Polymerase chain reaction and high-resolution melt development (PCR-HRM)

PCR-HRM was performed using "MeltDoctor HRM Master Mix" kit according to the optimized conditions described in the section of methodology. The pre-melt and post-melt zones applied during melting curve analysis were 76-76.6 °C and 81.9-82.5 °C, respectively. The PCR-HRM applied on CLR-S, CLR-R controls and on different ratios of CLR-H mixtures (plasmids) allowed obtaining five distinct curves either in normalized melting curves or in a difference plot. These curves allowed the distinction between each type of plasmid. The CLR-R and CLR-S plasmids showed curves with a single peak at Tm of 79.1 °C and 79.5 °C; respectively, while CLR-H mixtures (25 %R, 50 %R, and 75 %R) showed a difference in the curve's shape. Each of these plasmids produced two peaks that were more or less marked depending on the composition of each hetero-resistant group: CLR-H 25 %R, CLR-H50 %R, and CLR-H 75 %R. No peak was recorded with the negative control (Fig. 1).

3.2. Polymerase chain reaction and high-resolution melt technique applied on clinical samples

The developed PCR-HRM method was applied on 233 DNA extracts obtained in the clinical samples. The results showed five types of melting curve profiles corresponding to CLR-S, CLR-R, CLR-H 25 %R, CLR-H 75 %R, and a fifth group named "other variant". "Other variant" represents another category of a melt curve identified by HRM software, which did not belong neither to the sensitive or resistant, nor the hetero-resistant groups; however, when sequenced, it displayed the presence of a mutation in the nucleotide sequence outside the zone of interest.

