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Assessing Helicobacter pylori motility and biofilm formation in subinhibitory concentrations of antimicrobials

Caroline Aparecida Maggioni Fauro
Universidade Federal de Mato Grosso, Campus Sinop

Luanny Cristiny Moresco Tessari
Universidade Federal de Mato Grosso, Campus Sinop

Nohati Rhanda Freitas Dos Santos
Universidade Federal de Mato Grosso, Campus Sinop

Neocimar Saraiva Correia
Universidade Federal de Mato Grosso, Campus Sinop

Cibele Bonacorsi
Universidade Federal de Mato Grosso, Campus Sinop

Alexandre Melo Bailão
Universidade Federal de Goiás

Lilian Cristiane Baeza
Universidade Estadual do Oeste do Paraná

Newton Valério Verbisck
Embrapa Gado de Corte

Corresponding author
Fabiana Cristina Donofrio
Universidade Federal de Mato Grosso, Campus Sinop
fabiana.donofrio@ufmt.br

Abstract. Numerous studies have shown that subinhibitory concentrations of antimicrobials can alter bacterial virulence factors. This study evaluates motility and biofilm formation by H. pylori 43504 grown in subinhibitory concentrations of amoxicillin (AMX), clarithromycin (CLA), or tetracycline (TET). For the swimming and swarming motility assays, H. pylori 43504 suspensions were prepared with the strain alone or with the strain in AMX, CLA, or TET at ½ MIC. Next, the media were incubated at 37 ºC, under microaerophilia. To assess biofilm formation in the presence of one of the antimicrobials at subinhibitory antimicrobial concentrations, bacterial suspensions (109 CFU/mL) were prepared in 2.5% FBS containing AMX, CLA, or TET at ½ MIC. After incubation for 10 days, H. pylori 43504 grown in medium containing AMX, CLA, or TET at ½ MIC presented greater swimming motility and lower swarming motility than the non-treated strain. H. pylori 43504 grown in medium containing AMX, CLA, or TET at ½ MIC showed stronger biofilm production than the non-treated strain. Our results showed that AMX, CLA, or TET at subinhibitory concentrations favors H. pylori 43504 swimming motility and biofilm formation after incubation for 3 days. This may have clinical consequences and make the microorganism difficult to eradicate.
Keywords: H. pylori, biofilm, motility, subinhibitory concentrations of antimicrobials

Introduction

Helicobacter pylori is a Gram-negative, flagellated, spiral-shaped, microaerophilic bacterium that produces urease, catalase, and oxidase. It colonizes the gastric mucosa, causing diseases such as chronic gastritis, peptic or duodenal ulcer,

and lymphoid tissue lymphoma (CHMIELA and KUPCINSKAS, 2019). Currently, this bacterium is considered a public health concern because it is classified as class I carcinogen and is associated with gastric adenocarcinoma and non-Hodgkin lymphoma development (STERBENC et al., 2019). Infection by *H. pylori* and disease progression are closely associated with host factors and *H. pylori* pathogenicity due to expression of virulence factors like flagella, heat shock proteins, urease, superoxide dismutase, and catalase, among others, which are essential for bacterial colonization, invasion, and survival in the gastric mucosa (BAJ et al., 2021; GOMEZ-RAMIREZ et al., 2021; STERBENC et al., 2019).

In the gastric mucosa, *H. pylori* develops swimming motility and moves through an attractive chemical gradient containing amino acids, mucin, urea, and sodium bicarbonate until it establishes close contact with epithelial cells; adhesion occurs through adhesin BabA (bound to the Lewis-b blood group) and outer membrane proteins (OMPs) SabA, HopZ, AlpA, AlpB, and OipA, which facilitates mucosa colonization and biofilm formation (ABDOLLAHI and TADJROBEHKAR, 2012; GU, 2017; HATHROUBI et al., 2018; CHMIELA and KUPCINSKAS, 2019; STERBENC et al., 2019; GOMEZ-RAMIREZ et al., 2021). Proteins LuxS and autoinducer 2 (AI-2) are also essential for maintaining the bacterium in the gastric epithelium and influence bacterial motility and biofilm formation (COLE et al., 2004; RADER et al., 2011; WANG et al., 2019).

