Scientific Research Publishing

Open Journal of Medical Microbiology, 2025, 15(4), 273-289
https://www.scirp.org/journal/ojmm
ISSN Online: 2165-3380
ISSN Print: 2165-3372

Stability of *Helicobacter pylori* Resistance to Clarithromycin

Esther Nina Ontsira Ngoyi[1,2,3], Elvire Berthenet[4], Lucie Benejat[5], Claire Guilloteau[1,2], Jamunarani Vadivelu[6], Khean Lee Goh[6], Philippe Lehours[1,2,5], Ange Antoine Abena[3], Matthew D. Hitchings[4], Ben Pascoe[4], Sam Sheppard[4], Francis Mégraud[1,2,5]

[1]INSERM U1053, Université de Bordeaux, Bordeaux, France
[2]Bacteriology Laboratory, CHU de Bordeaux, Bordeaux, France
[3]Microbiology Laboratory, Marien Ngouabi University, Brazzaville, Republic of the Congo
[4]Department of Microbiology and Infectious Diseases, Medical School, Swansea University, Swansea, UK
[5]National Reference Center for Helicobacters and Campylobacters, Université de Bordeaux, Bordeaux, France
[6]Department of Medical Microbiology, University of Malaya, Kuala Lumpur, Malaysia
Email: esthernina2009@gmail.com

How to cite this paper: Ngoyi, E.N.O., Berthenet, E., Benejat, L., Guilloteau, C., Vadivelu, J., Goh, K.L., Lehours, P., Abena, A.A., Hitchings, M.D., Pascoe, B., Sheppard, S. and Mégraud, F. (2025) Stability of *Helicobacter pylori* Resistance to Clarithromycin. *Open Journal of Medical Microbiology*, **15**, 273-289.
https://doi.org/10.4236/ojmm.2025.154022

Received: October 13, 2025
Accepted: December 8, 2025
Published: December 11, 2025

Abstract

The aim of this work was to study the competition between *Helicobacter pylori* isolates susceptible to clarithromycin and their resistant mutants, in terms of growth, by co-culture over an extended time period and to look for compensatory mutations eventually occurring in resistant mutants by whole genome sequencing. A mixture of susceptible and resistant isolates to clarithromycin was detected in gastric biopsies from two patients by real-time FRET-PCR and the *H. pylori* isolates from these two patients were obtained by culture on blood agar with and without macrolide supplement and were named 3657S/3657R and 3695S/3695R. Random amplified polymorphism DNA confirmed that the susceptible and resistant isolates belonged to the same strain and sequencing showed that the 23S rDNA mutations were A2143G (isolate 3657) and A2142G (isolate 3695). The susceptible isolate and its resistant mutant were cultured in the short term in brucella broth where the resistant mutants grew faster, and co-cultured over the long term (30 - 40 days) with AGS gastric eucaryotic cells. Although after 15 days, the growth of the resistant mutant was better than the growth of the susceptible isolate in both pairs, after 30 or 40 days a difference was observed: the growth of the resistant mutant was still better for strain 3657 (R/S ratio: 1.7) but not for strain 3695 (R/S: 0.1). Whole genome sequencing indicated that resistant and susceptible isolates of the same strain clustered closely on a phylogenetic tree. However, concerning strain 3657, changes were detected in two genes between the resistant mutant studied at the start and the same mutant after 40 days of co-culture: HP-0316,

a hypothetical protein and HP-1342, a putative outer membrane protein. These changes may correspond to putative compensatory mutations. In conclusion, different molecular adaptations may occur following the selection of an *H. pylori* mutant resistant to macrolides, confirming the *in vivo* observation.

Keywords

H. pylori, Clarithromycin, Competition, Compensatory Mutation

1. Introduction

Helicobacter pylori (*H. pylori*), is a Gram-negative, microaerophilic spiral bacterium that colonizes the human gastric mucosa of more than 50% of the world's population. *H. pylori* was the first bacterial species proven to cause cancer and is classified as a group I carcinogen by the International Agency for Research on Cancer [1].

In 1993, an effective therapy was proposed based on the prescription of two antibiotics, clarithromycin and amoxicillin or metronidazole, and an anti-secretory drug, essentially a proton pump inhibitor (PPI) [2] [3]. This triple-therapy was found to be effective in large trials [4] and has been used worldwide. Eradication of the bacterium cures peptic ulcer disease, and the macrolide drug clarithromycin is a key component of combination therapies [5]. However, it became clear later that the success of these therapies decreased because *H. pylori*, like other bacteria, can become resistant to antibiotics, rendering the current use of this clarithromycin-based standard triple therapy unacceptable in many parts of the world [6]. Clarithromycin resistance, associated with single base mutations within the peptidyl transferase-encoding region of the 23S rRNA gene [7], is now the main cause of treatment failure. Wu in Taiwan region reported a resistance rate to clarithromycine than 18% [8]. This resistance is variable in different countries and can reach 32.18% [9]. Three mutations have been described in which the adenine residues at positions 2142 and 2143 (equivalent to *Escherichia coli* coordinates 2058 and 2059) are replaced with guanine (A2142G and A2143G) or cytosine (A2143C). They appear spontaneously *in vivo* and are then selected by exposition to subinhibitory concentrations of the antibiotic.

The evolution of antibiotic resistance in pathogenic bacteria is typically accompanied by fitness costs that are expressed in terms of reduced growth rate and reduced competitive ability and virulence [10] [11]. Fitness costs for maintaining resistance genes lead to selection against resistance when pathogen populations encounter antibiotic-free environments, as occurs during transmission between hosts or when antibiotic use is discontinued. As exposure to high doses of an antibiotic is transient, there is the possibility that resistance may only be maintained in the long term if resistant populations can offset the cost of resistance. Therefore, it appears important to know whether the adverse event of resistance in the per-

spective of long-term bacterial survival impacts fitness [12]. Indeed, reversion mutations may occur, and the biological cost caused by resistance can sometimes be mitigated [13]. This phenomenon can also be explained by the appearance of compensatory mutations, thereby restoring competitiveness in resistant mutants [11]. In contrast, when the development of resistance occurs at no biological cost and, in some cases, even with an advantage over the wild strain, the impact of the selective pressure favours the maintenance of resistant mutants in the microbial population [13].

Given that there is controversy with regard to the evolution of clarithromycin resistance in *H. pylori*, the aim of this work was to study the competition between *H. pylori* isolates susceptible to clarithromycin and their resistant mutants, in terms of growth, by co-culture over an extended time period and by whole genome sequencing to look for compensatory mutations in resistant mutants.