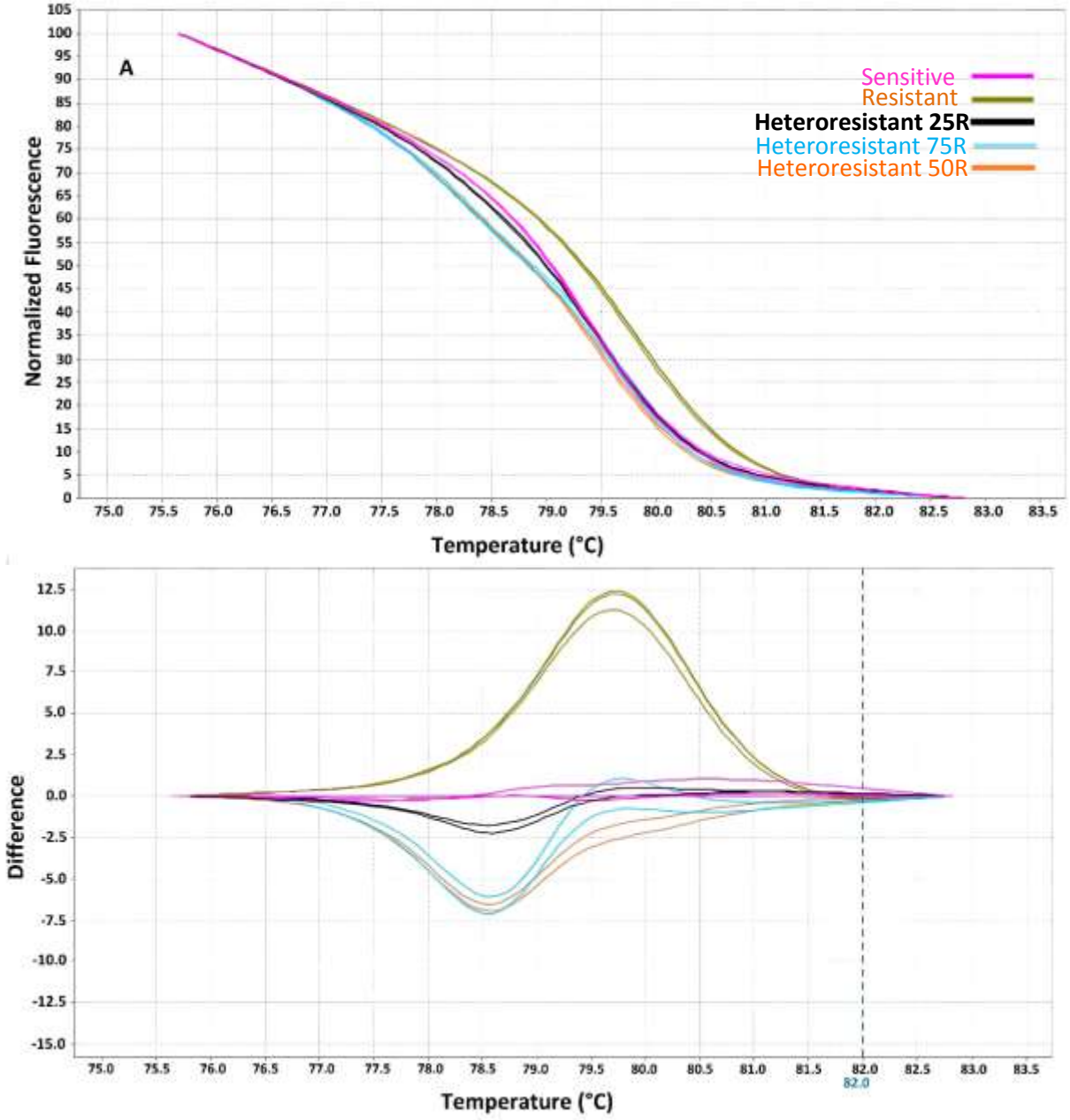

Fig. 1: High-resolution melting curves of CLR-sensitive, resistant, and hetero-resistant plasmids. Where; A: Normalized melt curves, B: Different plots

Among the 233 tested DNA samples, 83.7 % (195/233) showed a sensitive melt curve profile, 2.1 % (5/233) were classified with CLR-R profile, 3.4 % (8/233) classified as CLR-H 25 %R, and 1.3 % (3/233) of samples were classified as CLR-H 75 %R, while the remaining 9.4 % of samples (22/233) were classified as "other variants" (Table 2). The shapes of these different melting curve profiles were clearly distinguishable both in the normalized curves and the difference plots.

Table 2: Correlation between polymerase chain reaction coupled with a high-resolution melting technique (PCR-HRM) and sequencing results according to point mutations

		HRM						
		Sensitive	Resistant	Hetero-resistant 25 %R	Hetero-resistant 50 %R	Hetero-resistant 75 %R	Other variants	Total
Sequencing	No mutation	190 (96.4%)	-	1 (0.5%)	-	-	6 (3%)	197 (100%)
	A2143G	-	5 (71.4%)	1(14.3%)	-	1(14.3%)	-	7 (100%)
	A2143G + other mutation	-	-	-	-	-	2(100%)	2 (100%)
	A2143A/G heterozygotes	-	-	6 (85.7%)	-	1(14.3%)	-	7 (100%)
	A2143A/G + other mutation	-	-	-	-	1 (50%)	1 (50%)	2 (100%)
	C2131T	2(100%)	-	-	-	-	-	2 (100%)
	C2173T	2(22.2%)	-	-	-	-	7(77.8%)	9 (100%)
	A2180G	1(100%)	-	-	-	-	-	1 (100%)
	G2140A/G	-	-	-	-	-	3(100%)	3 (100%)
	A2180A/G	-	-	-	-	-	1(100%)	1 (100%)
	C2131T/C	-	-	-	-	-	1(100%)	1 (100%)
	G2107A	-	-	-	-	-	1(100%)	1 (100%)
	Total	195 (83.7%)	5 (2.1%)	8 (3.4%)	-	3(1.3%)	22(9.4%)	233 (100%)

Where; The empty cells imply no noted cases (0 %)

3.3. DNA sequencing

To verify the results obtained by PCR-HRM, all the amplified products were sequenced. None of the mutations occurring at position 2142 (*i.e. A2142G* or *A2142C*) were observed. Fig. (2) illustrates sequence analysis of the resistant and hetero-resistant profiles, which occurred predominantly at position 2143.

