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In vitro efficacy of combinations of antibiotics used in clinical practice on clinical isolates of *Helicobacter pylori*

Zahyra Kaouah¹ | Julien M. Buyck¹ | Maxime Pichon^{1,2} | Christophe Burucoa^{1,2}  |
 Laure Prouvensier¹ | Jeremy Moreau¹ | Sandrine Marchand^{1,3} | Julie Cremniter^{1,2}  |
 Nicolas Grégoire^{1,3} 

¹PHAR2, Inserm U1070, Université de Poitiers, Poitiers, France

²Département des Agents Infectieux, CHU de Poitiers, Poitiers, France

³Laboratoire de Toxicologie et de Pharmacocinétique, CHU de Poitiers, Poitiers, France

Correspondence

Nicolas Grégoire, Université de Poitiers, Pôle Biologie Santé, Bâtiment B36/B37, 1 Rue Georges Bonnet, TSA 51106-86073 Poitiers Cedex 9, France.
 Email: nicolas.gregoire@univ-poitiers.fr

Abstract

Background: The main antibiotics used against *Helicobacter pylori* have been chosen empirically over time, with few preclinical studies to provide support. The rise in resistance to some of these antibiotics is prompting a reassessment of their use. This work aimed to evaluate the in vitro efficacy of 2×2 combinations of the most widely used antibiotics against *H. pylori*.

Materials and Methods: J99 reference strains and 19 clinical isolates of *H. pylori* with various antibiotic resistance phenotypes were used. Minimum inhibitory concentrations were carried out using the microdilution method in 96-well plates. The activity of 15 possible combinations of two antibiotics including amoxicillin, clarithromycin (CLA), levofloxacin, rifampicin, tetracycline, and metronidazole was determined for all strains by the checkerboard method. A mean fractional inhibitory concentration index (FIC_{mean}) was calculated for each combination and strain and the type of pharmacodynamic interaction was considered as synergic if FIC_{mean} ≤ 0.5, additive if 0.5 < FIC_{mean} ≤ 1, indifferent if 1 < FIC_{mean} < 4 or antagonistic if FIC_{mean} ≥ 4.

Results: Most of the 285 pharmacodynamic interactions tested with clinical strains were close to additivity (average FIC_{mean} = 0.89 [0.38–1.28]). No interaction was found to be antagonistic. When two antibiotics to which a strain was resistant were combined, the concentrations required to inhibit bacterial growth were higher than their respective breakpoints.

Conclusion: The present results have shown that in vitro, the different antibiotics used in therapeutics have additive effects. The addition of the effects of two antibiotics to which a strain was resistant was not sufficient to inhibit bacterial growth. In probabilistic treatment, the choice of antibiotics to combine should therefore be based on the local epidemiology of resistance, and on susceptibility testing in the case of CLA therapy, so that at least one antibiotic to which the strain is susceptible is used.

KEYWORDS

antibiotic combinations, *Helicobacter pylori*, resistance

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1 | INTRODUCTION

Helicobacter pylori is a major human pathogen that colonizes the surface of the gastric epithelium.¹ The global prevalence of *H. pylori* is reaching 43.9% of the whole human population.² The consequences of *H. pylori* infections have been associated with the development of different gastrointestinal diseases, such as gastric ulcers, gastric cancer, mucosa-associated lymphoid tissue and lymphoma, and biliary tract cancer.^{3–5} *H. pylori* is the only bacterium classified as a class 1 carcinogen, with pathogen eradication resulting in a reduction of cancer risk.⁶

Helicobacter pylori infection usually persists for life and can be cleared only by antibiotic eradication treatment.⁷ Anti-*H. pylori* therapy involves multidrug regimens that consist of at least two antibiotics and proton pump inhibitors (PPI) with or without bismuth.^{8,9} A few anti-infective agents can be used against *H. pylori* including amoxicillin (AMX), clarithromycin (CLA), metronidazole (MTZ), tetracycline (TET), levofloxacin (LEV), and rifampicin (RIF).¹⁰

