**Helicobacter pylori** heteroresistance to clarithromycin in adults—New data by in situ detection and improved concept

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**Funding information**
 Ministry of Human Capacities (Hungary), Grant/Award Number: ÚNKP-18-3-I-SE-44 and ÚNKP-19-3-I-SE-73; Semmelweis University, Budapest, Hungary

**Abstract**

**Background:** Clarithromycin (Cla) heteroresistance of **Helicobacter pylori** (H pylori) infections is commonly assessed by comparing the resistance status of antrum and corpus biopsy samples and by demonstrating the discrepancy between them (inter-niche heteroresistance). However, fluorescence in situ hybridization (FISH) technique is capable of showing the synchronous presence of susceptible and resistant bacteria (intraniche heteroresistance), enabling the detection of heteroresistant **H pylori** populations within one biopsy sample.

**Materials and Methods:** Antrum and corpus biopsy specimens of 305 **H pylori**-infected patients were investigated with an RNA-targeted Cla-resistance FISH test. Anamnestic data were collected from the institutional electronic register. Prevalence rates of susceptible, homo- and heteroresistant cases were correlated with the anamnestic and clinicopathological data.

**Results:** Overall Cla-resistance rate was 23.9% (73 cases), consisting of 35 (11.5%) homo resistant and 38 (12.5%) heteroresistant cases. Thirty-five patients had at least one biopsy site where susceptible and resistant bacteria were present simultaneously. From this subset, 20 cases demonstrated intraniche heteroresistance on both sites. Prior Cla-based eradication attempts were more frequent in homo resistant than in susceptible and heteroresistant cases (P < .001, P < .001, respectively). Cla-containing therapy eradicated heteroresistant infections at a significantly lower rate in comparison with susceptible cases (P = .0112), but more effectively than homoresistants (P = .0393).

**Conclusions:** The most frequent type of Cla-heteroresistance is the coexistence of susceptible and resistant **H pylori** bacteria in the same location (intraniche heteroresistance). A previous Cla-based eradication attempt predisposes patients to homoresistant infection. Heteroresistance is characterized by a non-eradication-related background and intermediate characteristics in many respects when compared to susceptible and homoresistant cases.
1 | INTRODUCTION

The success-rate of Helicobacter pylori (H pylori) eradication therapies has been decreasing worldwide. Clarithromycin (Cla) is a cornerstone of first-line eradication regimens, and the development of resistance mechanisms against this antibiotic is an important factor in the escalation of untreatable infections.

Phenotypic and genotypic methods are available for identifying resistant bacteria. Cultured bacterial isolates can be evaluated with the E-test as the most frequently used method for detecting phenotypic resistance. The polymerase chain reaction (PCR) is the most widely applied technique in identifying bacterial point mutations leading to Cla resistance, both in routine diagnostic microbiology and for research purposes. Another, yet less known, tool for diagnosing genotypic resistance is fluorescence in situ hybridization (FISH), which, in microbiological smears or even in formalin-fixed, paraffin-embedded (FFPE) tissue samples, is capable of showing individual H pylori bacteria carrying point mutations causing Cla resistance.

Resistance can either be a homogeneous attribute within the bacterial population, or individual bacteria can show various responses to an antibiotic, known as heteroresistance. However, heteroresistance is not a well-defined entity and is mainly characterized from a phenotypic perspective. A Cla-homoresistant infection only contains resistant bacteria, while heteroresistance can manifest as either the coexistence of Cla-susceptible and Cla-resistant microbes in the same location (intr niche heteroresistance) or spatially separate, distinct subpopulations with different resistance attributes (susceptible, homoresistant, or mixed), known as inter niche heteroresistance. To assess the prevalence of heteroresistance among H pylori infections, several recent studies used the interniche-only approach since the widely used H pylori Cla-resistance detection methods only give yes or no results as they are not routinely adapted to reveal intraniche heteroresistance. Under these circumstances, heteroresistance was diagnosed if the resistance status of the two gastric regions differed from each other (resistant in antrum vs susceptible in corpus or susceptible in antrum vs resistant in corpus). As intraniche heteroresistance was reported to be frequent, we have found the FISH method to be the most appropriate Cla-susceptibility test for the present study, since this in situ technique is capable of visualizing and thereby identifying the coexistence of susceptible and resistant bacteria, moreover, culture cannot be applied to FFPE tissue blocks and the sensitivity of PCR may be impaired.

Accordingly, we performed a retrospective study on H pylori Cla-resistance data obtained with the diagnostic FISH method to assess the prevalence of heteroresistance, with special emphasis on infections comprising coexisting Cla-susceptible and Cla-resistant bacteria in the same biopsy sample. We aimed to correlate the Cla-resistance status with the anamnestic and clinicopathological data of the patients as well.

2 | METHODS

2.1 Patient selection, clinical data, and ethics

Patients who underwent an upper endoscopy procedure at the 1st Department of Surgery, Semmelweis University or at Ferencváros Health Centre, Budapest during the study period (2005-2017) were selected for this retrospective cross-sectional study. All histopathological work-up was performed at the 2nd Department of Pathology, Semmelweis University, Budapest. Cases with concurrent antrum and corpus biopsies of the same patients were collected from our institutional archive. Inclusion criteria stated that both antrum and corpus biopsy samples were positive for H pylori with immunohistochemistry, and formalin-fixed and paraffin-embedded tissue (FFPE) blocks with sufficient tissue from both sites of the stomach were available for further analyses. The medical records of the patients were reviewed by experienced medical doctors (KE, KI) using the electronic register of Semmelweis University. Cases with insufficient data about prior eradication status were excluded.