3.4. Comparison between sequencing and PCR-HRM results

The PCR-HRM and sequencing results were compared for the 233 tested clinical samples, and the obtained results are reported in Table (2). Of the 197 sequenced sensitive samples (showing no mutations), 190 cases (96.4 %) were also classified as CLR-S by HRM, six cases (3 %) classed as other variants, and a single case (0.5 %) was classed as CLR-H 25 %R. Of the seven sequenced samples carrying only the A2143G mutation, five samples (71.4 %) were also classified as CLR-R by HRM, while the remaining two samples (28.6 %) were classified as CLR-H (one

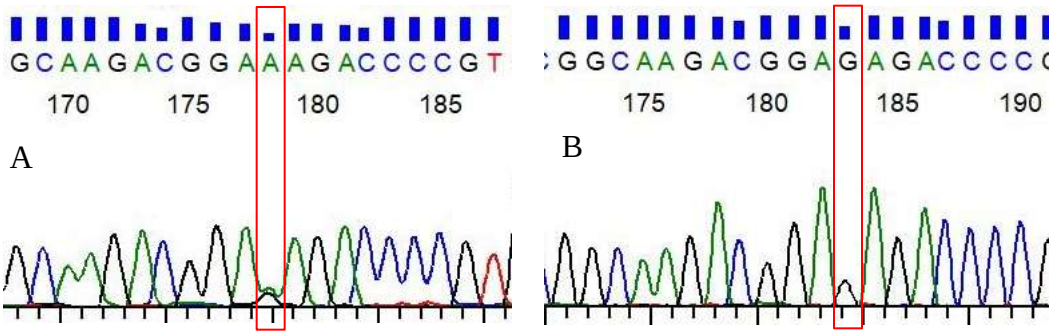

Fig. 2: Electropherogram of samples with different CLR-resistant profiles. Where; A: *A2143A/G*, CLR-hetero-resistant sample; B: *A2143G*, CLR-resistant sample

sample as CLR-H 25 %R and the other as CLR-H 75 %R). Other mutations were also observed in this study, including *C2131T*, *C2173T*, *A2180G*, *G2107A*, *G2140A/G*, *A2180A/G*, and *C2131T/C*, which occurred either singly or combined. When analyzed by PCR-HRM, the samples harboring these mutations were classified as "other variants" or as CLR-S.

3.5. Limit of detection (LOD)

The LOD (sensitivity) of the developed PCR-HRM technique was carried out by analysis of serial dilutions of the DNA templates. The lowest concentration of DNA identified correctly was 0.95×10^{-5} ng/ µl.

3.6. Polymerase chain reaction and high-resolution melt specificity and sensitivity

To determine the sensitivity and specificity of the developed PCR-HRM technique, a contingency table was drawn in SPSS using results obtained from PCR-HRM and sequencing. The sequencing results were considered as references; however, *H. pylori* DNA samples classified in "other variant" group using PCR-HRM were excluded from the analysis. Thus, 211 samples only were tested. The obtained results are presented in Table (3). Results showed that the newly developed PCR-HRM technique had a sensitivity of 100 % and a specificity of 99.5 %. The PPV was 93.8 % and NPV was 100 %, while the Kappa coefficient concordance (K) was 0.96.

4. Discussion

Over the past two decades, there has been a noticeable rise in antibiotic resistance among the microorganisms, particularly *H. pylori*. This trend posed a growing challenge in managing its infections worldwide. In fact, the previously highly-effective first-line triple therapy (comprising CLR, AMX, and PPI) has been authorized in few regions only where CLR resistance rates remained below 15 % (Katelaris *et al*., 2023; Malfertheiner *et al*., 2023). Even levofloxacin-based triple therapy typically used in rescue treatment after the first-line therapy failure, is hindered by widespread of fluoroquinolone resistance (Katelaris *et al*., 2023; Malfertheiner *et al*., 2023). Consequently, bismuth quadruple therapy (*i.e*., proton pump inhibitors, bismuth, tetracycline, and metronidazole) and concomitant bismuth-free quadruple therapy, including proton pump inhibitors (PPI) and three antibiotics; mainly clarithromycin, amoxicillin, and metronidazole or tinidazole, has emerged as viable first-line treatment options; especially in areas with high antibiotic resistance rates (Katelaris *et al*., 2023; Malfertheiner *et al*., 2023). Therefore, the choice of a therapeutic protocol in a given geographical area is heavily influenced by

Table 3: Contingency between polymerase chain reaction coupled with a high-resolution melting technique (PCR-HRM) and sequencing results

		Sequencing		
		CLR-Resistant	CLR-Sensitive	Total
PCR-HRM	CLR-Resistant	15	1[a]	16
	CLR-Sensitive	0	195	195
	Total	15	196	211

Where; [a]: The case that is resistant by PCR-HRM and sensitive by sequencing is a hetero-resistant 25 %R

antibiotic resistance rate of the circulating *H. pylori* strains notably CLR resistance.