Treatment for *H. pylori* eradication is based on the guidelines of the V Maastricht Consensus report (revised in 2018) and IV Brazilian Consensus report on H. pylori (2018) and updated guidelines of the European and American Societies of Gastroenterology. The treatment consists in combining two antibiotics (usually clarithromycin and amoxicillin, tetracycline, or furazolidone) and a proton-pump inhibitor (PPI) (omeprazole, lansoprazole, or pantoprazole) and bismuth salts (HSU et al., 2018).

Various studies have reported that antibiotics and concentrations below the Minimum Inhibitory Concentration (MIC) may intervene and alter expression of bacterium virulence factors, biofilm formation, morphology, bacterial growth, cytoplasmic pH regulation, and synthesis of repair proteins and heat shock proteins (functioning as chaperones) (BERNARDO et al., 2004; VALLEDOR and JORRÍN, 2011; DONOFRIO et al., 2014; DONOFRIO et al., 2015). Studies conducted by Donofrio and colleagues in 2014 and 2015 showed that, when treated with subinhibitory amoxicillin concentrations, *H. pylori* 43504 presents higher expression of heat shock proteins (Hsp70kDa and Hsp60kDa), superoxide dismutase, moonlighting enolase, and neutrophil-activating protein. Under this stress conditions, the bacterium increases nitric oxide induction in macrophages and produces proteins that act as antioxidants, ensuring bacterial survival and contributing to colonization and pathogenesis. Bearing these findings in mind, this study evaluates the motility phenotype and biofilm formation by H. pylori treated with subinhibitory amoxicillin, clarithromycin, or tetracycline concentrations (½ MIC).

Materials and Methods

Bacterial strain, medium, and growth

H. pylori strain ATCC 43504 (amoxicillin-sensitive and metronidazole-resistant) isolated from human gastric antrum, from a patient with gastritis, was used to determine the growth curve. First, *H. pylori* 43504 was cultivated in agar microplates containing Columbia agar (Kasvi, Curitiba, PR, Brazil) added with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and incubated at 37 °C and under 10% CO2 atmosphere (Zenith Lab Inc., Jiangsu, China) for 72 h. Then, the colonies were placed in brain-heart-infusion (BHI) broth (HiMedia Laboratories, Mumbai, India) supplemented with 10% FBS until a suspension with 10^6 CFU/mL (UDO620 0.10) was achieved. Next, the microplate wells (96 flat-bottom wells) (Kasvi, Curitiba, PR, Brazil) were filled with 200 µL of bacterial suspension and incubated for 24, 48, 72, 96, and 120 h. The growth curve was determined by the difference in absorbance at 620 nm (Biolisa Reader, Quibasa, Belo Horizonte, MG, Brazil) read before and after incubation at 37 °C and under 10% CO_2 atmosphere for 24, 48, 72, 96, and 120 h. Tests were carried out in triplicate and repeated three times. The negative control was 200 µL of BHI broth supplemented with 10% FBS.

Susceptibility test

The Minimum Inhibitory Concentration (MIC) was determined by an adapted microplate microdilution method (BrCAST/EUCAST, 2019). Clarithromycin (CLA) (KLARICID 500 mg - Abbott Laboratórios do Brasil Ltda, São Paulo, SP, Brazil), amoxicillin (AMX) trihydrate (Laboratório Germed Farmacêutica 500 mg S/A, Campinas, SP, Brazil), or tetracycline (TET) (Laboratório Prati – Donaduzzi 300 mg, Toledo, PR, Brazil) was solubilized in ultrapure sterile water at 1 mg/mL, and the resulting solution was filtered through a syringe filter with 0.22 µm membrane (Sigma-Aldrich, St. Louis, USA), divided into aliquots, and frozen at -20 ºC until use.