2. Material and Methods

Gastric biopsies were obtained at the National Reference Center for Helicobacters and Campylobacters in Bordeaux, France, where they were grown in 1 ml of brucella broth for culture and molecular tests.

2.1. Isolation of a Susceptible Strain and Its Resistant Mutant

a) ***Detection of point mutations associated with clarithromycin resistance and determination of biopsies with susceptible and resistant genotypes***

A small biopsy fragment was digested in 20 µl of proteinase K (Qiagen SA, Courtaboeuf, France) with 180 µl of lysis buffer (Qiagen). DNA extraction was performed by using a MagNA Pure LC DNA isolation kit I (Qiagen). Detection of *H. pylori* and mutations in the 23S rRNA gene associated with clarithromycin resistance was performed by real-time PCR, as previously described [14]. The method included amplification of a fragment of the *H. pylori* 23S rRNA gene coupled with a simultaneous detection of the amplicon by probe hybridization, followed by a melting curve analysis of the amplicons which allows the observation of resistant and susceptible genotypes [15] [16]. This PCR also enabled the identification of biopsies containing both susceptible and resistant genotypes. Finally, strains from two patients with susceptible and resistant genotypes were obtained and named 3657-S/3657-R and 3695-S/3695-R.

b) ***Culture and separation of susceptible and resistant mutants***

In each clinical case with both susceptible and resistant genotypes (3657-S/3657-R and 3695-S/3695-R), biopsies were cultured on Mueller Hinton agar plates supplemented with 10% sheep blood and Polyvitex in a microaerobic atmosphere at 37˚C. Clarithromycin resistant mutants were selected by plating 10^9 colony forming units (CFU) on the same medium containing 1 µg/ml of clarithromycin and clarithromycin susceptible strains were selected by plating 10^9 CFU on the medium without clarithromycin**.** In both cases, each colony was subcultured and the minimal inhibitory concentrations (MIC) of clarithromycin against *H.*

pylori were determined by agar diffusion using E-test clarithromycin strips and real-time PCR in order to confirm susceptibility or resistance. Resistant mutants and susceptible isolates were single-colonies purified on a selective plate and then immediately frozen at −80˚C.

c) ***Random amplified polymorphism DNA* (*RAPD*)**

Molecular typing (RAPD) was carried out using primer 1254 (CCGCAGCCAA) and primer 1290 (GTGGATGCGA) [17]. RAPD was carried out to determine whether each susceptible isolate and its resistant mutant belonged to the same strain, when the strips showed an identical profile between the susceptible isolate and its resistant mutant, after migration on an agarose gel.

2.2. Determination of the Type of Mutation

DNA sequencing

Sequencing of the 23S rRNA gene which is associated with clarithromycin resistance was performed to determine the mutation type: A2142G or A2143G or A2143C, by sequencing (Applied Biosystems, version 3.1 or 1.1).

2.3. Competition between the Susceptible Isolate and Its Resistant Mutant

a) ***Culture in brucella broth***

Isolates 3657-S/3657-R and 3695-S/3695-R were grown separately over a short period of time (48 hours) in macrolide-free brucella broth supplemented with 10% serum. Quantification was performed after 3, 6, 9, 25, 32 and 48 hours.

b) Competition in co-culture with eukaryotic cells

To assess the difference in the bacterial growth rate over a long time course, co-culture on the AGS gastric cell line was carried out for 30 or 40 days. Suspensions of similar opacity of the susceptible strain and its resistant mutant were used to infect the cells at a multiplicity of infection (MOI) of 2. Sequential passage of cells was carried out twice a week corresponding to 8 passage for 3695 isolate and 10 passage for 3657 isolates. The quantity of each isolate (susceptible and resistant) in CFU, was determined at the beginning of the experiment and at days 15 and 30 for strain 3695 and at days 15 and 40 for strain 3657, by culture on Mueller Hinton agar supplemented with 10% sheep blood and polyvitex with and without erythromycin and incubated in a microaerobic atmosphere. The CFU ratio between the susceptible strain and its resistant mutant was then recorded.

Then, Prepare a 0.5 MF suspension of each isolate in Brucella broth in a test tube;

- Perform two dilutions (10^{-1} and 10^{-2}) in 15 ml flat-bottomed tubes: 4.5 ml of Brucella broth + 0.5 ml of bacterial suspension;
- Distribute 4.5 ml of the previously prepared Brucella broth (100 ml of broth + 5 ml of fetal calf serum) into 16 tubes (4 tubes for isolate 3657 S, 4 tubes for isolate 3657 R, 4 tubes for isolate 3695 S and 4 tubes for isolate 3695 R);
- Distribute 500 µl of bacterial suspension into the 8 tubes;

- Remove tube 1 (t0), culture it, and culture the other tubes with shaking in a microaerobic atmosphere;
- Remove the tubes at 3 hours, 6 hours, 9 hours, 12 hours, 24 hours, 36 hours and 48 hours and, from 1 ml of suspension, culture the pure suspension and the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions in triplicate on MH agar + 10% sheep blood + Polyvitex (100 µl to spread with a sterile spreader);
- Also culture the uninoculated negative control tube;
- Dry the plates under a laminar flow hood;
- Incubate the plates in a microaerobic atmosphere for 5 to 6 days, then count the bacteria and calculate the ratio of resistant bacteria/sensitive bacteria.

The cell culture media were prepared by mixing the following reagents:

- 45 ml of DEMXII;
- 5 ml of fetal calf serum (serum D, see in the freezer);
- 50 µl of vancomycin (see in the freezer, microtubes labeled V);
- Filter this entire mixture with a 0.22 filter and syringe by screwing it into another 15 ml tube (remove the plunger before filtering).

Cell detachment, recovery, and flask preparation were performed as follows:

- Recover the medium contained in each flask and discard it in the waste container;
- Rinse the cells with PBS (10 ml), then recover all the supernatant and discard it in the waste container;
- Add 3 ml of trypsin; - Incubate at 37˚C for 5 minutes;
- Stop the trypsin action by adding 7 ml of medium;
- Transfer everything into a small 15 ml conical tube;
- Centrifuge (program 6, *i.e.*, 1300 rpm for 3 min);
- Discard the supernatant;
- Resuspend the cells in 5 ml;
- Cell count: 15 µl of trypan blue + 15 µl of cells, take 10 µl and place in the counting chamber, the cell counter indicates: 1.13×10^6 cells per ml, *i.e.*, 5.65×10^6 cells in 5 ml;
- Prepare 4 flasks (3 to be infected the next day and 1 negative control), by placing 1.13 million cells in each, *i.e.*, 1 ml.