Various methods have been developed to identify CLR-R *H. pylori* isolates (Medakina *et al*., 2023). Traditional phenotypic techniques such as agar diffusion (disc diffusion or E-test) are commonly used. However, due to *H. pylori's* slow growth rate, these methods are time-consuming and expensive, making them impractical for large-scale or routine analysis (Jearth *et al*., 2023; Danchuk *et al*., 2024). Molecular methods that detect mutations associated with CLR resistance (Fernandez-Caso *et al*., 2022) offer faster results and high sensitivity compared to traditional techniques. Yet, their costs remain a barrier to widespread adoption; particularly in regions with limited resources.

In an attempt to overcome this challenge, we developed a PCR-HRM technique for detecting *H. pylori* CLR resistance. The newly designed primer HpHR-S, when used with the HPY-A primer (Ménard *et al*., 2002) allowed specific amplification of a region of 108 pb harboring *A2143* mutation of 23S rRNA gene. On applying a melt temperature of 60 °C, HRM analysis of PCR products obtained in *H. pylori* CLR-S, CLR-R, and CLR-H DNA, generated distinct plots, thus enabling clear differentiation among these variants (*i.e*., sensitive, resistant, hetero-resistant or other variants). The obtained results confirmed the PCR-HRM technique's ability to identify CLR hetero-resistant strains, whatever the ratio R/S was. To evaluate practical effectiveness of the new developed technique, it was applied to several clinical samples and the obtained results were compared to those of sequencing.

Sequencing results showed that the majority of studied samples (197/233) exhibited a CLR-S profile. The others samples (36/233) showed some mutations with predominance of *A2143G* (18/36; 50 %), which is widely known for its significant therapeutic implications. Other mutations have been identified in this study such as *C2131T*, *C2173T*, *A2140A/G*, *A2180G*, *A2180A/G*, *C2131T/C*, and *G2107A*, occurring either individually or in combination. The mutations *C2131T* and *C2173T* have recently been reported in several resistant strains. The *C2131T* mutation has been documented in two previous studies, occurring in phenotypically resistant strains; however, it appeared in a single sample alongside with

A2143G mutation, which is known to confer resistance (Chu *et al*., 2020; Zhang *et al*., 2020). Similarly, the *C2173T* mutation has been detected in a single case among many phenotypically resistant strains, but it is uncertain whether this mutation occurred alone in the sample or in combination with another known resistance-conferring mutation (Domanovich-Asor *et al*., 2020). Consequently, samples harboring these two mutations (*C2131T*, *C2173T*) along with the other mutations detected for the first time in this study had been classified as sensitive.

Among the sequenced CLR-S samples, 96.4 % were also identified as CLR-S using PCR-HRM. The remaining samples were categorized as "other variants" (3 %) or as CLR-H 25 %R (0.5 %). Presence of the last category could be attributed to limited capability of sequencing to detect heterozygote profiles; particularly in cases of low ratios of mutant/ wild-type strains. In contrast, PCR-HRM, being a highly sensitive technique, analyzed the thermal dissociation of amplicons during the fusion step and enabled detection of various variants even at small ratios. Insufficiency of sequencing to detect hetero-resistant (heterozygote) isolates has been previously reported and documented by Eilertson *et al*., (2014), who revealed that deep sequencing exhibits better sensitivity than Sanger sequencing for detecting the hetero-resistance isolates. Enhanced capability of PCR-HRM to detect CLR-H *H. pylori* samples and surpassing the limitations of sequencing, has offered significant potential for optimizing therapy and improving eradication rates by addressing treatment failure attributed to hetero-resistant strains.