Three hundred and five patients met these criteria, including 175 females and 130 males. The mean age was 54.66 years [SD: ±14.88]. Endoscopic findings were recorded in each case (see details below). Prior treatment of H pylori infection was documented in 53 cases (17.38%), including 35 patients (66.04%) with two or more previous eradication therapies. To analyze the success rates of Cla-containing and non-clarithromycin-based H pylori eradication regimens, administered eradication medicines and the results of 6-week control 13C-urea breath tests (UBT) or endoscopic biopsies were also recorded where available. Eradication was considered successful if the UBT test or Helicobacter pylori immunohistochemistry was negative.

The study was designed in accordance with the ethics guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Semmelweis University (#97/2012).

2.2 Routine histologic work-up and assessment

Standardized protocol was followed for the diagnostic work-up of gastric biopsy specimens. Accordingly, two or three antrum and two corpus samples per patient were fixed in 10% buffered formalin. After paraffin embedding, 3- to-4-μm sections were prepared and stained with H&E, and Alcian blue-periodic acid-Schiff. Structural alterations of the gastric tissue were assessed in accordance with the updated Sydney system. Histologic findings were analyzed again by an experienced pathologist (LG) and recorded in each case (see details below).
Immunohistochemistry was used to diagnose and grade *H. pylori* infection status. Three freshly cut 4-μm sections from each antral and corpus biopsy specimen were mounted on a coated glass slide. After deparaffinization and heat pretreatment, tissue samples were incubated with polyclonal rabbit anti-*Helicobacter pylori* primary antibody (clone B0471, Dako, Glostrup, Denmark; 1:350 dilution) for 32 minutes at 42°C, followed by a detection step using the UltraView DAB Detection Kit in the Ventana BenchMark XT automated immunohistochemistry staining system (Ventana Medical Systems, Inc Tucson, AZ, USA). The specimens were counterstained with hematoxylin. The density of the *H. pylori* bacteria was assessed semi-quantitatively on all of the IHC slides according to the updated Sydney system as mild (1+; low density, scattered distribution), moderate (2+; moderate density, small groups of bacteria), or severe (3+; high density, large groups/fields of *H. pylori*) infection.

### 2.3 | Fluorescence in situ hybridization and resistance status

From each biopsy specimen, one 4-μm tissue section was freshly cut. Antral and corpus sections of each case were separately mounted on two different coated glass slides. After deparaffinization, microwave pretreatment was performed at 400 W for 10 minutes in Vector Antigen Unmasking Solution H-3300 (Vector Laboratories, Burlingame, CA, USA). FISH staining was performed by using the BACTFish *H. pylori* Combi Kit (Izinta Kft., Budapest, Hungary) as previously described by Kocsmár et al. In summary, specimens were incubated with DNA hybridization solution containing specific probes to identify *H. pylori* (Hpy-1 targeting 16S-rRNA, labeled with green fluorochrome FITC/fluorescein-isothiocyanate/) and clarithromycin resistance (ClaR1-3 targeting 23S-rRNA, labeled with orange-red fluorescent Cy3) for 90 minutes at 46°C. Posthybridization wash ensued for 15 minutes at 46°C. The slides were mounted with DAPI mounting medium (Vector Laboratories, Burlingame, CA, USA). Specific signals were examined with a Leica DMRXA (Leica Microsystems, Wetzlar, Germany) epifluorescence microscope equipped with DAPI, Spectrum Green and Spectrum Orange filters (Vysis, Downers Grove, IL, USA) throughout the slides. The image documentation was handled with a Leica DFC365 FX monochrome camera and Leica CW4000 FISH software.

In susceptible bacteria, bacterial ribosomes of *H. pylori* are labeled only by the species-specific (Hpy-1) probe (none of the resistance-specific probes is hybridized to the bacterial rRNA). Thus, susceptible *H. pylori* bacteria appear in green in fluorescence microscopy images. Clarithromycin-resistant *H. pylori* bacteria are labeled by both species-specific (Hpy-1) probe and one of the three resistance-specific probes (ClaR1-3). Therefore, Cla-resistant bacteria exhibit yellow fluorescence on multichannel composite images because of the mixed green and red specific signals of the same bacterial cell.

### 2.4 | Investigated parameters and statistical analysis

The variation of the following parameters was investigated: sex (male/female), age (in years), Cla-resistance status (susceptible/heteroresistant/homoresistant/homo-resistant), endoscopic findings (presence/absence of gastroesophageal reflux disease [GERD], erosive gastritis, peptic ulcer, gastric polyp), histologic findings (presence/absence of active chronic gastritis, intestinal metaplasia, gastric carcinoma), bacterial density (1+/2+/3+), activity (presence/absence of active chronic gastritis), structural alterations of gastric mucosa (since the number of the cases showing a specific lesion of the different *H. pylori*-related mucosal changes was too low in our cohort to investigate the individual alterations separately, we collected and handled these together as "structural alterations" of the mucosa, which refers to the presence of any/absence of all of the following: intestinal metaplasia, gastric polyp, mucosal atrophy, dysplasia, gastric carcinoma, ulceration), prior eradication therapy (none/one/two or more), and eradication therapy (clarithromycin-containing regimen/non-clarithromycin-containing regimen: successful/unsuccessful).

All data management and statistical analyses were carried out in R software version 3.5.1. Continuous variables were described as mean, range, and SD, and categorical variables as frequencies. For statistical analysis, the following methods were used: Fisher’s exact test, chi-square test, McNemar’s test, Kruskal-Wallis test and Spearman rank correlation.

All statistical tests were two-sided, and P values were considered significant when P < .05.