Cell infection with the 3657 S/3657R isolate pair

Preparation of a suspension with isolate 3657 S and another suspension with isolate

3657 R. Calibration of the suspensions to an OD of 1 using a spectrophotometer.

Calculation of the volume to inoculate into the flasks with an MOI of 1 (for 1.13 million cells), knowing that an OD of 1 corresponds to 2×10^8 bacteria per 1 ml, which corresponds to a volume of 22 µl (for an MOI of 2).

Cell infection with the 3695 S/3695R isolate pair

Preparation of a suspension with isolate 3657 S and another suspension with isolate

3657 R. Calibration of the suspensions to an OD of 1 using a spectrophotometer. Calculation of the volume to inoculate into the flasks with an MOI of 1 (for 1.13

million cells), knowing that an OD of 1 corresponds to 2×10^8 bacteria per 1 ml, which corresponds to a volume of 22 µl (for an MOI of 2).

Cell passaging

The infected cells were passaged twice a week for at least 30 days (1 month) and 40 days, and new cells were infected with the previously infected cells.

2.4. Whole Genome Sequencing

a) ***Genome sequencing***

DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Nucleic acid content was quantified and assessed with a Nanodrop spectrophotometer prior to normalization and sequencing. NexteraXT (v3 Technology, 250bp paired-end) libraries were prepared and high-throughput genome sequencing was performed using an Illumina MiSeqSystem benchtop sequencer (Illumina, San Diego, CA, USA). Short reads were assembled *de novo* using SPAdes (version 3.0.0) and were evaluated using QUAST [18].

b) ***Data base archiving, annotation and phylogeny***

Contiguous genome sequences were imported and archived on the Bacterial Isolate Genome sequence database (BIGSdb) [19]. Putative coding sequences were identified for each assembled genome using the rapid annotation server RAST [20]. A pan-genome was constructed from all loci identified in at least one of the isolates used in this study using a reference pan-genome approach [21]. This derived list was used to interrogate each of the genomes analyzed in this study (BLASTN). Genes were defined as present by a match of greater than 70% identity over 50% or more of the locus length. Gene-by-gene alignments were exported to investigate single nucleotide polymorphism (SNP) variation. A neighbour-joining tree of the start and end isolates for both resistant and susceptible versions of the two strains was constructed using FastTree2 [22] and annotated with Evolview [23] according to the clarithromycin resistance profile and day of isolation. Individual SNPs in genes highlighted as potential hosts for compensatory mutations were analyzed from gene-by-gene alignments using Bioedit.

3. Results

3.1. Characterization of the Strains

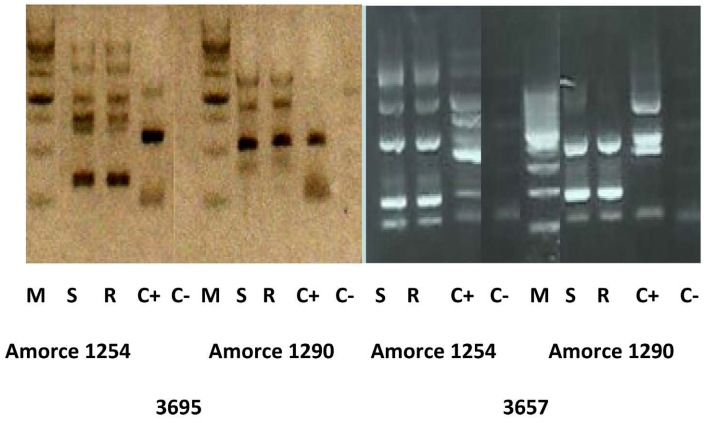
Each susceptible isolate and its respective clarithromycin resistant mutant were identified by culture and PCR and named 3657-S, 3657-R and 3695-S and 3695-R, respectively.

The MIC for both susceptible isolates was below the limit of 0.125 µg/ml and the 3695 resistant mutant MIC was higher than that of the 3657 resistant mutant (see **Table 1**).

The RAPD showed that in the two cases, the susceptible isolate and its resistant mutants had the same strain profile (see **Figure 1**).

Table 1. Clarithromycin minimal inhibitory concentration (MIC) measured in the 2 versions (labeled R for Resistant and S for Susceptible) of 2 clinical isolates (3657 and 3695).

Isolate	MIC (mg/ml)
3657 S	<0.000125
3657 R	0.004
3695 S	<0.000125
3695 R	0.032



S (Susceptible isolate), R (resistant isolate), M (molecular 1 Kb mass standard), C+ (external control, another *H. pylori* strain), C– (negative control).

Figure 1. Representative RAPD patterns of *H. pylori* 3695-S/3695-R and 3657-S/3657-R on 2% agarose gel.

Sequencing of the 23S rRNA gene of the resistant mutant showed an A2142G mutation in the 3695-R isolate and an A2143G mutation in the 3657-R isolate.

3.2. Culture in Brucella Broth

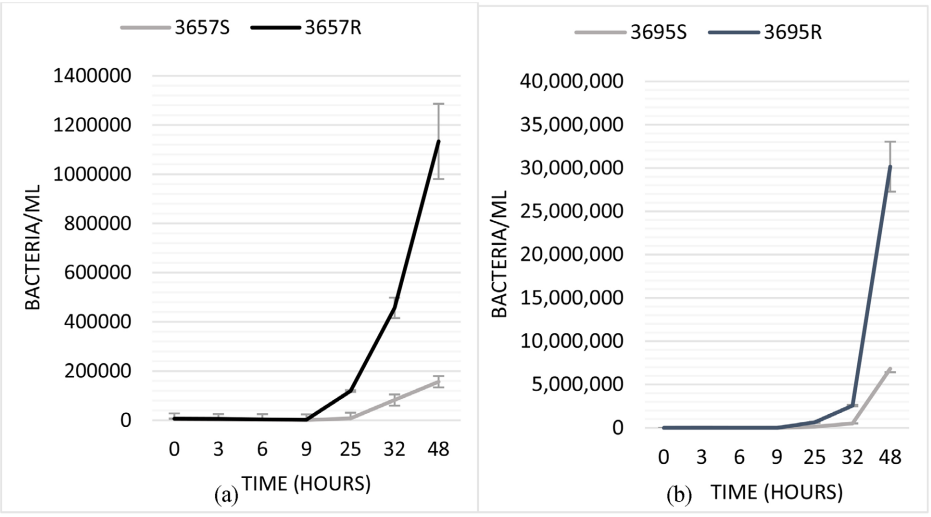


Figure 2. (a) Growth rate of *H. pylori* 3657-S and 3657-R isolates in short term culture (R/S ratio at 48 hours = 7.2); (b) Growth rate of *H. pylori* 3695-S and 3695-R isolates in short term culture (R/S ratio at 48 hours = 3.4).