Treatment of primary *H. pylori* infections is largely probabilistic; however, susceptibility-guided therapy is recommended after initial treatment failures or in the case of CLA-containing therapy.⁹ In 2018, it was shown by a multicountry European study that, the increasing failure rate of eradication treatment due to the appearance of resistant *H. pylori* strains contributes to the worldwide prevalence of this infection. The *H. pylori* resistance rates were 21% for CLA, 16% for LEV, and 39% for MTZ and were significantly higher in Central/Western and Southern than in the Northern European countries.¹¹

The therapeutic arsenal has been built up empirically over time and few in vitro studies have been conducted to confirm the rationale for the treatments currently used, and the emergence of resistance prompts a reassessment of this arsenal.¹² This work aimed to evaluate the in vitro efficacy of 2×2 combinations with AMX, LEV, CLA, RIF, TET, and MTZ.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

The reference strain J99, susceptible for all the antibiotics tested, was used.¹³ Nineteen clinical isolates were selected to cover a variety of phenotypes. The clinical isolates were recovered from gastric biopsies from patients of Poitiers university hospital, department of infectious agents (the age range was 30–70 years), frozen at –80°C in a mixture of nutritive broth (Oxoid, Basingstoke Hampshire, UK) with 50% glycerol after routine analysis. Isolates were identified as *H. pylori* based on spiral morphology observed after Gram staining and positive activities for urease, catalase, and oxidase. Their antibiotic susceptibility was routinely tested by E-test (bioMérieux, Marcy l'Etoile, France) and interpreted in accordance to the recommendations of the European Committee for Antimicrobial Susceptibility Testing (EUCAST).¹⁴ After thawing, all the clinical isolates were

grown on Campylobacter agar consisting in Columbia agar plates supplemented with 5% sheep blood and Skirrow antibiotic supplement (Oxoid, Basingstoke Hampshire, United Kingdom) under microaerobic conditions by using the Genbox Microaer (bioMérieux, Marcy l'Etoile, France) in an anaerobic jar at 37°C for 24 to 48 h. For minimum inhibitory concentrations (MICs) and checkerboard experiments by microdilution, an inoculum was prepared by resuspending colonies from Columbia agar plates in Brucella Broth (Condalab, Madrid, Spain) supplemented with 10% of inactivated fetal calf serum (eurobio Scientific, les Ulis, France) (BB10%) to reach an optical density of 0.4 (corresponding to approximately 10⁷ CFU/mL).¹⁵

2.2 | Minimum inhibitory concentrations (MICs)

Minimum inhibitory concentrations were determined according to EUCAST recommendations using the microdilution method. Twofold dilutions in BB10% of all antibiotics were performed in 100 μL of BB10% to obtain a final range of concentrations until 256 mg/L. The initial bacterial inoculum was adjusted to 10⁶ CFU/mL, then 100 μL were transferred to each well to reach a final inoculum concentration of approximately 5×10⁵ CFU/mL. The plates contained also positive and negative controls, corresponding to BB10% with bacteria and BB10% alone, respectively. Plates were incubated in a microaerobic atmosphere at 37°C for 24–48 h under shaking at 150 rpm. The experiments were carried out in duplicate and the phenotypic status was interpreted according to the Breakpoint values defined by the EUCAST¹⁴: clinical isolates were classified as susceptible when the MIC of AMX was ≤0.125 mg/L, CLA ≤0.25 mg/L, LEV ≤1 mg/L, TET ≤1 mg/L, RIF ≤1 mg/L, and MTZ ≤8 mg/L.

2.3 | Checkerboard assay

A two-dimensional, two-agent broth microdilution checkerboard titration method with 96-well plates was used to study the interaction between antibiotics.¹⁶ The antibiotics of the combination were 1:2 serially diluted in 100 μL of BB10%, after which 100 μL of a bacterial suspension corresponding to 10⁶ CFU/mL was added to each well. For each antibiotic, the concentrations tested ranged from 0.125 to 8 times the MIC. The plates were incubated for 48 h under microaerobic conditions at 37°C and shaking. Interactions between antibiotics were then evaluated using the fractional inhibitory concentration (FIC) index as follows.