### 3 | RESULTS

#### 3.1 | Overall resistance and intragastric Cla-resistance pattern

All of the cases included in this study (n = 305) were analyzed using the FISH method to diagnose their Cla-resistance status. Cla-susceptible infection was diagnosed in 232 cases (76.1%). Cla-resistant *H. pylori* was detected in a total of 73 patients (23.9%), including 65 cases (21.3%) with resistant bacteria in antrum biopsy samples and 64 patients (21%) with resistance-positive bacteria in corpus. From the 73 Cla-resistant cases, 35 homoresistant (11.5%) and 38 heteroresistant (12.5%) infections were found. Intragastric Cla-resistance patterns of the heteroresistant cases (various combinations of antrum and corpus FISH results manifesting in heteroresistance) are shown in Figure 1 and Table 1. Thirty-five patients had at least one biopsy site where susceptible and resistant bacteria were present simultaneously. The Cla-resistance status of antrum and corpus specimens differed in 18 out of 38 heteroresistant patients (47.4%).

#### 3.2 | Interpatient differences of *Helicobacter pylori* infections according to Cla-resistance status

Cla resistance was significantly more frequent in female patients (P < .001, Fisher’s exact test). Significant differences were also observed in gender distribution between susceptible, homo-
heteroresistant cases ($P = .0346, 3 \times 2$ Fisher’s exact test) as the susceptibles were characterized by a balanced male/female ratio, the homoresistant subgroup exhibited strong female predominance, while heteroresistants represent an intermediate group between the two with moderate female predominance (homoresistant-susceptible: $P = .027$, heteroresistant-susceptible and heteroresistant-homoresistant comparisons revealed no significant difference: $P = .165$ and $P = .457$, respectively; Fisher’s exact test). By examining *H pylori*-related alterations (considering both the endoscopic and histologic diagnoses), differences were observed in the prevalence of peptic ulcer disease and GERD depending on the resistance status. The presence of resistance alleles in non-ulcer cases was significantly higher than in patients suffering from peptic ulcer disease ($P = .02513$, Fisher’s exact test) with no difference between homo- and heteroresistant cases. Gastroesophageal reflux disease was significantly more common in Cla-resistant infections ($P = .023$, Fisher’s exact test). Significant differences were also found among susceptible, homo- and heteroresistant subgroups ($P = .045, 3 \times 2$ Fisher’s exact test) as GERD was most frequent in homoresistant cases and least in susceptible ones, while heteroresistants correspond to an intermediate subgroup (homoresistant-susceptible $P = .030$, heteroresistant-susceptible, and heteroresistant-homoresistant differences were not significant in pairwise comparisons: $P = .482$ and $P = .226$, respectively; Fisher’s exact test). The results of the comparisons are shown in Table 2.

### 3.3 Intrapatient differences of *Helicobacter pylori* infections according to Cla-resistance status

Both in the total cohort, in patients infected by susceptible bacteria, and in heteroresistant cases, the bacterial density of antral and corpus tissue samples showed only a weak positive correlation (Table 3). However, the bacterial densities of the two gastric regions were more similar in homoresistant cases as a moderately positive correlation coefficient was found (Table 3). A comparison of cases with $1+ vs 2+/3+$ densities revealed that $2+/3+$ infections are slightly more frequent in the antrum (in the total cohort; Fisher’s exact test, $P = .041$).

Active inflammation was mostly localized to the antrum, resulting in a significant difference between the activities of the two gastric regions, both in the total cohort as well as in the susceptible and heteroresistant cases (Table 3). When activity was present in only one stomach region, antral mucosa was also affected more frequently than corpus, in the total cohort, in susceptible cases, and in heteroresistant infections (Table 3). Cases with active inflammation involving the antral and corpus region simultaneously were more frequent in homoresistant infections in comparison with heteroresistant ones (Table 3). *H pylori*-related mucosal structural alterations showed statistically significant antral predominance in the total cohort, in susceptible, and in homoresistant cases (Table 3). In heteroresistant cases with mucosal structural alterations, no statistically significant difference was identified between the two gastric regions due to the low event numbers of this subgroup, but the trend was similar (Table 3).

Analogous results were observed by investigating cases where structural alteration was found in only one of the two regions: Antral mucosa was also more frequently affected, both in the total cohort, in susceptible infections and in homoresistant cases (Table 3).

### 3.4 Eradication anamnesis of the patients

By analyzing the anamnestic data of our patients, prior eradication therapy was significantly more frequent among both hom- and heteroresistant cases than in susceptible patients ($P < .001$ and $P = .00329$, respectively, $\chi^2$-test). A comparison of hom- and heteroresistant subgroups showed that prior eradication attempts are found significantly more frequently in the anamnesis of patients with homoresistant infections ($P < .001, \chi^2$-test). This tendency was
### TABLE 2 General cohort characteristics as well as endoscopic and histologic findings in the total cohort, clarithromycin susceptible, homo- and heteroresistant cases

|                     | Total        | Susceptible | Homoresistant | Heteroresistant | P       |
|---------------------|--------------|-------------|---------------|-----------------|---------|
|                     | n(%) or median (SD) | n(%) or median (SD) | n(%) or median (SD) | n(%) or median (SD) |         |
| Total               | 305 (100%)   | 232 (76.07%) | 35 (11.48%)   | 38 (12.46%)     |         |
| Sex                 |              |             |               |                 |         |
| Male                | 130 (42.62%) | 108 (46.55%) | 9 (25.71%)    | 13 (34.21%)     | .0346   |
| Female              | 175 (57.38%) | 124 (53.45%) | 26 (74.29%)   | 25 (65.79%)     |         |
| Age (range 21-87)   | 54.66 (±14.88) | 54.83 (±15.27) | 54.74 (±13.08) | 53.55 (±14.29) | .9112   |
| Endoscopic findings |              |             |               |                 |         |
| GERD                | 237 (77.70%) | 173 (74.57%) | 32 (91.43%)   | 32 (84.21%)     | .0448   |
| Erosive gastritis   | 26 (8.52%)   | 22 (9.48%)   | 2 (5.71%)     | 2 (5.26%)       | .6841   |
| Peptic ulcer        | 20 (6.56%)   | 20 (8.62%)   | 0 (0.0%)      | 0 (0.0%)        | .0251   |
| Gastric polyp       | 5 (1.64%)    | 3 (1.29%)    | 0 (0.0%)      | 2 (5.26%)       | .2162   |
| Histologic findings |              |             |               |                 |         |
| Active chronic gastritis | 276 (90.49%) | 211 (90.95%) | 33 (94.29%)   | 32 (84.21%)     | .3526   |
| Intestinal metaplasia | 63 (20.66%) | 52 (22.41%)  | 7 (20.0%)     | 4 (10.53%)      | .2763   |
| Carcinoma           | 3 (0.98%)    | 2 (0.86%)    | 0 (0.0%)      | 1 (2.63%)       | .5613   |

Note: P values correspond to results of 3 × 2 Fisher’s exact test for comparing susceptible, homo- and heteroresistant subgroups, except the age where Kruskal-Wallis test was used.