Resistant mutants grew faster than the susceptible isolates in short term broth culture (48 hours) in both cases (3657 and 3695 strains), (see Figure 2(a) and Figure 2(b)).

3.3. Co-Culture with AGS Cells

For both pairs of isolates (susceptible and resistant), the growth of the resistant mutant at 15 days was better than that of its susceptible counterpart. However, at 30 or 40 days a difference was observed. The growth rate of the 3657-R mutant still remained higher than that of its susceptible counterpart while the contrary was observed for 3695 (Figure 3(a) and Figure 3(b)).

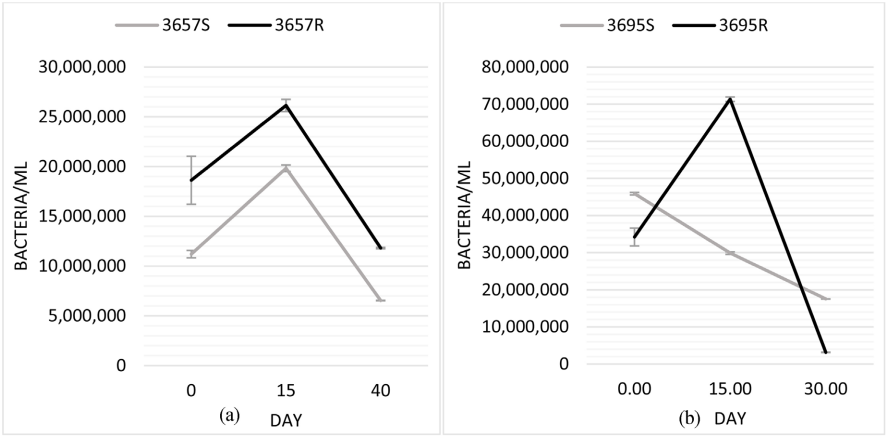


Figure 3. (a) Growth rate of *H. pylori* 3695-S and 3695-R (R/S ratio at day15 = 4; R/S ratio in long term culture (day 30 = 0.1); (b) Growth rate of *H. pylori* 3657-S and 3657-R (R/S ratio at day 15 = 1; R/S ratio in long term culture (day 40 = 1.7).

3.4. Whole Genome Sequencing

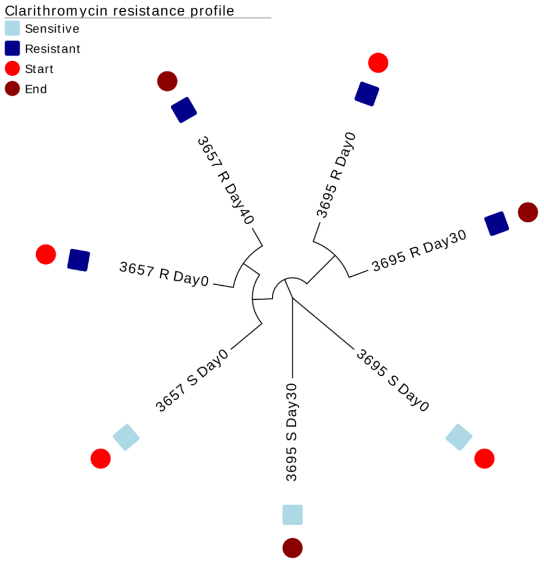


Figure 4. Circular cladogram view of a neighbour-joining tree built on a 1,508,595 bp alignment of the resistant and susceptible versions of strains 3657 and 3695.

The mean number of contigs for the 11 isolates tested was 50.5 giving rise to a mean total length of 1,648,591 bp. The pan genome included 1538 genes and the core genome alignment 1,508,595 bp. Resistant and susceptible isolates clustered closely on a phylogenic tree (Figure 4). Concerning the resistant isolate 3657-R, notable changes occurred in two genes: *HP*_0316 (hypothetical protein) and *HP*_1342 (putative outer membrane protein) between the start isolate (R0) and the isolate obtained after 40 days of cellular co-culture (R40). The alignments are presented in Table 2 and Table 3. Potential compensatory mutations may have occurred in these two genes. In contrast, no major changes in genes, no any significant mutation appeared in the resistant isolate 3695-R after 30 days of co-culture (Table 4 to Table 5).

Table 2. Hp 1342 alignments: strain 3657 (modifications corresponding to putative outer membrane protein).