$$FIC_i = FIC_A + FIC_B = \frac{MIC_{A/B}}{MIC_A} + \frac{MIC_{B/A}}{MIC_B}, \quad (1)$$

where, MIC_A was the MIC of A in the absence of B, MIC_B was the MIC of B in the absence of A, MIC_{A/B} was the MIC of A in the presence of B and MIC_{B/A} was the MIC of B in the presence of A (Figure S1).¹⁷

A FIC_{mean} was calculated for each combination and bacterial strain by averaging the FIC_i values from different ratio of concentrations of antibiotics A and B for each well without bacterial growth

adjacent to a well with visible bacterial growth¹⁸ (Figure S1). The combination was considered synergistic for $FIC_{mean} \leq 0.5$; additive for $0.5 < FIC_{mean} \leq 1$; indifferent for $1 < FIC_{mean} < 4$; and antagonistic for $FIC_{mean} \geq 4$, according to EUCAST definition.¹⁷

2.4 | Whole genome sequencing

Clinical strains of *H. pylori* were subcultured twice on Campylobacter agar plates (24–48 h incubation in microaerobic conditions at 37°C) prior to DNA extraction using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany) followed by quantification using a Nanodrop One (Thermo Fisher Scientific). Extracted DNA (2 µg) was prepared using the NEBNext Companion Module for Oxford Nanopore Technologies (New England Biolabs, Ipswich, MA, USA) and then encoded using the Native Barcoding Expansion EXP-NBD114 kit (ONT, Oxford, UK). 1.5 µg of barcoded DNA was ligated using the SQK-LSK109 ligation sequencing kit (ONT) with enrichment for fragments ≥ 3 kb and purification using NucleoMag NGS Clean-up and Size Select magnetic beads (Macherey-Nagel, Düren, Germany). The library was loaded onto a FLO-MIN106 R9.4.1 flow cell (ONT) and sequenced with a MinION Mk1b (ONT) for 24 h. Base calling was performed using a high-precision template and (de-multiplexed, barcoded and cut adapter) using guppy. Using filtlong (v0.2.1), the worst 5% of reads or ≤ 1 kb were removed and assembly was performed using flye (v2.9-b1769).^{19,20} Medaka (v1.6.0) and Kleborate (v2.2.0) were used for final polishing and MLST.²¹ Prokka (v1.14.6) and Snippy (v4.6.0) were used to annotate and compare the genomes respectively.^{22,23} Results were visualized using Geneious prime software (v2022.0.1). Sequences of antibiotic resistance-related genes were retrieved from the annotated genome of *H. pylori* 26,695 (accessible at GenBank accession number CP003904.1), considering only mutations in genes associated with ≥ 1 resistance.^{9,24} Sequences from this study are available under SRA accession number PRJNA1013907.

2.5 | Antibiotics and reagents

Antibiotics (i.e., AMX, CLA, LEV, RIF, TET, and MTZ) were purchased from Merck–Sigma-Aldrich (Saint-Quentin-Fallavier, France). Stock solutions were prepared in appropriate solvent according to the manufacturer's recommendation and frozen at -20°C .

3 | RESULTS

3.1 | Phenotypic and genotypic resistance of *H. pylori* strains

Results of the susceptibility testing are presented in Table 1. Eight of nineteen (8/19; 42%) isolates were susceptible to all antibiotics tested, and seven were resistant to one antibiotic (7/19, 37%). Four

(4/19; 21%) were resistant to more than one antibiotic (C8, C10, C22, and C23).

The isolate C22 was multiresistant to all antibiotics except RIF and AMX. However, its MIC to AMX was equal to clinical breakpoint (0.125 mg/L), as C4 and C10 isolates.

Four isolates were resistant to CLA. Within the resistance genes evaluated (23S rDNA, rpl22 and in infB genes), only the A2143G mutation (23S rDNA gene) was found, in each of the four isolates.

Five isolates were resistant to LEV and showed resistance-associated mutations in the gyrA a quinolone resistance-determining region (QRDR): N87I or D91(Y/N).^{25,26} No described mutations for the gyrB gene have been detected.