Initially, *H. pylori* 43504 was cultivated as described above. Then, the colonies were placed in BHI broth supplemented with 10% FBS until a *H. pylori* 43504 suspension corresponding to 10^6 CFU/mL (UDO_{620} 0.10) was achieved. The microplate wells (96 flat-bottom wells) were filled with 100 µL of BHI broth supplemented with 10% FBS containing CLA, AMX, or TET at concentrations between 0.0078 and 2.0 µg/mL, followed by addition of 100 µL of bacterial suspension. Growth inhibition, induced by one of the tested antimicrobials, was determined by the difference in absorbance at 620 nm read before and after incubation at 37 °C and under 10% CO_2 atmosphere for 72 h. Tests were

carried out in triplicate and repeated three times. The growth control was 100 µL of the same bacterial suspension added to 100 µL of BHI broth supplemented with 10% FBS. The dose-response curve was employed to determine which CLA, AMX, and TET concentrations should be used in the motility assays.

Motility assays

H. pylori 43504 suspensions in BHI broth supplemented with com 5% FBS and containing 10^9 CFU/mL (equivalent to standard 4 of the McFarland scale, UDO_{620} 0.40) were prepared and added with AMX, CLA, or TET at ½ MIC. A control suspension without antimicrobial was also prepared. To evaluate the swimming motility phenotype, 5 µL of each suspension was inoculated into culture medium containing 0.3% bacteriological agar (HiMedia Laboratories, Mumbai, India), 1% peptone (HiMedia Laboratories, Mumbai, India), and 5% yeast extract (HiMedia Laboratories, Mumbai, India) supplemented with 5% FBS. To evaluate the swarming motility phenotype, 5 µL of each suspension was added to the surface of a microplate containing 0.5% agar culture medium, 1% peptone, and 5% yeast extract supplemented with 5% FBS, and heated at 40 ºC for 5 min. Then, each suspension was incubated at 37 ºC under 10% CO^2 atmosphere for 10 days. Tthe growth halo diameter (in millimeters, mm) was measured with a pachymeter every days (SPERANDIO et al., 2003; MOREIRA et al., 2010) and classified according to Abdouchakour et al. (2018). Results are expressed as percentage (%) of motility on days 3, 6, and 10, obtained from the mean and standard deviation of three independent assays, conducted in triplicate; the negative control (untreated strain) was considered as 100%.

Biofilm Analysis by Staining

For the biofilm formation assays, *H. pylori* suspensions with 10^9 CFU/mL (UDO620 0.40) in BHI broth supplemented with 2.5% FBS added with CLA, AMX, or TET at ½ MIC were prepared. Then, 200 µL of each suspension was added to the wells of a microplate (96 flat-bottom wells) at 37 °C under 10% CO_2 atmosphere for 1, 3, and 6 days. Tests were carried out in six replicates and repeated three times. The negative control consisted of BHI broth supplemented with 2.5% FBS.

Biofilm mass was quantified by crystal violet staining. After incubation, the microplate wells were washed three times with sterile phosphate buffer saline solution (Sigma-Aldrich, St. Louis, USA), to remove non-adhered bacterial cells, and fixed with absolute ethanol (INLAB, Diadema, SP) at room temperature for 30 min. Then, the microplate wells were washed again and stained with 200 µL of 1% crystal violet (Laborclin, Pinhais, PR, Brazil) for 5 min. After that, the microplate wells were washed once again to remove the dye. Next, the biofilm was resuspended in 300 µL of acetic acid (INLAB, Diadema, SP) at 33% and homogenized for 1 min, to ensure homogeneity of the stained material. Finally, 200 µL of each sample was transferred to a new microplate, and the optical density of the bacterial biofilms was quantified with the aid of a microplate reader at a wavelength of 492 nm. The biofilm formation ability of the strain treated with AMX, CLA, or TET at ½ MIC and the control, without antimicrobial, was classified according to STEPANOVIC et al. (2000).

Statistical analysis

The results obtained for the tested antimicrobials were analyzed by ANOVA followed by multiple comparison according to the TUKEY test; the program Graph Pad Instat version 8 was used (GraphPad, San Diego, CA, USA); significance was set at $p < 0.05$.