>9533|Hp_3657R_1_0:1175265-1177421 + id9532_1086
----------ATGAAAATCAAAAAATCCCTCTTG
CTCTCTCTTTCTCTCATGGCTTCATTATCAAGGGCTGAAGATGACGGATTTTACATGAGC
GTGGGCTATCAGATCGGTGAAGCGGTTCAAAAAGTGAAAAACACTGGAGCATTGCAAAAT
CTTGCAGACAGATACGATAACTTAAGCAACCTTTTAAACCAATACAACTACCTTAACTCT
TTAGTCAATCTAGCCAGTACGCCCAGTGCGATCACGGGTGCGATTGACAATCTAAGCTCA
AGCGCCATTAATCTCACTAGCGCTACCACCACTTCCCCCGCCTATCAAGCTGTGGCTTTA
GCACTCAATGCCGCTGTGGGCATGTGGCAAGTCATAGCCTTTGGCATCAGCTGTGGCCCT
GGCCCCAATCTTGGCCCAGAACATTTAGAAAATGGGGGCGTTCGATCGTTTGACAACACG
CCAAACTACAGCTACAACACCGGTAGCGGAACGACCACCACCACTTGTAATGGAGCTAGT
AATGTAGGGCCCAATGGCATTCTATCTAGCAGCGAATACCAGGTTCTCAATACCGCTTAT
CAAACTATCCAAACCGCTTTAAACCAAAACCAAGGAGGCGGGATGCCTGCCTTGAATGG-
--CTCTAAAAATATGATAGTCAATAT----------CAATCAAACTTTCACAAGAAAC
CCTACCACAGAATACACTTACCCTGATGGGAATGGCAA----------TTATTATTCA
GGCGGTTCATCAATCCCAATCCAGCTAAAGATTAGCAGCGTCAATGACGCTGAAAACCTT
TTGCAACAAGCCGCTACTATCATCAATGTCCTTACCACCCAAAACCCGCATGT------G
AATGGTGGCGGTGGGGCATGGGGGTTTAGCGGCAAGACCGGGAATGTGATGGATATTTTT
GGCCCTTCTTTTAACGCTATTAACGAGATGATTAAAAACGCTCAAACAGCCCTAGCAAAA
ACCCAACAGCTTAACGCTAATGAAAACGCCCAAATCACGCAACCCAACAATTTCAACCCC
TACACTTCTGAAGACAAAGGGTTCGCTCAAGAAATGCTCAATAGAGCTAACGCTCAAGCA
GAGATTTTAAATTTAGCCCAACAAGTAGCGGACAATTTCCACAGCATTCAAGGGCCTATC
CAACAAGATCTAGAAGAATGCACCGCAGGATCGGCTGGTGTGATTAACGACAACACTTAT
GGTTCAGGCTGCGCGTTTGTGAAGGAAACTTTAAACTCTTTGGTGCAACACACCGCTTAT
TATGGCAATCAGGTCAATCAAGAGAAAGCTTTGGCTCAAACCATTTTGAATTTTAAAGAA
GCCCTTAATACCCTAAACAAAGACTCTACAGCGATTAATAATAGTATCTCTCACTTGCCT
AACGCTAAATCTCTTCAAAACATGACGCATTCCACTCAAAACCCTAATTCCCCAGAAGGT
TTGCTCACTTATTCTTTGGATACCAACAAATACAACCAGCTCCAAAATATCACGCAAGAA
TTAGGCAAGAACCCTTTTAGGCGTATCGGCGTGATTGACTATCAAACCAACAATGGGGCG
ATGAACGGCATCGGCGTGCAAGTGGGCTATAAGCAATTCTTTGGCAAAAAAAGAAGGTGC
GGGTTAAGGTATTACGGCTTTTTTGATTACAACCATGCGTTCATTAAATCCAGCTTTTTT
AACTCGGCTTCTGATGTGTGGACTTATGGGGTGGGAATGGACGCTCTCTATAACTTCATC
AATGATAAAAACACCAACTTTTTAGGCAAGAATAACAAGCTTTCTGTGGGGCTTTTTGGT
GGCTTTGTGCTAGCTGGGACTTCGTGGCTTAATTCTGAATTTGTGAATTTGAACATGGTG
GGTAATATCTATAGCGCTAAGATGAATGTGGCTAATTTCCAATTTTTATTCAATTTAGGC
TTGAGGATGAATCTCGCTAGGCCTAAGAAAAAAAACAGCGATCATGCCGCTCAGCATGGC

ATGGAATTGGGCGTGAAAATCCCTACCATTAACACGGATTATTACTCTTTCATGGGGGCT
GAACTCAAATACAGAAGGCTCTATAGCGTGTATCTCAATTATGTGTTTGCTTACTAG
>9534|Hp_3657R_1_40:1175265-1177421 + id9532_1086
----------ATGAAAATCAAAAAATCCCTCTTG
CTCTCTCTTTCTCTCATGGCTTCATTATCAAGAGCTGAAGATGACGGATTTTACATGAGC
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CTTGCAGACAGATACGATAACTTAAGCAACCTTTTAAACCAATACAACTACCTTAACTCT
TTAGTCAATCTAGCCAGTACGCCCAGTGCGATCACGGGTGCGATTGACAATCTAAGCTCA
AGCGCCATTAATCTCACTAGCGCTACCACCACTTCCCCCGCCTATCAAGCTGTGGCTTTA
GCACTCAATGCCGCTGTGGGCATGTGGCAAGTCATAGCCTTTGGCATCAGCTGTGGCCCT
GGCCCCAATCTTGGCCCAGAACATTTAGAAAATGGGGGCGTTCGATCGTTTGACAACACG
CCAAACTACAGCTACAACACCGGTAGCGGAACGACCACCACCACTTGTAATGGAGCTAGT
AATGTAGGGCCCAATGGCATTCTATCTAGCAGCGAATACCAGGTTCTCAATACCGCTTAT
CAAACTATCCAAACCGCTTTAAACCAAAACCAAGGAGGCGGGATGCCTGCCTTGAATGG-
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CCTACCACAGAATACACTTACCCTGATGGGAATGGCAA----------TTATTATTCA
GGCGGTTCATCAATCCCAATCCAGCTAAAGATTAGCAGCGTCAATGACGCTGAAAACCTT
TTGCAACAAGCCGCTACTATCATCAATGTCCTTACCACCCAAAACCCGCATGT------G
AATGGTGGCGGTGGGGCATGGGGGTTTAGCGGCAAGACCGGGAATGTGATGGATATTTTT
GGCCCTTCTTTTAACGCTATTAACGAGATGATTAAAAACGCTCAAACAGCCCTAGCAAAA
ACCCAACAGCTTAACGCTAATGAAAACGCCCAAATCACGCAACCCAACAATTTCAACCCC
TACACTTCTGAAGACAAAGGGTTCGCTCAAGAAATGCTCAATAGAGCTAACGCTCAAGCA
GAGATTTTAAATTTAGCCCAACAAGTAGCGGACAATTTCCACAGCATTCAAGGGCCTATC
CAACAAGATCTAGAAGAATGCACCGCAGGATCGGCTGGTGTGATTAACGACAACACTTAT
GGTTCAGGCTGCGCGTTTGTGAAGGAAACTTTAAACTCTTTGGTGCAACACACCGCTTAT
TATGGCAATCAGGTCAATCAAGAGAAAGCTTTGGCTCAAACCATTTTGAATTTTAAAGAA
GCCCTTAATACCCTAAACAAAGACTCTACAGCGATTAATAATAGTATCTCTCACTTGCCT
AACGCTAAATCTCTTCAAAACATGACGCATTCCACTCAAAACCCTAATTCCCCAGAAGGT
TTGCTCACTTATTCTTTGGATACCAACAAATACAACCAGCTCCAAAATATCACGCAAGAA
TTAGGCAAGAACCCTTTTAGGCGTATCGGCGTGATTGACTATCAAACCAACAATGGGGCG
ATGAACGGCATCGGCGTGCAAGTGGGCTATAAGCAATTCTTTGGCAAAAAAAGAAGGTGC
GGGTTAAGGTATTACGGCTTTTTTGATTACAACCATGCGTTCATTAAATCCAGCTTTTTT
AACTCGGCTTCTGATGTGTGGACTTATGGGGTGGGAATGGACGCTCTCTATAACTTCATC
AATGATAAAAACACCAACTTTTTAGGCAAGAATAACAAGCTTTCTGTGGGGCTTTTTGGT
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GGTAATATCTATAGCGCTAAGATGAATGTGGCTAATTTCCAATTTTTATTCAATTTAGGC
TTGAGGATGAATCTCGCTAGGCCTAAGAAAAAAAACAGCGATCATGCCGCTCAGCATGGC
ATGGAATTGGGCGTGAAAATCCCTACCATTAACACGGATTATTACTCTTTCATGGGGGCT
GAACTCAAATACAGAAGGCTCTATAGCGTGTATCTCAATTATGTGTTTGCTTACTAG

Table 3. Hp 0316 alignments: strain 3657 (corresponding to hypothetical protein).