Abbreviations: GERD, Gastroesophageal reflux disease.

### TABLE 3 Bacterial density, inflammatory activity, and Helicobacter pylori -related mucosal structural alterations in the total cohort as well as in clarithromycin susceptible, homo- and heteroresistant cases (A+/ A−: positive/ negative in the antrum; C+/ C−: positive/ negative in the corpus; only A/ C: the activity or alteration was present only in the antrum/ corpus [identical with A+ C− and A− C+, respectively])

|                     | Total       | Susceptible | Homoresistant | Heteroresistant | P         |
|---------------------|-------------|-------------|---------------|-----------------|-----------|
|                     | n(%) or median (SD) | n(%) or median (SD) | n(%) or median (SD) | n(%) or median (SD) |         |
| Density             |             |             |               |                 |           |
| Antrum              | 1+: 67      | 1+: 54      | 1+: 4         | 1+: 9           | ρ = 0.4657 |
|                     | 2+: 111      | 2+: 81      | 2+: 15        | 2+: 15          | ρ = 0.4488 |
|                     | 3+: 127      | 3+: 97      | 3+: 16        | 3+: 14          | ρ = 0.5867 |
| Corpus              | 1+: 90      | 1+: 70      | 1+: 6         | 1+: 14          | P < .001a |
|                     | 2+: 113      | 2+: 80      | 2+: 12        | 2+: 13          | P < .001a |
|                     | 3+: 102      | 3+: 74      | 3+: 17        | 3+: 11          | P = .006a |
| Activity            |             |             |               |                 |           |
| A+ C+               | 209/305     | 160/232     | 29/35         | 20/38           | P < .001b |
| A− C+               | 10/305      | 7/232       | 1/35          | 0/35            | P = .3711b |
| A+ C−               | 62/305      | 48/232      | 4/35          | 10/38           | P = .0433b |
| A− C−               | 24/305      | 17/232      | 1/35          | 6/38            | P = .0248c |
| Only A              | 62/305      | 48/232      | 4/35          | 10/38           | P = .0433b |
| Only C              | 10/305      | 7/232       | 1/35          | 2/38            | P = .0248c |
| Mucosal alteration  |             |             |               |                 |           |
| A+ C+               | 36/305      | 32/232      | 2/35          | 2/38            | P < .001b |
| A− C+               | 10/305      | 9/232       | 0/35          | 1/38            | P = .0412b |
| A+ C−               | 62/305      | 51/232      | 6/35          | 5/38            | P = .2207b |
| A− C−               | 197/305     | 140/232     | 27/35         | 30/38           | P = .1997c |
| Only A              | 62/305      | 51/232      | 6/35          | 5/38            | P = .025c |
| Only C              | 10/305      | 9/232       | 0/35          | 1/38            | P = .1997c |

*Spearman rank correlation (ρ: correlation coefficient).

**McNemar test.

Fisher’s exact test.*
also confirmed by investigating the number of previous eradication therapies, since two or more prior eradication attempts were found significantly more frequently in both the homo- and heteroresistant subgroups than in susceptible patients (Table 4). More than half of the homoresistant cases (54%) had two or more prior eradication attempts, contrary to the heteroresistant subgroup where it was significantly less, only 21% (Table 4).

3.5 | Eradication rates

Detailed eradication protocol data and the results of control UBT tests or endoscopic biopsies were available in 19 heteroresistant, 20 homoresistant, and 70 Cla-susceptible cases. By analyzing the success rates of Cla-containing H pylori eradication regimens, significant differences were observed between heteroresistant, homoresistant, and susceptible infections (Table 5). H pylori bacteria were successfully eradicated in 93% (57/61) of susceptible cases while, by contrast, Cla-containing therapy failed to cure the infection in the vast majority (85%, 11/13) of the homoresistant subgroup (P < .0001, Fisher exact test). Heteroresistant infections represented an intermediate category since Cla-containing eradication therapy succeeded in the majority (6/10) of these, but it was significantly lower in comparison with susceptible cases (P = .0112, Fisher exact test) and higher than in homoresistant ones (P = .0393, Fisher exact test).

Similar significant differences were not observed among heteroresistant, homoresistant, and susceptible infections treated by non-Cla-containing H pylori eradication regimens, mainly due to the low case numbers (Table 5).

4 | DISCUSSION

We performed a retrospective study on clarithromycin heteroresistance of H pylori infection in previously unsuccessfully eradicated and eradication-naive patients. Our approach included not only the evaluation of Cla-resistance status differences between antrum and corpus samples, but the detection of the simultaneous presence of susceptible and resistant H pylori in the same gastric location as well (intraniche heteroresistance). To our knowledge, this is one of the largest reports on clarithromycin heteroresistance of H pylori and the first that used the FISH method to detect genotypic resistance in adult patients.20

In accordance with other published series, the presence of Cla-susceptible and resistant bacteria in the same host is common.11-16,21 However, the different methodologies applied make it difficult to compare our results on the prevalence of heteroresistance with other studies. Namely, other authors performed susceptibility testing with the E-test or PCR, or combinations thereof.11-16,21 Moreover, in most of these studies, heteroresistance was defined as a difference in antibiotic resistance status between antral and corpus biopsy samples (intraniche-only approach) which omit the possibility of intraniche heteroresistance. However, these studies demonstrated the need to examine both antrum and corpus biopsies by susceptibility testing in general. We used the Cla-susceptibility FISH method, which is capable of showing the simultaneous presence of susceptible and resistant H pylori bacteria within the same biopsy sample, making a more complex diagnosis of heteroresistance possible. In our cohort, heteroresistance was detected in more than half of the resistant cases (38/73; 52%) with a very high prevalence of the intraniche-type heteroresistance (35/38; 92%) which frequently occurred in both the antral and corpus regions of the stomach (20/35; 57%).