Most of the clinical samples carrying *A2143G* mutation only were identified as resistant (71.4 %) or hetero-resistant (28.6 %) by PCR-HRM. However, two *A2143G* samples and a single *A2143A/G* heterozygous sample that carried other mutations in outside of the region of interest were classified as other variants by HRM software. These data, in addition to the fact that all samples harboring others mutations other than *A2143* were accurately classified as "other variants" or sensitive by PCR-HRM, confirmed the ability of PCR-HRM to detect variants even at small ratios. PCR-HRM effectively distinguished the "hetero-resistant profile" (indicating a mixed infection or hetero-resistant strain) from CLR-R and CLR-S strains, in both of plasmids and biological samples. The PCR-HRM showed high sensitivity and specificity, a limit of detection of 0.95×10^{-5} ng/ µl, and K coefficient concordance of 0.96.

In *H. pylori* positive samples, the sequences of samples identified as CLR-R or CLR-H using PCR-HRM exhibited exclusively *A2143* mutation. This suggests that PCR-HRM can effectively be utilized in screening for *H. pylori* CLR-R/CLR-S samples. Meanwhile, only samples classified as other variants must be sequenced to determine their CLR profile.

Sequencing showed exclusively the presence of *A2143G* mutation, which represented the mutation that led to therapeutic failure in the majority of cases. The *A2142G* and *A2142C* mutations were not detected in this series and didn't allow testing the ability of PCR-HRM to detect them, which constituted a limitation of this study. However, since the mutations that confer resistances to CLR were very close together (at positions 2142 and 2143), the presence of either mutation would likely result in a practically identical plot profile.

Using PCR-RFLP method, absence of *A2142G* has been also noted in another region of Morocco (Casablanca) (Essaidi *et al*., 2022). Likewise, predominance of *A2143G* has been observed in neighboring countries (Djennane-Hadibi *et al*., 2016; Mormeneo Bayo *et al*., 2023). These results supported the hypothesis of De Francesco *et al*., (2014) study, which suggested that there is a shift in the epidemiology of *H. pylori* CLR-mutations.

In this study, the determined *H. pylori* CLR resistance rate using PCR-HRM was 7.6 % (16/211), considering both CLR-R and CLR-H samples and excluding the "other variants" samples. This rate was comparable to that obtained by sequencing 7.7 % (18/233) and was similar to that obtained in Germany

(10.9 %) by Hofreuter et al., (2021), but was much lower than those observed in France (20.9 %), China (35.9 %), Korea (28.8 %), and Egypt (40 %) (Mégraud *et al*., 2020; Park *et al*., 2020; Jiang *et al*., 2022; Metwally *et al*., 2022). It's also lower than the rates noted in other Moroccan regions such as Rabat (28.8 %) (Bouihat *et al*., 2017) and Casablanca (14.6 %) (Essaidi *et al*., 2022). The fact that the obtained *H. pylori* CLR resistance rate was considerably lower than the Maastricht consensus threshold rate of 15-20 % suggested the adequacy of *H. pylori* first line triple therapy for infection treatment. However, these findings should be interpreted cautiously, since this study was conducted in a limited geographic area and did not account for potential changes in CLR resistance over time. This constitutes a limitation of this study and sampling in other geographical area is still needed to confirm this data; especially because *H. pylori* resistance rate vary widely from one geographical area to another and even within regions of the same country (Boyanova *et al*., 2023).

So, in the national cancer control strategy, management of *H. pylori* infection must not be neglected, because it's a key of gastric cancer prevention (Yan *et al*., 2022). The results of this study confirmed that implementation of an adequate *H. pylori* eradication protocols needs an extensive overview and monitoring of *H. pylori* antibiotics resistance evolution as recommended by several other studies conducted by Malfertheiner *et al*., (2022); Gómez-Ruiz de Arbulo *et al*., (2023). Thus, in an attempt to help in generalization of *H. pylori* CLR resistance test, we developed a molecular technique based on PCR-HRM. This technique showed several benefits for *H. pylori* CLR resistance screening: as it was highly sensitive, reliable, specific, fast (less than 2 h of run), easy to implement, less expensive, and well suited for routine analysis of large samples.

Conclusion

The currently developed PCR-HRM technique used for detecting *H. pylori* CLR resistance showed high specificity compared to sequencing method. It allowed screening of *H. pylori* CLR resistant, sensitive, and hetero-resistant samples, respectively. A good concordance between the results obtained using this technique and those obtained by sequencing was noted with *H. pylori* sensitive and resistant profiles. This technique seemed to be more sensitive in detecting *H. pylori* CLR-H than sequencing. Our results confirmed that PCR-HRM is suitable and can be used in routine analysis for diagnosis of *H. pylori* and detection of CLR resistance in a single run.