>9533|Hp_3657R_1_0:285568-285984 + HP_0316
----------ATGCCTAACACCACCGCAGAAAAAGACTACACCCAATACAGCGAAAAA
CAGCTTTTTAATTTAATCCATCAATTAGAGCGAAAAATCAAAAAGATGCAAAATGATAGA
GTTTCTTTTAAAGAAAAAATGGCTAAAGAATTGGAAAAAAGGGATCAAAACTTTAAGGAT
AAAATAGACGCATTAAATGAACTCTTGCAAAAAATCAGCCAAGCTTTTGATGATAAAAGA
GATTGCTGTTTTGGGCATGAGATCCCAAATATTGAAACGCAACAAGCTATGAGAGATGCG
TATAACAAAGAGACAGATTTGTTCGTTGAGGATTTTTCTAGTTATAGCAATGAGAGAAAA
AAGGCTTTAGGTGTTGAAGCTCAATCTTAAAAAATCTTTTCAAAAA----------
>9534|Hp_3657R_1_40:285568-285984 + HP_0316
----------ACCACCGCAGAAAAAGACTACACCCAATACAGCGAAAAA

CAGCTTTTTAATTTAATGAATAAGAAAGAACAAGAACTTGAAAAGCTA-AAAGGGATAGG
GCATATCTTGAAAAGAAAATGGCTAAAGAATTGGAAAAAAGGGATCAAAACTTTAAGGAT
AAAATAGACGCATTAAATGAACTCTTGCAAAAAATCAGCCAAGCTTTTGATGATAAAAGA
GATTGCTGTTTTGGGCATGAGATCCCAAATATTGAAACGCAACAAGCTATGAGAGATGCG
TATAACAAAGAGACAGATTTGTTCGTTGAGGATTTTTCTAGTTATAGCAATGAGAGAAAA
AAGGCTTTAGGTGTTGAAGCTCAATCTTAAAAAATCTTTTCAAAAA----------

Table 4. Hp 0316 alignments: strain 3695.

>9536|Hp_3695R_1_0:285568-285984 + HP_0316
AAGGAATAAAGCATGCCA---AACACCACCAACAAAGATTACACAAAATACAGCCAAAGA
CAGCTTTTTAACCTCATGAATAAAAAAGAACAAGAAATTGAAAAGCTAAAAAGGGATAGG
GCGTATCTTGAAAAGAAAATGGCTAAAGAGCTAGAAAAAAAAGATAAAGGTTTCACTATC
AAGTTAGAAGAATTGAACAAAATCTTACAAGAGATCAACAAAAGAGCTAAT---------
GATTGTTGTTTTGGGCATGAAACCCCAAACATTCAAACGCAACAAGCCATGAGAGAAGCA
GATAACAAAGAAACAGATTTGTTCGTTGAGGATTTTTCTAGTTATAGCAATGAGAGAAAA
AAGGCTTTAGGTGTTGAAGCTCAATCTTAAAAAATCTTTTCAAAAAGATTTTGATAA
>9543|Hp_3695R_2_30:285568-285984 + HP_0316
----------ATGCCA---AACACCACCAACAAAGATTACACAAAATACAGCCAAAGA
CAGCTTTTTAACCTCATGAATAAAAAAGAACAAGAAATTGAAAAGCTAAAAAGGGATAGG
GCGTATCTTGAAAAGAAAATGGCTAAAGAGCTAGAAAAAAAAGATAAAGGTTTCACTATC
AAGTTAGAAGAATTGAACAAAATCTTACAAGAGATCAACAAAAGAGCTAAT---------
GATTGTTGTTTTGGGCATGAAACCCCAAACATTCAAACGCAACAAGCCATGAGAGAAGCA
GATAACAAAGAAACAGATTTGTTCGTTGAGGATTTTTCTAGTTATAGCAATGAGAGAAAA
AAGGCTTTAGGTGTTGAAGCTCAATCTTAAAAAATCTTTTCAAAA----------

Table 5. Hp 1342 alignments: strain 3695.

>9536|Hp_3695R_1_0:1175265-1177421 + id9532_1086
TTTGTATCAAAGAAAAATCTAATAAAAGGAAACAACATGAAAATCAAAAAATCCCTCTTT
CTCTCTCTTTCTCTCATGGCTTCATTATCAAGGGCTGAAGACGATGGATTTTACATGAGC
GTGGGCTATCAGATCGGCGAAGCGGTTTAACAAGTGAAAAACACGGGAGCGATACAGAAT
CTTGCAGACAGATACGATAACTTGAGCAACCTTTTAAACCAATACAACTACCTTAACTCT
TTAGTCAATCTAGCCAGCACACCGAGCGCGATCACCGGTGCGATTGACAATTTAAGCTCA
AGCGCCATTAACCTCACTAGCGCTACCACCACTTCCCCCGCCTATCAAGCTGTGGCTTTA
GCGCTCAACGCCGCTGTGGGCATGTGGCAAGTCATAGCCCTTTTTATTGGCTGTGGTCCT
GGCCCTA----------CCAACAATCAAAGCTACCAATCGTTTGGTAACACA
CCAGCCCTTAAT----------GGGACCACCACCACTTGCAATCAAGCGTAT
GGGACAGGCCCCAATGGCATCCTATCCATTGACGAATACCAAAAACTCAACCAAGCTTAT
CAAATCATCCAAACCGCTTTAAACCAAAATCAAGGAGGCGGGATGCCTGCCTTGAATGG-
--CTCTAAAAATATGGTAGTCAATAT----------CAATCAAACTTTCACAAGAAAC
CCTACAACAGAATACACTTACCCCGATGGGAATGGCAA----------TTATTATTCA
GGCGGGACTCCACTCCCAATCCAGCTAAAGATTAGCAGCGTCAATGACGCTGAAAACCTT
TTGCAACAAGCCGCCACTATCATGCAAGTCCTTACCACTCAAAACCCGCATGT------G
AATGGTGGCGGTGGGGCATGGGGTTTTGGCGGCAAGACCGGGAATGTGATGGATATTTTT
GGCGATAGTTTTAACGCTATTAACGAAATGATCAAAAACGCTCAAGCCGTTTTAGAAAAA
ACCCAACAGCTTAACGCTAATGAAAACACCCAAATCACGCAACCAGACAATTTCAACCCC
TACACTTCTAAAAATAAGCAATTCGCTCAAGAAATGCTCAACAGAGCTAACGCTCAAGCA
GAGATTTTAAATTTAGCTAAGCAAGTAGCGGACAATTTCCACAGCATTCAAGGGCCTATT
CAAGGGGATTTGGAAGAATGTAAAGCAGGATCGGCTGGCGTGATTAACGACAACACTTAT