Most studies found a lower prevalence of heteroresistance in comparison with ours.12,13,21 The different methodologies should not necessarily provide divergent results since the frequently used PCR and E-test methods give the opportunity to detect bacteria with discrepant resistance status within one sample, as was demonstrated in several studies.22-26 However, this approach did

| TABLE 4 Prevalence of clarithromycin susceptible, homo- and heteroresistant Helicobacter pylori infections in correlation with the eradication anamnesis of the patients |
|---|---|---|---|
| | Susceptible | Homoresistant | Heteroresistant |
| No previous eradication therapy | 212 (91.38%) | 12 (34.29%) | 28 (73.68%) |
| Susc-Homo | P < .001 | CI of OR: 8.16-51.12 |
| Susc-Hetero | P = .0036 | CI of OR: 1.42-9.48 |
| Homo-Hetero | P < .001 | CI of OR: 0.06-0.57 |
| One previous eradication therapy | 12 (5.17%) | 4 (11.43%) | 2 (5.26%) |
| Susc-Homo | P = .2403 | CI of OR: 0.12-1.92 |
| Susc-Hetero | P = 1.0000 | CI of OR: 0.21-9.40 |
| Homo-Hetero | P = .4177 | CI of OR: 0.31-27.02 |
| Two or more previous eradication therapies | 8 (3.45%) | 19 (54.29%) | 8 (21.05%) |
| Susc-Homo | P < .001 | CI of OR: 0.01-0.09 |
| Susc-Hetero | P < .001 | CI of OR: 0.04-0.45 |
| Homo-Hetero | P = .004 | CI of OR: 1.44-14.31 |

Abbreviations: CI of OR, confidence interval of odds ratio; Hetero, clarithromycin heteroresistance; Homo, clarithromycin homoresistance; Susc, clarithromycin susceptible infection.
TABLE 5  Eradication rates depending on the clarithromycin resistance status of the Helicobacter pylori infection

|                     | Successful eradication | Unsuccessful eradication | Comparisons (Fisher’s exact test) | CI of OR       |
|---------------------|------------------------|--------------------------|-----------------------------------|----------------|
|                     | Cla-containing regimen |                         |                                   |                |
| Homoresistant       | 2                      | 11                       | Homo-Hetero: \( P = 0.0393 \)      | 1.1535 59.0051 |
| Heteroresistant     | 6                      | 4                        | Hetero-Susc: \( P = 0.0112 \)      | 0.0208 0.5326  |
| Susceptible         | 57                     | 4                        | Homo-Susc: \( P < 0.0001 \)        | 0.0021 0.0784  |
|                     |                        |                          |                                   |                |
|                     | Non-Cla regimen        |                         |                                   |                |
| Homoresistant       | 3                      | 4                        | Homo-Hetero: \( P = 1.0000 \)      | 0.0867 5.1272  |
| Heteroresistant     | 3                      | 6                        | Hetero-Susc: \( P = 0.3469 \)      | 0.0352 1.7748  |
| Susceptible         | 6                      | 3                        | Homo-Susc: \( P = 0.6145 \)        | 0.0488 2.8841  |

Abbreviations: CI of OR, confidence interval of odds ratio; Hetero, clarithromycin heteroresistance; Homo, clarithromycin homoresistance; Susc, clarithromycin susceptible infection.

not become routine practice in microbiological diagnostic laboratories since the general clinical view only requires a susceptible-resistant discrimination of the cases. Accordingly, many others assessed heteroresistance with the interniche-only approach, which underestimates the heteroresistance rates. This may explain why our study revealed a higher prevalence, in accordance with others using methods capable of detecting intraniche heteroresistance, too.

Conversely, one interesting question emerges: How many heteroresistant cases do we miss with methods omitting the intraniche type? We found 21/38 (55%) heteroresistant cases in which no susceptible infection was detected at any site, but one or both regions contained a mixed population of susceptible and resistant bacteria (antrum/corpus resistance status: 1 homo/hetero, 0 hetero/homo, 20 hetero/hetero). Therefore, interniche-only methodologies can reduce the rate of heteroresistance by more than 50%.

We have also experienced in clinical practice that in certain cases, endoscopic biopsy sampling was only performed from the antral region, or the Cla-susceptibility test was only ordered from the antral tissue sample. Accordingly, we examined how many resistance/heteroresistance cases we might miss with a Cla-susceptibility test using antral samples only. In total, 8/38 (21%) heteroresistant cases exhibited an antral susceptible/corpus resistant combination (including two homo- and six heteroresistant corpus samples), suggesting that one-fifth of the resistant cases can remain hidden with the antrum-only approach. Conversely, a similar proportion, nine antral resistant/corpus susceptible heteroresistant cases were found (24%; including 1 homo- and 8 heteroresistant antral samples). This finding contradicts some earlier studies in which the Cla-resistance rate was found to be different between regions of the stomach (higher in corpus, higher in fundus, or in antrum).