Acknowledgments

The authors like to express their deep gratitude to all funders and staff members of the Gastroenterology unit and Anatomy-Pathology department for their help in collection and preparation of specimens.

Conflict of interests

The authors report there are no competing interests to declare.

Funding source

This work was supported by the National Center for Scientific and Technical Research (CNRST) and the "Ministère de l'éducation nationale, de la formation professionnelle, de l'enseignement supérieur et de la recherche scientifique" Morocco (Ministry of National Education, Vocational Training, Higher Education and Scientific Research) (Grant number: 03P/2016); the University Hospital Hassan II of Fez (Grant number: PR04-12 13) and Laboratory of Human Pathology Biomedicine and Environment of the Faculty of Medicine and Pharmacy of Fez.

Ethical approval

This study was ethically approved on 22 April (2009) by the Institutional Review Board of the Hassan II University Hospital of Fez, Morocco, with the Ethical permission number (03AV/2009). All participants included in the study provided his/ her written informed consents on study participation.

Author's Contributions

SOZ: Conceptualization, Investigation, Formal analysis, Writing, original draft, Writing, review & editing and Visualization; BB: Conceptualization, Investigation, Formal analysis, Writing, original draft, Writing, review & editing and Visualization. MEK: Investigation, Writing, review & editing and Visualization; DB: Investigation, Writing, review & editing and Visualization; SAI: Investigation, Writing, review & editing and Visualization; LC: Investigation, Writing, review & editing and Visualization; MEA: Investigation, Writing, review & editing and Visualization.

5. References

Bouihat, N.; Burucoa, C.; Benkirane, A.; Seddik, H.; Sentissi, S.; Al Bouzidi, A. et al. (2017). *Helicobacter pylori* primary antibiotic resistance in 2015 in Morocco: A phenotypic and genotypic prospective and multicenter study. Microbial Drug Resistance. 23(6): 727-732. https://doi.org/10.1089/mdr.2016.0264.

Boyanova, L.; Hadzhiyski, P.; Gergova, R. and Markovska, R. (2023). Evolution of *Helicobacter pylori* resistance to antibiotics: A topic of increasing concern. Antibiotics. 12(2): 332. https://doi.org/10.3390/antibiotics12020332.

Broeders, S.; Huber, I.; Grohmann, L.; Berben,G.; Taverniers, I.; Mazzara, M. et al. (2014). Guidelines for validation of qualitative real-time PCR methods. Trends in Food Science and Technology. 37(2): 115-126. https://doi.org/10.1016/j.tifs.2014.03.008.

Chu, A.; Wang, D.; Guo, Q.; Lv, Z.; Yuan, Y. and Gong, Y. (2020). Molecular detection of *H. pylori* antibiotic-resistant genes and molecular docking analysis. FASEB Journal. 34(1): 610-618. https://doi.org/10.1096/fj.201900774R.

Danchuk, S.N.; Solomon, O.E.; Kohl, T.A.; Dreyer, V.; Barilar, I.; Utpatel, C. et al. (2024). Challenging the gold standard: The limitations of molecular assays for detection of *Mycobacterium tuberculosis* heteroresistance. Thorax. 79(7): 670-675. https://doi.org/10.1136/thorax-2023-220202.

De Francesco, V.D.; Zullo, A.; Giorgio, F.; Saracino, I.; Zaccaro, C.; Hassan, C. et al. (2014). Change of point mutations in *Helicobacter pylori* rRNA associated with clarithromycin resistance in Italy. Journal of Medical Microbiology. 63(Pt 3): 453-457. https://doi.org/10.1099/jmm.0.067942-0.

Djennane-Hadibi, F.; Bachtarzi, M.; Layaida, K.; Ali Arous, N.; Nakmouche, M.; Saadi, B. et al. (2016). High-level primary clarithromycin resistance of *Helicobacter pylori* in Algiers, Algeria: A prospective multicenter molecular study. Microbial Drug Resistance. 22(3): 223-226. https://doi.org/10.1089/mdr.2015.0209.