Seven isolates were resistant to MTZ and had mutations in the rdxA gene (but these mutations have also been observed for some susceptible isolates) (data not shown).

The multiresistant isolate (C22) was also the only one resistant to TET and had the corresponding mutation AGA926-928TTC in 16S rDNA gene.²⁶

No isolate was resistant to RIF.

3.2 | Checkerboards assays

The values of FIC_{mean} are presented in Table 2. Interactions were mainly additive or indifferent ($0.5 < FIC_{mean} < 4$), although synergies ($FIC_{mean} \leq 0.5$) were observed sporadically for some strains and combinations of antibiotics. No interaction was found to be antagonistic for any strain ($FIC_{mean} \geq 4$).

The mean of FIC_{mean} for the 19 isolates showed that the MTZ/RIF combination had the lowest FIC_{mean} values ($=0.64$), while the LEV/AMX combination had the worst ($=1.04$). When considering the whole combinations, combinations containing MTZ showed the lower range of FIC_{mean} (0.64–0.93) whereas the combinations with LEV showed the highest range of FIC_{mean} (0.95–1.04). Concerning the multiresistant strain C22, the combination of the AMX with either CLA, RIF, or TET showed a FIC_{mean} close to or even synergistic. Moreover, there was no relationship between the resistance profile of the isolate and the type of pharmacodynamic interaction (no more synergy for strains resistant to at least one antibiotic, highlighted in gray).

To present the interactions for each pair of concentrations, FIC_B has been plotted as a function of FIC_A (Figure 1) for the 19 *H. pylori* isolates and J99 reference strain. Isoles corresponding to the limit defining synergy ($FIC_A + FIC_B = 0.5$) and additivity ($FIC_A + FIC_B = 1$) have been represented. This representation showed that for some strains and some pairs of concentrations the interactions were synergistic (below the green isobole) although FIC_{mean} (Table 2) corresponded to an additive interaction (e.g., combination RIF/MTZ). Note that for some strains and antibiotic concentrations, the points lied above the blue additivity line (e.g., combination CLA/LEV). These cases corresponded to FIC_B values of 1 ($MIC_{B/A}/MIC_B = 1$) while the corresponding FIC_A value was nonzero ($MIC_{A/B}/MIC_A > 0$), that is, cases where the MIC of B was unchanged in the presence

TABLE 1 MIC determination (mg/L) and mutations of genes involved in resistance for clarithromycin, levofloxacin and tetracycline²⁴ for the J99 reference strain and 19 *H. pylori* isolates.

Isolates	MIC (mg/L)						Resistance-associated mutations		
	AMX (≤0.125)	RIF (≤1)	LEV (≤1)	CLA (≤0.25)	TET (≤1)	MTZ (≤8)	CLA (23SrDNA)	LEV (<i>gyrA</i>)	TET (16S rDNA)
J99	0.032	0.25	0.125	0.032	0.032	4			
C1	0.032	0.25	0.25	128	0.032	4	A2143G		
C2	0.0078	0.125	0.125	0.032	0.125	0.25			
C3	0.0078	0.5	0.125	0.0156	0.032	4			
C4	0.125	0.25	0.25	0.0156	0.032	1			
C5	0.0156	0.25	0.125	0.0156	0.032	0.5			
C7	0.0078	0.25	0.125	0.032	0.063	64			
C8	0.063	0.5	4	16	0.0078	4	A2143G	D91Y	
C9	0.0156	0.25	0.5	0.063	0.0157	4			
C10	0.125	0.25	4	0.125	0.125	64		D91N	
C11	0.032	0.25	0.5	0.032	0.063	0.5			
C13	0.0078	0.5	0.125	0.0157	0.032	128			
C14	0.0078	0.5	32	0.032	0.063	4		N87I	
C15	0.0078	0.5	0.25	0.032	0.063	4			
C16	0.063	0.5	0.25	0.032	0.5	4			
C17	0.032	0.5	0.25	0.032	0.063	16			
C19	0.0156	0.5	2	0.032	0.063	2		D91N	
C20	0.0156	0.5	0.25	0.032	0.032	16			
C22	0.125	0.5	64	128	4	256	A2143G	N87I	AGA926-928TTC
C23	0.032	0.5	1	128	0.063	128	A2143G		

Note: Values in bold are interpreted as resistant according to Ca SFM 2021,³⁷ breakpoints are reported below antibiotic abbreviations.