In other respects, however, we observed gastric regional differences (Table 3, Table S1). Antral and corpus bacterial densities showed a weak/moderate positive correlation, but slightly more 2+/3+ antral infections were found in the total cohort. Though active gastritis was commonly found on both sites, it was significantly more frequent in the antrum if only one region was affected. *H pylori*-related mucosal alterations (including atrophy, intestinal metaplasia) also occurred predominantly in the antral region. These observations fit well with the generally accepted concept that *H pylori* infection and its complications primarily affect the antrum. However, Cla-resistance status-related regional differences were also observed as active gastritis showed antral predominance in the susceptible and heteroresistant cases, but not in homoresistant ones. By explaining this, we found that homoresistant infections more frequently caused active inflammation simultaneously on both sites of the stomach, and the highest correlation between antral and corpus bacterial densities in homoresistant cases. As the eradication anamnesis data suggest, homoresistance is most probably the consequence of previous unsuccessful eradication attempts. Therefore, homoresistant bacterial populations may represent *H pylori* infections with increased fitness resulting in a higher colonization and bacterial density of corpus. Moreover, repeated eradication attempts and the related long-term proton-pump inhibitor use may result in suppression of acid production in the corpus, which can also contribute to the spread of *H pylori* from the antrum to the corpus and consequently a shift from antral- to corpus-predominant gastritis and atrophy. However, in our cohort, no association was observed between increased corpus colonization and atrophy, at least partly due to the low number of cases. As we have previously shown, higher bacterial densities correlate directly with the occurrence of active gastritis explaining the more frequent corpus activity in our homoresistant cases.

We have also found resistance status-dependent differences regarding the prevalence of gastroesophageal reflux disease and peptic ulcers. GERD was most frequently observed in homoresistant cases and less prevalent among susceptible ones, while patients with heteroresistant infections displayed an intermediate rate of this disease. By contrast, peptic ulcers were exclusively found in susceptible cases. This contradicts previously published data since others observed no statistically significant difference between ulcer patients with Cla-susceptible and Cla-resistant *H pylori* infection.

Several studies proved that females exhibit a higher prevalence of Cla resistance, which is also strongly supported by our results. However, our data refines this picture, as the discrepancy between males and females was highest in homoresistant cases, with correspondingly high frequency of two or more prior eradication treatments.
less in heteroresistants and most balanced in susceptible ones, where no previous eradication therapy was typically found. This suggests that homoresistance is mostly caused by unsuccessful eradication attempts, whereas heteroresistance seems to have a non-eradication-related background. This latter factor can be due to either the use of clarithromycin for non-eradication purposes, or multiple infections.\textsuperscript{11-16,21,35} Heteroresistance caused by multiple infections is characteristic of populations with a simultaneously high prevalence of \textit{H pylori} and Cla resistance, but not for Hungary.\textsuperscript{25} Moreover, several studies demonstrated higher macrolide consumption among females including both eradication and use for other purposes.\textsuperscript{36,37} Thus besides methodological differences and possible geographical variations, higher previous clarithromycin consumption in females for non-eradication purposes may be a further factor contributing to the higher heteroresistance rate experienced by our cohort in comparison with other studies.\textsuperscript{12,13,14,21}

There is no consensus on the optimal eradication protocol of heteroresistant \textit{H pylori} infections. Our results, in agreement with published data from De Francesco et al\textsuperscript{26} indicate that heteroresistant cases represent a distinct subgroup with an intermediate cure rate of \textit{H pylori} infection when treated by a clarithromycin-containing eradication regimen (Table 5). Namely, compared to homoresistant infections in which clarithromycin is inefficient, the majority of the Cla-heteroresistant cases were successfully eradicated by using Cla-containing therapeutic protocols, but with a higher failure rate than in susceptible patients where Cla-based therapy is outstanding. As non-Cla-containing protocols can also fail the eradication in heteroresistant cases, further investigations are needed to clarify the exact role of clarithromycin and other antibiotics in the treatment of Cla-heteroresistant infections including the potential efficacy of clarithromycin-containing quadruple therapies. Accordingly, further discussions and studies would be desirable on the optimal therapeutic strategies for the heteroresistant patients who represent about half of the clarithromycin-resistant cases.

An additional limitation is that our study is retrospective, and despite the relatively high number of involved patients, sequentially splitting the cohort along different aspects resulted in low event numbers in the final sub-subgroups, as manifested in no relevant results or low statistical power in certain comparisons. Another limitation is that genetic clarithromycin susceptibility tests (including FISH) detecting the three most frequent point mutations of 23S rRNA are unable to diagnose clarithromycin resistance caused by uncommon genetic mechanisms.\textsuperscript{28-41} On the other hand, point mutations detected by genetic methods do not always manifest in phenotypic resistance.\textsuperscript{26,40,42,43} However, Jüttner et al\textsuperscript{44} proved that the Cla-susceptibility FISH method and E-test provide nearly identical results in gastroenterology practice, moreover, heteroresistance-related sampling errors can be responsible for the few discrepant cases.

In summary, heteroresistant \textit{H pylori} infections represent a distinct subgroup which displays intermediate characteristics in many regards as compared to the susceptible and homoresistant cases. Heteroresistance is associated with a non-eradication-related background, and the majority of these infections can be eradicated by clarithromycin-containing regimens as well. On the contrary, homoresistance is mostly caused by prior unsuccessful eradication attempts and, therefore, may represent \textit{H pylori} infections with increased fitness resulting in a higher corpus colonization. Susceptible and homoresistant/heteroresistant \textit{H pylori} subpopulations may exist simultaneously at different sites of the stomach (intrachore heteroresistance). Hence, multiple sampling is necessary and the clarithromycin susceptibility testing of each \textit{H pylori} positive antral and corpus biopsy specimen is recommended to avoid false-susceptible results. We can also conclude that the correct and accurate determination of resistance status requires methods capable of identifying the coexistence of Cla-susceptible and Cla-resistant bacteria in the same sample (intrachore heteroresistance). Finally, our results can help further tailor the strategy of the eradication therapies. This is true especially in regions where the clarithromycin resistance rate exceeds 15\% but—as in our cohort—the majority of the infections are still susceptible and these patients can benefit from clarithromycin-containing regimens by using appropriate susceptibility testing. Moreover, homoresistant cases certainly need non-clarithromycin-based protocols. Heteroresistant \textit{H pylori} infections shall be reported as resistant to clarithromycin, and non-clarithromycin-containing regimens are still the first-choice treatment preferences for these patients. Nevertheless, personalized therapeutic options may be considered in certain heteroresistant cases, including the additional use of clarithromycin as well. However, further studies would be desirable to determine the most effective eradication strategies of heteroresistant \textit{H pylori} infections since these represent about half of the clarithromycin-resistant cases.