GGTTCAGGTTGCGCGTTTGTGAAGGAAACTTTAAACTCTTTGGAGCAACACACCGCTTAT
TATGGCAATCAGGTCAATCAGGATAGGGCTTTATCTCAAACCATTTTGAATTTTAAAGAA
GCCCTTAATACTTTAGGGAACGACTCAAAAGCGATCAATAGCGGTATTTCTCACTTGCCT
AACGCTAAATCTCTTCAAAACATGACGCATGCCACTCAAAACCCTAATTCCCCAGAAGGT
TTGCTCACTTATTCTTTGGATACCAACAAGTACAACCAATTCCAAACCATCGCGCAAGAA
TTAGGCAAGAACCCCTTTAGGCGCATCGGCGTGATTGACTATCAAAACAATAACGGGGCG
ATGAACGGGATCGGCGTGCAAGCGGGCTATAAGCAATTCTTTGGTAAAAAAAGGAATTGG
GGGTTAAGGTATTACGGCTTTTTTGATTACAACCACGCTTATATCAAATCTAATTTTTTT
AACTCGGCTTCTGACGTGTGGACTTATGGGGTGGGCATGGACGCTCTTTATAACTTTATC
AACGATAAAAACACTAACTTCTTAGGCAAAAATAACAAGCTTTCCGTGGGGCTTTTTGGT
GGCTTTGCGTTAGCTGGGACTTCGTGGCTCAATTCCCAACAAGTGAATTTGACCATGATG
AATGGCATTTATAACGCTAATGTCAGCACTTCTAACTTCCAATTTTTGTTTGATTTAGGC
TTGAGAATGAATCTTGCTAGGCCCAAGAAAAAAGACAGCGATCATACCGCTCAGCATGGC
ATTGAACTAGGCTTTAAAATCCCCACCATTAACACGGACTATTATTCTTTCATGGGTGCG
AAACTAGAATACAGAAGGATGTATAGCCTTTTCCTCAATTATGTGTTTGCTTACTAG
GCGTATCTTGAAAAGAAAATGGCTAAAGAGCTAGAAAAAAAAGATAAAGGTTTCACTATC
AAGTTAGAAGAATTGAACAAAATCTTACAAGAGATCAACAAAAGAGCTAAT---------
GATTGTTGTTTTGGGCATGAAACCCCAAACATTCAAACGCAACAAGCCATGAGAGAAGCA
GATAACAAAGAAACAGATTTGTTCGTTGAGGATTTTTCTAGTTATAGCAATGAGAGAAAA
AAGGCTTTAGGTGTTGAAGCTCAATCTTAAAAAATCTTTTCAAAA----------
>9543|Hp_3695R_2_30:1175265-1177421 + id9532_1086
TTTGTATCAAAGAAAAATCTAATAAAAGGAAACAACATGAAAATCAAAAAATCCCTCTTT
CTCTCTCTTTCTCTCATGGCTTCATTATCAAGGGCTGAAGACGATGGATTTTACATGAGC
GTGGGCTATCAGATCGGCGAAGCGGTTTAACAAGTGAAAAACACGGGAGCGATACAGAAT
CTTGCAGACAGATACGATAACTTGAGCAACCTTTTAAACCAATACAACTACCTTAACTCT
TTAGTCAATCTAGCCAGCACACCGAGCGCGATCACCGGTGCGATTGACAATTTAAGCTCA
AGCGCCATTAACCTCACTAGCGCTACCACCACTTCCCCCGCCTATCAAGCTGTGGCTTTA
GCGCTCAACGCCGCTGTGGGCATGTGGCAAGTCATAGCCCTTTTTATTGGCTGTGGTCCT
GGCCCTA----------CCAACAATCAAAGCTACCAATCGTTTGGTAACACA
CCAGCCCTTAAT----------GGGACCACCACCACTTGCAATCAAGCGTAT
GGGACAGGCCCCAATGGCATCCTATCCATTGACGAATACCAAAAACTCAACCAAGCTTAT
CAAATCATCCAAACCGCTTTAAACCAAAATCAAGGAGGCGGGATGCCTGCCTTGAATGG-
--CTCTAAAAATATGGTAGTCAATAT----------CAATCAAACTTTCACAAGAAAC
CCTACAACAGAATACACTTACCCCGATGGGAATGGCAA----------TTATTATTCA
GGCGGGACTCCACTCCCAATCCAGCTAAAGATTAGCAGCGTCAATGACGCTGAAAACCTT
TTGCAACAAGCCGCCACTATCATGCAAGTCCTTACCACTCAAAACCCGCATGT------G
AATGGTGGCGGTGGGGCATGGGGTTTTGGCGGCAAGACCGGGAATGTGATGGATATTTTT
GGCGATAGTTTTAACGCTATTAACGAAATGATCAAAAACGCTCAAGCCGTTTTAGAAAAA
ACCCAACAGCTTAACGCTAATGAAAACACCCAAATCACGCAACCAGACAATTTCAACCCC
TACACTTCTAAAAATAAGCAATTCGCTCAAGAAATGCTCAACAGAGCTAACGCTCAAGCA
GAGATTTTAAATTTAGCTAAGCAAGTAGCGGACAATTTCCACAGCATTCAAGGGCCTATT
CAAGGGGATTTGGAAGAATGTAAAGCAGGATCGGCTGGCGTGATTAACGACAACACTTAT
GGTTCAGGTTGCGCGTTTGTGAAGGAAACTTTAAACTCTTTGGAGCAACACACCGCTTAT
TATGGCAATCAGGTCAATCAGGATAGGGCTTTATCTCAAACCATTTTGAATTTTAAAGAA
GCCCTTAATACTTTAGGGAACGACTCAAAAGCGATCAATAGCGGTATTTCTCACTTGCCT
AACGCTAAATCTCTTCAAAACATGACGCATGCCACTCAAAACCCTAATTCCCCAGAAGGT
TTGCTCACTTATTCTTTGGATACCAACAAGTACAACCAATTCCAAACCATCGCGCAAGAA