Abbreviations: AMX, amoxicillin; CLA, clarithromycin; LEV, levofloxacin; MIC, Minimum inhibitory concentrations; MTZ, metronidazole; RIF, rifampicin; TET, tetracycline.

of that specific concentration of A (and vice versa). However, the MIC of one antibiotic was never increased in the presence one other antibiotic.

Following the example of β -lactam/ β -lactamase inhibitor combinations,²⁷ the MIC values of an antibiotic A (to which the bacteria was resistant) have been plotted as a function of the concentration of antibiotic B (to which the bacteria was not necessarily resistant) ($MIC_{A/B}$ as defined in Equation 1 as a function of the concentration of B, Figures S2–S5). This makes it possible to assess whether, in the presence of a certain concentration of B, susceptibility to antibiotic A was restored, that is, whether the MIC for antibiotic A fell below its breakpoint. It can be seen in these figures that if the strain was resistant to antibiotic B (the graphs framed in red, Figures S2–S5), the concentrations of B required to decrease the MIC of A below its respective breakpoint (red line, Figures S2–S5) were always higher than the breakpoint of B (blue line, Figures S2–S5). In other words, to inhibit bacterial growth, the combination of two antibiotics to which the bacteria were resistant always required concentrations higher than the breakpoints. As for example, for CLA-resistant isolates (Figure S2), for C1 or C8 strain which was susceptible to MTZ, the CLA MIC fell below its breakpoint (horizontal red line, 0.25 mg/L)

with concentrations of MTZ below its own breakpoint (vertical blue line, 8 mg/L). By contrast, for C22, which was resistant to both antibiotics, the CLA MIC fell below its breakpoint with concentrations of MTZ much higher than its own breakpoint (>8 mg/L).

4 | DISCUSSION

To the best of our knowledge, this study is the first to have evaluated in vitro different double combinations of antibiotics used to treat *H. pylori* on a relatively large number of clinical isolates. The phenotypes of the selected isolates were varied, to make extrapolation to clinical conditions more robust. The results showed that for the six antibiotics tested, the in vitro effects in double combinations were mainly additive or indifferent. On average, the lowest FIC_{mean} values were observed for MTZ (range 0.64–0.93 depending on the combination), while the highest were observed for LEV (range 0.93–1.04 depending on the combination). Looking in detail, it could be observed that the type of pharmacodynamic interaction could vary according to concentration and that synergies have been observed for certain concentration ranges. Moreover, it can be noted that there

TABLE 2 FIC_{mean} of each combination for J99 reference strain and the 19 *H. pylori* isolates.