ACKNOWLEDGEMENTS

This study was partly supported by The New National Excellence Program (ÚNKP-18-3-1SE-44 and ÚNKP-19-3-1SE-73) of the Ministry of Human Capacities (Hungary) as well as with a Start-up grant of Semmelweis University, Budapest, Hungary.

DISCLOSURES OF INTERESTS

The authors declare no conflict of interest.

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REFERENCES

1. Yeo YH, Shiu S-I, Ho HJ, et al. First-line Helicobacter pylori eradication therapies in countries with high and low clarithromycin resistance: a systematic review and network meta-analysis. Gut. 2018;67(1):20-27.
2. Malfertheiner P, Megraud F, O’Morain CA, et al. Management of Helicobacter pylori infection—the Maastricht V/Florence Consensus Report. Gut. 2017;66(1):6-30.
3. Megraud F. H pylori antibiotic resistance: prevalence, importance, and advances in testing. Gut. 2004;53(9):1374-1384.
4. Graham DY, Fischbach L. Helicobacter pylori treatment in the era of increasing antibiotic resistance. Gut. 2010;59(8):1143-1153.
5. Thung I, Aramin H, Vavinskaya V, et al. Review article: the global emergence of Helicobacter pylori antibiotic resistance. Aliment Pharmacol Ther. 2016;43(4):514-533.
6. Mégraud F, Lehour P, Helicobacter pylori detection and antimicrobial susceptibility testing. Clin Microbiol Rev. 2007;20(2):280-322.
7. Monno R, Giorgio F, Carmine P, Soleo L, Cinquepalmi V, Ierardi E. Helicobacter pylori clarithromycin resistance detected by Etest and TaqMan real-time polymerase chain reaction: a comparative study. APMIS. 2012;120(9):712-717.
8. Trebesius K, Pantelik K, Strobel S, et al. Rapid and specific detection of Helicobacter pylori macrolide resistance in gastric tissue by fluorescent in situ hybridisation. Gut. 2000;46(5):608-614.
9. El-Halwawy OM, Valvano MA. Antimicrobial heteroresistance: an emerging field in need of clarity. Clin Microbiol Rev. 2015;28(1):191-207.
10. Matteo MJ, Granados G, Olmos M, Wonaga A, Catalano M. Helicobacter pylori amoxicillin heteroresistance due to point mutations in PBP-1A in isogenic isolates. J Antimicrob Chemother. 2008;61(3):474-477.
11. Farzi N, Behzad C, Hasani Z, Aleeouyeh M, Zojaji H, Zali MR. Characterization of clarithromycin heteroresistance among Helicobacter pylori strains isolated from the antrum and corpus of the stomach. Folia Microbiol. 2019;64(2):143-151.
12. Bilgili C, Stadlima A, Makristhatis A, et al. Prospective multicentre clinical study on inter- and intrapatient genetic variability for antimicrobial resistance of Helicobacter pylori. Clin Microbiol Infect. 2018;24(3):267-272.
13. Selgrad M, Tammer I, Langner C, et al. Different antibiotic susceptibility between antrum and corpus of the stomach, a possible reason for treatment failure of Helicobacter pylori infection. World J Gastroenterol. 2014;20(43):16245-16251.
14. Marzio L, Cellini L, Amitrano M, et al. Helicobacter pylori isolates from proximal and distal stomach of patients never treated and already treated show genetic variability and discordant antibiotic resistance. Eur J Gastro Hepatol. 2011;23(6):467-472.
15. Rimbara E, Noguchi N, Tanabe K, Kawai T, Matsumoto Y, Sasatsu M. Susceptibilities to clarithromycin, amoxicillin and metronidazole of Helicobacter pylori isolates from the antrum and corpus in Tokyo, Japan, 1995–2001. Clin Microbiol Infect. 2005;11(4):307-311.
16. Kim JJ, Kim JG, Kwon DH. Mixed-infection of antibiotic susceptible and resistant Helicobacter pylori isolates in a single patient and underestimation of antimicrobial susceptibility testing. Helicobacter. 2003;8(3):202-206.
17. Rüssmann H, Adler K, Haas R, Gebert B, Koletzko S, Heesemann J. Rapid and accurate determination of genotypic clarithromycin resistance in cultured Helicobacter pylori by fluorescent in situ hybridization. J Clin Microbiol. 2001;39(11):4142-4144.
18. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney system. International workshop on the histopathology of gastritis, Houston 1994. Am J Surg Pathol. 1996;20(10):1161-1181.
19. Kocsmary É, Szirtes I, Kramer Z, et al. Sensitivity of Helicobacter pylori detection by Giemsa staining is poor in comparison with immunohistochemistry and fluorescent in situ hybridization and strongly depends on inflammatory activity. Helicobacter. 2017;22(4):e12387.
20. Caristo E, Parola A, Rapa A, et al. Clarithromycin resistance of Helicobacter pylori strains isolated from children’ gastric antrum and fundus as assessed by fluorescent in-situ hybridization and culture on four-sector agar plates. Helicobacter. 2008;13(6):557-563.
21. Kao C-Y, Lee A-Y, Huang A-H, et al. Heteroresistance of Helicobacter pylori from the same patient prior to antibiotic treatment. Infect Genet Evol. 2014;23:196-202.
22. Maeda S, Yoshida H, Matsunaga H, et al. Detection of clarithromycin-resistant Helicobacter pylori strains by a preferential homoduplex formation assay. J Clin Microbiol. 2000;38(1):210-214.
23. van der Ende A, van Doorn L J L, Rooijakers S, Feller M, Tytgat GNJ, Dankert J. Clarithromycin-susceptible and -resistant Helicobacter pylori isolates with identical randomly amplified polymorphic DNA-PCR genotypes cultured from single gastric biopsy specimens prior to antibiotic therapy. J Clin Microbiol. 2001;39(7):2648-2651.
24. Buruca G, Garnier M, Silvain C, Fauchère J-L. Quadruplex real-time PCR assay using allele-specific specification primers for detection of mutations conferring clarithromycin resistance to Helicobacter pylori. J Clin Microbiol. 2008;46(7):2320-2326.
25. Sun L, Talarico S, Yao L, et al. Droplet digital PCR-based detection of clarithromycin resistance in Helicobacter pylori isolates reveals frequent heteroresistance. J Clin Microbiol. 2018;56(9):e00019-e118.
26. De Francesco V, Zullo A, Ierardi E, et al. Phenotypic and genotypic Helicobacter pylori clarithromycin resistance and therapeutic outcome: benefits and limits. J Antimicrob Chemother. 2010;65(2):327-332.
27. Ayala G, Galván-Portillo M, Chihu L, et al. Resistance to antibiotics and characterization of Helicobacter pylori strains isolated from antrum and body from adults in Mexico. Microb Drug Resist. 2011;17(2):149-155.
28. Yousfi MM, Reddy R, Osato MS, Graham DY. Is Antrum or Corpus the Best Site for Culture of Helicobacter pylori? Helicobacter. 1996;1(2):88-91.
29. Moayyedi P, Wason C, Peacock R, et al. Changing patterns of Helicobacter pylori gastritis in long-standing acid suppression. Helicobacter. 2000;5(4):206-214.
30. Duck WM, Sobel J, Prucker JM, et al. Antimicrobial resistance incidence and risk factors among Helicobacter pylori-infected persons, United States. Emerg Infect Dis. 2004;10(6):1088-1094.
31. De Francesco V, Margiotta M, Zullo A, et al. Prevalence of primary clarithromycin resistance in Helicobacter pylori strains over a 15 year period in Italy. J Antimicrob Chemother. 2007;59(4):783-785.
32. Xia HX, Buckley M, Keane CT, O’Morain CA. Clarithromycin resistance in Helicobacter pylori: prevalence in untreated dyspeptic patients and stability in vitro. J Antimicrob Chemother. 1996;37(3):473-481.
33. De Francesco V, Giorgio F, Ierardi E, et al. Primary clarithromycin resistance in Helicobacter pylori: the Multicentric Italian Clarithromycin Resistance Observational (MICRO) study. J Gastrointestin Liver Dis. 2011;20(3):235-239.
34. Fasciana T, Calà C, Bonura C, et al. Resistance to clarithromycin and genotypes in Helicobacter pylori strains isolated in Sicily. J Med Microbiol. 2015;64(11):1408-1414.
35. Ben Mansour K, Fendri C, Battik H, et al. Multiple and mixed Helicobacter pylori infections: comparison of two epidemiological situations in Tunisia and France. Infect Genet Evol. 2016;37:43-48.
36. Schröder W, Sommer H, Gladstone BP, et al. Gender differences in antibiotic prescribing in the community: a systematic review and meta-analysis. J Antimicrob Chemother. 2016;71(7):1800-1806.
37. Megraud F, Coenen S, Versporten A, et al. Helicobacter pylori resistance to antibiotics in Europe and its relationship to antibiotic consumption. Gut. 2013;62(1):34-42.
38. Liu M, Douthwaite S. Activity of the ketolide telithromycin is refractory to Erm monomethylation of bacterial tRNA. Antimicrob Agents Chemother. 2002;46(6):1629-1633.
39. Webber MA, Piddock LJ. The importance of efflux pumps in bacterial antibiotic resistance. J Antimicrob Chemother. 2003;51(1):9-11.
40. Mamelli L, Amoros J-P, Pagès J-M, Boilla J-M. A phenylalanine-arginine β-naphthylamide sensitive multidrug efflux pump involved in intrinsic and acquired resistance of Campylobacter to macrolides. *Int J Antimicrob Agents*. 2003;22(3):237-241.