Domanovich-Asor, T.; Motro, Y.; Khalfin, B.; Craddock, H.A.; Peretz, A. and Moran-Gilad, J. (2020). Genomic analysis of antimicrobial resistance genotype-to-phenotype agreement in *Helicobacter pylori*. Microorganisms. 9(1): 2. https://doi.org/10.3390/microorganisms9010002.

Eilertson, B.; Maruri, F.; Blackman, A.; Herrera, M.; Samuels, D.C. and Sterling, T.R. (2014). High proportion of heteroresistance in *gyrA* and *gyrB* in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. Antimicrobial Agents and Chemotherapy. 58(6): 3270-3275. https://doi.org/10.1128/AAC.02066-13.

Essaidi, I.; Bounder, G.; Jouimyi, R.M.; Boura, H.; Elyounsi, I.; Kheir, F.Z. et al. (2022). Comparative study of *Helicobacter pylori* resistance to clarithromycin and metronidazole and its association with epidemiological factors in a Moroccan population. Asian Pacific Journal of Cancer Prevention. 23(8): 2755-2761. https://doi.org/10.31557/APJCP.2022.23.8.2755.

Fernandez-Caso, B.; Miqueleiz, A.; Valdez, V.B. and Alarcón, T. (2022). Are molecular methods helpful for the diagnosis of *Helicobacter pylori* infection and for the prediction of its antimicrobial

resistance? Frontiers in Microbiology. 13: 962063. https://doi.org/10.3389/fmicb.2022.962063.

Gómez-Ruiz de Arbulo, M.; Tamayo, E.; Bujanda, L.; Mendibil, L.; Mendiola, J.; Cilla, G. et al. (2023). Surveillance of *Helicobacter pylori* resistance over 22 Years (2000-2021) in Northern Spain. Journal of Global Antimicrobial Resistance. 34: 127-133. https://doi.org/10.1016/j.jgar.2023.07.002.

Hofreuter, D.; Behrendt, J.; Franz, A.; Meyer, J.; Jansen, A.; Bluemel, B. et al. (2021). Antimicrobial resistance of *Helicobacter pylori* in an eastern German region. Helicobacter. 26(1): e12765. https://doi.org/10.1111/hel.12765. Epub 2020 Oct 17.

Jearth, V.; Rath, M.M.; Chatterjee, A.; Kale, A. and Panigrahi, M.K. (2023). Drug-resistant *Helicobacter pylori*: Diagnosis and evidence-based approach. Diagnostics. 13(18): 2944. https://doi.org/10.3390/diagnostics13182944.

Jiang, Z.; Qian, X.; Wang, Z.; Dong, Y.; Pan, Y.; Zhang., Z. et al. (2022). Antibiotic resistance of *Helicobacter pylori* isolated from patients in Nanjing, China: A cross-section study from 2018 to 2021. Frontiers in Cellular and Infection Microbiology. 12: 1-8. https://doi.org/10.3389/fcimb.2022.970630.

Katelaris, P.; Hunt, R.; Bazzoli, F.; Cohen, H.; Fock, K.M.; Gemilyan, M. et al. (2023). *Helicobacter pylori* World Gastroenterology Organization Global Guideline. Journal of Clinical Gastroenterology. 57(2): 111-126. https://doi.org/10.1097/MCG.0000000000001719.

Li, Y.; Choi, H.; Leung, K.; Jiang, F.; Graham, D.Y. and Leung, W.K. (2023). Global prevalence of *Helicobacter pylori* infection between 1980 and 2022: a systematic review and meta-analysis. The Lancet Gastroenterology and Hepatology. 8(6): 553-564. https://doi.org/10.1016/S2468-1253(23)00070-5.

Malfertheiner, P.; Camargo, M.C.; El-Omar, E.; Liou, J.M.; Peek, R.; Schulz, C. et al. (2023). *Helicobacter pylori* infection. Nature Reviews Disease Primers. 9(1): 19. https://doi.org/10.1038/s41572-023-00431-8.

Malfertheiner, P.; Megraud, F.; Rokkas, T.; Gisbert, J.P.; Liou, J.M.; Schulz, C. et al. (2022). Management of *Helicobacter pylori* infection: the Maastricht VI/Florence consensus report. Gut. 327745. https://doi.org/10.1136/gutjnl-2022-327745.