	FIC _{mean} combinations with CLA				FIC _{mean} combinations with RIF				FIC _{mean} combinations with TET				FIC _{mean} combinations with AMX						
	LEV	AMX	RIF	MTZ	TET	LEV	AMX	TET	MTZ	LEV	AMX	MTZ	LEV	AMX	MTZ	LEV	AMX	MTZ	
Reference strain																			
J99	1.38	1.08	0.93	0.59	0.33	1.05	1.05	1.04	0.52	0.75	1.06	0.83	1.04	0.99	0.59				
Clinical isolates																			
C1	1.18	0.41	0.89	1.00	1.15	0.64	0.77	0.94	0.54	1.09	0.78	0.82	0.82	0.75	0.92				
C7	0.50	0.89	0.90	1.00	0.62	1.18	1.17	1.05	0.79	1.10	0.85	0.59	0.78	0.91	1.21				
C8	1.21	1.04	0.77	0.80	0.99	0.94	1.05	1.10	0.89	0.74	1.12	0.40	1.04	0.86	1.18				
C10	0.99	0.80	0.92	0.65	0.93	0.43	1.19	0.66	0.49	1.21	0.76	0.96	1.11	0.91	1.04				
C13	1.04	0.79	0.69	0.72	0.63	1.18	0.86	0.88	0.65	1.05	1.28	0.67	1.23	1.09	1.10				
C14	0.84	0.70	0.60	0.80	0.78	0.99	0.89	1.21	0.89	0.98	1.25	0.81	1.21	1.23	0.98				
C17	0.84	0.91	0.71	0.75	0.73	0.94	1.06	0.93	0.64	0.77	1.10	0.70	0.86	0.98	1.23				
C19	0.98	0.90	0.71	0.86	0.71	0.99	0.66	0.93	0.65	1.14	0.89	0.80	1.19	1.26	1.05				
C20	0.87	0.47	0.52	0.59	0.50	1.11	1.21	1.12	0.62	1.21	1.11	0.77	1.12	0.66	0.51				
C22	1.16	0.59	0.77	0.77	1.17	0.84	0.54	0.80	0.69	0.77	0.47	0.73	1.12	0.78	0.84				
C23	0.87	0.89	1.04	0.60	1.19	1.19	1.17	0.99	0.38	0.74	0.85	0.93	1.05	0.97	0.93				
C2	1.28	0.46	0.83	1.05	1.16	1.11	1.05	1.21	0.77	1.10	0.72	0.96	1.06	0.63	1.07				
C3	1.22	0.45	1.18	0.71	0.91	1.19	0.68	1.04	0.62	0.98	1.11	0.74	0.97	0.54	0.47				
C4	1.24	1.05	0.95	1.16	0.92	0.84	1.04	0.66	0.61	1.21	0.72	1.08	1.17	1.09	0.87				
C5	0.92	0.98	0.65	0.66	0.86	0.98	1.25	0.69	0.40	0.77	0.81	0.62	0.64	0.56	0.89				
C9	1.22	0.56	1.14	0.90	0.79	0.71	1.05	0.88	0.80	1.04	1.26	0.73	0.99	0.94	0.61				
C11	1.08	1.04	0.81	0.72	0.91	0.91	0.58	0.95	0.62	1.04	0.81	0.63	1.19	0.60	0.99				
C15	1.04	0.77	0.84	1.10	0.91	1.09	1.11	0.94	0.45	0.86	1.14	0.66	1.18	1.19	0.95				
C16	0.93	0.93	0.66	0.87	0.83	0.84	0.82	0.64	0.57	1.16	0.87	0.92	0.98	0.99	0.82				
Average (FIC _{mean})	1.02	0.77	0.82	0.83	0.88	0.95	0.95	0.93	0.64	1.00	0.94	0.76	1.04	0.89	0.93				
Interaction	IND	ADD	ADD	ADD	ADD	ADD	ADD	ADD	ADD	ADD	ADD	ADD	IND	ADD	ADD				

Note: Combination were considered synergistic for FIC_{mean} ≤ 0.5 (values highlighted in green), additive (ADD) for 0.5 < FIC_{mean} ≤ 1 (values highlighted in yellow), indifferent (IND) for 1 < FIC_{mean} < 4 (values highlighted in pink).¹⁷ Isolates highlighted in gray were resistant to at least one antibiotic. J99 values were not included in the determination of Mean (FIC_{mean}).

Abbreviations: AMX, amoxicillin; CLA, clarithromycin; FIC, fractional inhibitory concentration; LEV, levofloxacin; MIC, Minimum inhibitory concentrations; MTZ, metronidazole; RIF, rifampicin; TET, tetracycline.