TTAGGCAAGAACCCCTTTAGGCGCATCGGCGTGATTGACTATCAAAACAATAACGGGGCG
ATGAACGGGATCGGCGTGCAAGCGGGCTATAAGCAATTCTTTGGTAAAAAAAGGAATTGG
GGGTTAAGGTATTACGGCTTTTTTGATTACAACCACGCTTATATCAAATCTAATTTTTTT
AACTCGGCTTCTGACGTGTGGACTTATGGGGTGGGCATGGACGCTCTTTATAACTTTATC
AACGATAAAAACACTAACTTCTTAGGCAAAAATAACAAGCTTTCCGTGGGGCTTTTTGGT
GGCTTTGCGTTAGCTGGGACTTCGTGGCTCAATTCCCAACAAGTGAATTTGACCATGATG
AATGGCATTTATAACGCTAATGTCAGCACTTCTAACTTCCAATTTTTGTTTGATTTAGGC
TTGAGAATGAATCTTGCTAGGCCCAAGAAAAAAGACAGCGATCATACCGCTCAGCATGGC
ATTGAACTAGGCTTTAAAATCCCCACCATTAACACGGACTATTATTCTTTCATGGGTGCG
AAACTAGAATACAGAAGGATGTATAGCCTTTTCCTCAATTATGTGTTTGCTTACTAG

4. Discussion

There are several reports showing that *in vitro* the A2142G and A2143G mutations had no significant effect on the growth rate of *H. pylori* under macrolide-free conditions in a short term culture [24] [25]. Our study confirms these data, despite the small size of our sample pairs due to the difficulty of obtaining both a sensitive and a resistant pair simultaneously in clinical samples. However, no long term cultures had been performed. In a previous study on one strain (lasting 10 days), a biological cost was reported for the resistant mutant [26]. The authors recommended a long-term study to better appreciate what can be observed *in vivo*, given that *in vivo* growth is slower compared to that *in vitro*. We were able to co-culture the susceptible and resistant isolates of the same strains for 30 or 40 days and found opposite results for the two strains.

During co-culture of resistant and susceptible variants of the strains with AGS cells, differences were observed in the way the two strain couples evolved. A high R/S ratio at day 15 was observed for 3695 confirming the better growth of the resistant mutant, but after day 30, the ratio decreased indicating less growth of the resistant strain compared to the susceptible isolate. This result implies that the resistant mutant, despite being fitter in the short term, is not stable on a long-term scale, and is outcompeted by the susceptible isolate. In contrast, in strain 3657, the resistant mutant was stable at both day 15 and day 40 (R/S ratio = 1.7). These results can be explained by the fact that the resistance in 3695 strain has a biological cost (R/S ratio at day 30 = 0.1) while this is not the case for 3657. This difference between the two couples of strains could also be explained by the type of mutation (A2143 for 3657 and A2142 for 3695) as was shown for other bacteria. Spectinomycin-resistant (Spcr) clonal variants of *Chlamydia psittaci* mutations in the 16S rRNA gene at positions 1191 and 1193 were associated with a marked impairment of biological fitness, and the bacteria were severely outcompeted by the wild-type parent. In contrast, mutations at position 1192 had minor effects on the bacterial life cycle, allowing the resistant isolates to compete more efficiently with the wild-type strain. Thus, mutations with a wide range of fitness costs can be selected allowing a prediction and monitoring of the emergence of antibiotic resistance in *Chlamydia* [27]. A positive impact of resistance on growth has also been shown: the introduction of the *qnr*A3 gene which confers a low level of re-

sistance to fluoroquinolones into small *E. coli* plasmids enhances their growth [28]. The authors suggest that the regulation of protein-DNA bonds exerted by the *qnr*A3 gene constitutes a selective advantage for bacteria that have acquired this gene, which would have initially contributed to their emergence. In contrast, the *qnr*A3 gene carried on a large conjugative plasmid induces a biological cost, retarding the growth of the strain which carries it and hence presumably its dissemination.

By performing whole genome sequencing, potential compensatory mutations were found in the resistant isolate 3657 which maintained its pre-eminence over its susceptible counterpart, however, this was not the case for the resistant isolate 3695-R. This could explain the differences in competitiveness between the two isolates. It is possible that this is a mutation affecting fitness, however, it is essential that this experiment be repeated with several pairs of sensitive and resistant strains in order to confirm the results. Such a situation has already been observed with other bacteria. In *Pseudomonas aeruginosa* mutants resistant to quinolones and cultured in vitro, compensatory mutations occur with a high frequency and help restore competitiveness [11]. For *S. aureus* mutants resistant to rifampicin, the competitiveness of a mutant having a substitution in the sub H481N subunit of RNA polymerase is 80%, but a second mutation in the same gene (S529L) achieves a competitiveness of 99% while increasing the level of resistance [29].

A remarkable evolution was also described in the context of isoniazid resistance of *Mycobacterium tuberculosis* strains: the resistance is related to a mutation in the *kat*G gene which results in the loss of catalase activity and the absence of virulence. However, the majority of resistant isolates carrying this mutation also contain a mutation in the promoter *ahp*C gene, leading to a rise in alkyl hydroxyperoxidase reductase production, which compensates for the loss of catalase and restores the virulence [11].

Unfortunately, the putative compensatory mutations observed in our case concern 2 genes for which the function is not yet known which prevents us from proposing an explanation for this result.

5. Conclusions

The competition between *H. pylori* isolates susceptible to clarithromycin and their resistant mutants, by co-culture over an extended time period, showed in one of the strains that after a long-term co-culture, the susceptible isolate took over the resistant isolate; to the contrary, in the other strain, the resistant isolate remained in the majority. Major changes in the genome were observed in the case of predominant resistant isolate. The disappearance of the resistant isolate following exposure to clarithromycin can be linked to the A2142G mutation in the 23S rRNA gene. But another study with a large pair isolates sensible/resistant is necessary to confirm our results.

The clarithromycin resistance profile was determined using an MIC measure. The start isolate corresponds to isolation of the strains of a co-culture experiment

with AGS cells on Day 0. The end isolate corresponds to Day 40 and Day 30 of a co-culture experiment with AGS cells for strains 3657 and 3695, respectively.

Limitations

This study has several limitations, regarding the small size of our sample pairs due to the difficulty of obtaining both a sensitive and a resistant pair simultaneously in clinical samples.

Conflicts of Interest

The authors had no conflict of interest.

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