41. Kocazeybek B, Sakli MK, Yuksel P, et al. Comparison of new and classical point mutations associated with clarithromycin resistance in *Helicobacter pylori* strains isolated from dyspeptic patients and their effects on phenotypic clarithromycin resistance. *J Med Microbiol*. 2019;68(4):566-573.

42. Oleastro M, Ménard A, Santos A, et al. Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in *Helicobacter pylori*. *J Clin Microbiol*. 2003;41(1):397-402.

43. Morris JM, Reasonover AL, Bruce MG, et al. Evaluation of sea-FAST, a rapid fluorescent in situ hybridization test, for detection of *Helicobacter pylori* and resistance to clarithromycin in paraffin-embedded biopsy sections. *J Clin Microbiol*. 2005;43(7):3494-3496.

44. Jüttner S, Vieth M, Miehlke S, et al. Reliable detection of macrolide-resistant *Helicobacter pylori* via fluorescence in situ hybridization in formalin-fixed tissue. *Mod Pathol*. 2004;17(6):684-689.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Kocsmár É, Kocsmár I, Buzás GM, et al. *Helicobacter pylori* heteroresistance to clarithromycin in adults—New data by in situ detection and improved concept. *Helicobacter*. 2019;00:e12670. [https://doi.org/10.1111/hel.12670](https://doi.org/10.1111/hel.12670)