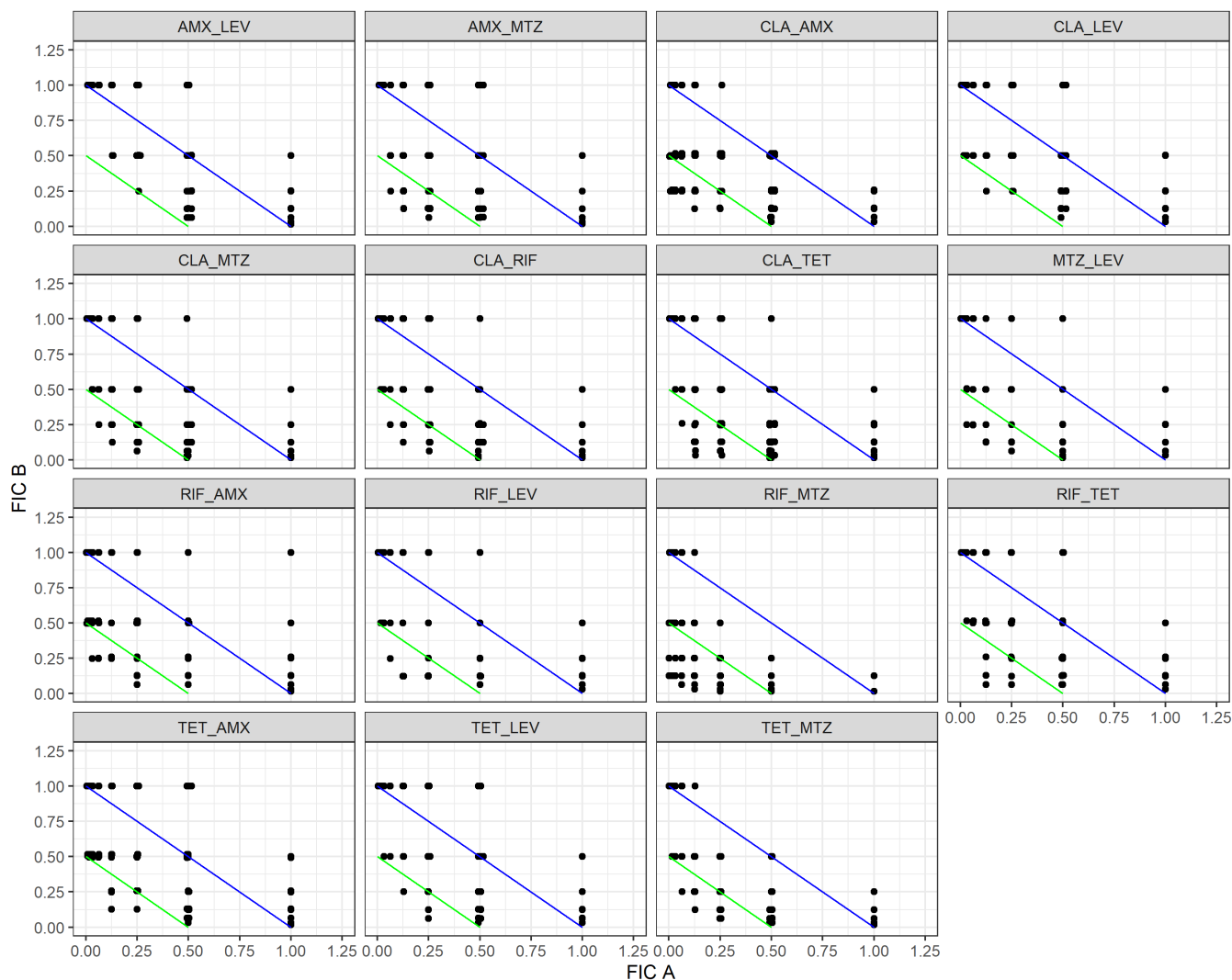


FIGURE 1 Fractional inhibitory concentration (FIC) B versus FIC A of all two-antibiotic combinations for the reference strain J99 and the 19 clinical isolates. FIC A and FIC B were defined as follows: $FIC_i = FIC_A + FIC_B = \frac{MIC_{A,combo}}{MIC_A} + \frac{MIC_{B,combo}}{MIC_B}$ (cf text for details). The names of the two antibiotics A and B in combination are indicated above each panel in the form "ATB A_ATB B." The dots indicate the different pairs of FIC A and FIC B values observed during checkerboard experiments for the 20 strains (for each strain, several FIC_i values were observed). Green isobole corresponds to the upper limit of the synergy zone, that is, all the values below this isobole were considered as synergistic. Values between the green isobole and the blue isobole, which is the upper limit of the additivity, were considered as additive. Values above the blue isobole were considered as indifferent.

were no links between the observed FIC_{mean} values, the phenotypes (S or R) and/or the resistance genotypes identified.