Marques, A.T.; Vítor, J.M.B.; Santos, A.; Oleastro, M. and Vale, F.F. (2020). Trends in *Helicobacter pylori* resistance to clarithromycin: From phenotypic to genomic approaches. Microbial Genomics. 6(3): e000344. https://doi.org/10.1099/mgen.0.000344.

Medakina, I.; Tsapkova, L.; Polyakova, V.; Nikolaev, S.; Yanova, T. and Dekhnich, N. (2023). *Helicobacter pylori* antibiotic resistance: Molecular basis and diagnostic methods. International Journal of Molecular Sciences. 24(11): 9433. https://doi.org/10.3390/ijms24119433.

Mégraud, F.; Alix, A.; Charron, P.; Bénéjat, L.; Ducournau, A.; Bessède, E. et al. (2020). Survey of the antimicrobial resistance of *Helicobacter pylori* in France in 2018 and evolution during the previous 5 years. Helicobacter. 26(1): e12767. https://doi.org/10.1111/hel.12767.

Ménard, A.; Santos, A.; Mégraud, F. and Oleastro, M. (2002). PCR-restriction fragment length polymorphism can also detect point mutation *A2142C* in the 23S rRNA gene, associated with *Helicobacter pylori* resistance to clarithromycin. Antimicrobial Agents and Chemotherapy. 46(4): 1156-1157. https://doi.org/10.1128/AAC.46.4.1156-1157.2002.

Metwally, M.; Ragab, R.; Abdel Hamid, H.S.; Emara, N. and Elkholy, H. (2022). *Helicobacter pylori* antibiotic resistance in Egypt: A single-center study. Infection and Drug Resistance. 15: 5905-5913. https://doi.org/10.2147/IDR.S386082.

Monaghan, T.F.; Rahman, S.N.; Agudelo, C.W.; Wein, A.J.; Lazar, J.M. and Everaert, K. et al. (2021). Foundational statistical principles in medical

research: Sensitivity, specificity, positive predictive value, and negative predictive value. Medicina. 57(5): 503. https://doi.org/10.3390/medicina57050503.

Morehead, M.S. and Scarbrough, C. (2018). Emergence of global antibiotic resistance. Primary Care. 45(3): 467-484. https://doi.org/10.1016/j.pop.2018.05.006.

Mormeneo Bayo, S.; Bellés Bellés, A.; Vázquez Gómez, D.; Planella de Rubinat, M.; Bayas Pastor, D.C.; Morales Portillo, A. et al. (2023). Antibiotic susceptibility and clarithromycin resistance determinants in *Helicobacter pylori* in the Northeast of Spain: A one-year prospective study. Antibiotics. 12: 356. https://doi.org/10.3390/antibiotics12020356.

Mülhardt, C. and Beese, E.W. (2007). Book chapter: Fundamental Methods in Molecular Biology and Genomics. Elsevier. pp. 11-36. https://doi.org/10.1016/b978-012088546-6/50004-x.

Park, J.Y.; Shin, T.S.; Kim, J.H.; Yoon, H.J.; Kim, B.J. and Kim, J.G. (2020). The prevalence of multidrug resistance of *Helicobacter pylori* and its impact on eradication in Korea from 2017 to 2019: A single-center study. Antibiotics. 9: 646. https://doi.org/10.3390/antibiotics9100646.

Salvatori, S.; Marafini, I.; Laudisi, F.; Monteleone, G. and Stolfi, C. (2023). *Helicobacter pylori* and gastric cancer. International Journal of Molecular Sciences. 24(3): 2895. https://doi.org/10.3390/ijms24032895.

Yan, L.; Chen, Y.; Chen, F.; Tao, T.; Hu, Z.; Wang, J. et al. (2022). Effect of *Helicobacter pylori* eradication on gastric cancer prevention: Updated report from a randomized controlled trial with 26.5 years of follow-up. Gastroenterology. 163(1): 154-162.e3. https://doi.org/10.1053/j.gastro.2022.03.039.

Zhang, Y.; Wen, Y.; Xiao, Q.; Zheng, W.; Long, G.; Chen, B. et al. (2020). Mutations in the antibiotic target genes related to clarithromycin, metronidazole and levofloxacin resistance in *Helicobacter pylori* strains from children in China. Infection and Drug Resistance. 13: 311-322. https://doi.org/10.2147/IDR.S235615.