When two antibiotics to which the strain was resistant were combined, the combined effects were insufficient to inhibit bacterial growth at concentrations below the breakpoints. This suggests that, for probabilistic treatment, the epidemiology of *H. pylori* resistance should prevail in the choice of antibiotics to combine, and that combinations with only antibiotics for which the risk of resistance is high (CLA, MTZ, and LEV) should be avoided.

The frequency of resistance to MTZ and CLA is high, which justifies combining them with other antibiotics for probabilistic treatment. MTZ is used in the first line of treatment in the United States, Asia-Pacific and Europe.¹⁰ The low rate of resistance to AMX, RIF, or TET reinforces the value of using these antibiotics in combination

with MTZ.^{28,29} For the five MTZ-resistant but CLA-susceptible isolates, low concentrations of CLA restored MTZ susceptibility in each case, which was not the case for CLA-resistant strains. However, the risk of an *H. pylori* strains being resistant to both CLA and MTZ (dual resistance) is relatively high over 15% in certain regions, limiting the value of combining these two antibiotics.¹⁰ If CLA and MTZ are combined, it is preferable to add a third antibiotic such as AMX as is the case for first-line concomitant treatment in Europe.^{9,30} According to our results, the use of TET in combination with MTZ, as in the case of bismuth quadritherapy, should be effective against MTZ-resistant strains. Likewise, RIF-based combined therapies represent a potential and attractive strategy for *H. pylori* eradication.^{29,31}

In vitro studies of the pharmacodynamic effects of antibiotics on *H. pylori* have their limitations but are nonetheless useful in guiding

their clinical use. There is relatively little *in vitro* data on *H. pylori* in the scientific literature. This is mainly due to the difficulties to cultivate *H. pylori*, especially in liquid media. Using a liquid microdilution method (96-well plate) to culture *H. pylori* enabled to carry out MIC and checkerboard experiments, in accordance with the EUCAST reference method.³² The MIC results with the microdilution method were comparable to those of routine E-tests carried out at the hospital (data not shown), except for one MIC for MTZ, in which it has already been shown that MTZ *in vitro* test are not reproducible.^{33,34} The *in vitro* results we obtained for MTZ should therefore be treated with caution.

The present study has some limitations. First, PPIs are an integral part of the therapies used in practice; here, the action of PPIs on the combinations was not considered. Nevertheless, the culture medium was at pH 7, thus indirectly compensating for the absence of PPIs even if a possible effect of PPIs, independent of pH, cannot be excluded. Second, triple antibiotics combinations have to be tested to see if synergy is more important or not, the present study only focusing on double combinations. Third, the bismuth effect was not evaluated by design. Synergism between bismuth salts and antibiotics is known, for example MTZ-resistant *H. pylori* isolates became susceptible when MTZ and bismuth were administered together.³⁵ Similarly, it was shown that CLA-resistant isolates were susceptible when CLA was combined with ranitidine and bismuth citrate.³⁶ Fourthly, checkerboard experiments measure the effect of combinations at a given time (30h here). Time-kill assays, by measuring the effect of combinations at different times, provide dynamic information on the combination, and in particular on any prevention of resistance emergence. In a companion paper, we present time-kill assay results for the AMX/CLA and AMX/LEV combinations, which complement the checkerboard results presented here.

In conclusion, the present results have shown that *in vitro*, on a varied panel of clinical isolates, the different antibiotics used in therapeutics have additive effects. However, the combined effects of two antibiotics to which a strain was resistant were not sufficient to prevent bacterial growth. In probabilistic treatment, the choice of antibiotics to combine should therefore be based on the epidemiology of resistance, so that at least one antibiotic to which the strain is susceptible is used.

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CONFLICT OF INTEREST STATEMENT

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ORCID

Christophe Burucoa  <https://orcid.org/0000-0001-6796-3731>

Julie Cremniter  <https://orcid.org/0000-0002-2249-8394>

Nicolas Grégoire  <https://orcid.org/0000-0001-6436-3757>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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