Results and discussion

The Growth curve and anti-H. pylori activity

The *H. pylori* growth curve showed that the bacterial biomass increased exponentially between 24 and 48 h; the stationary phase started after incubation for 96 h (Figure 1A). These findings agreed with the results of other papers (BENAISSA et al., 1996; MERRELL et al., 2003). The effect of the tested antimicrobials on *H. pylori* growth allowed us to determine MICs of 0.125, 0.0313, and 1.0 µg/mL for AMX, CLA, and TET, respectively. Therefore, ½ MIC of 0.0625, 0.015, and 0.5 µg/mL were used for AMX, CLA, and TET during the assays (Figure 1B). Our results agreed with CLSI (2015) and BrCAST/EUCAST (2021) and epidemiological cutoff points, which differ between wild-type and reduced-sensitivity isolates, more specifically AMX ≤ 0.125 µg/mL, CLA ≤ 0.25 µg/mL, and TET ≤ 1.0 µg/mL. e results and discussion should be written continuously using the current literature to support and argue the results obtained.

Evaluation of Swimming and Swarming Motility

In the swimming motility assay, non-treated *H. pylori* (NT) presented motility zone with mean diameter of 6.75, 7.25, and 9.25 mm after incubation for 3, 6, and 10 days, respectively, which corresponded to weak motility. *H. pylori* grown in AMX, CLA, or TET at ½ MIC presented motility zone with mean diameter of 8, 5.5, and 6.5 mm after incubation for 3 days, respectively, corresponding to weak motility; 12, 13, and 13 mm after incubation for 6 day, respectively, corresponding to weak motility; and 23.5, 23.25, and 23.75 mm after incubation for 10 days, respectively, corresponding to moderate motility (Figures 2A and 3). After incubation for 10 days, *H. pylori* grown in AMX, CLA, or TET at ½ MIC presented 154% ($p < 0.001$), 151.4% ($p < 0.001$), and 135.1% ($p < 0.01$) higher swimming motility than NT, respectively. After incubation for 3 and 6 days, *H. pylori* grown in AMX, CLA, or TET at ½ MIC presented increased swimming phenotype, without statistical significance (Figure 2B).

In the swarming motility assay, non-treated H. pylori presented motility zone with mean diameter

of 7.25, 8.5, and 10.75 mm after incubation for 3, 6, and 10 days, respectively, which corresponded to weak motility. *H. pylori* grown in AMX, CLA, or TET at ½ MIC presented motility zone with mean diameter of 4.75, 5, and 4.75 mm after incubation for 3 days, respectively; 6, 5, and 5.5 mm after incubation for 6 days, respectively; and 9, 5, and 8 mm after incubation for 10 days, respectively (Figures 4A and 5). Therefore, the classification was weak motility. Concerning swarming motility, *H. pylori* grown in AMX, CLA, or TET at ½ MIC presented decreased phenotype as compared to NT for all the incubation times, without statistical significance. The exception was *H. pylori* grown in CLA at ½ MIC, which presented 53.5% decreased phenotype ($p < 0.01$) as compared to NT after incubation for 10 days (Figure 4B).

Various studies have shown that motility is an important virulence factor for H. pylori and is strongly associated with chemotaxis and physical or chemical environmental factors such as temperature, pH, microaerophilia, amino acids, mucin, urea, sodium bicarbonate, and low concentrations of antimicrobials. These factors drive flagellar motility and movement type, to facilitate gastric mucosa colonization and biofilm formation (RAJAGOPALA et al., 2007; ABDOLLAHI and TADJROBEHKAR, 2012; ATTARAN and FALSAFI, 2017; HATHROUBI; ZEREBINSKI; OTTEMANNA, 2018).

Our results showed higher H. pylori swimming motility for the microorganism grown in AMX, CLA, or TET at ½ MIC for 10 days as compared to NT ($p < 0.001$). In contrast, we found lower H. pylori swarming motility for the microorganism grown in AMX, CLA, or TET at ½ MIC for 10 days as compared to NT. These results suggested that H. pylori grown in subinhibitory AMX, CLA, or TET concentration have improved individual translocation with counterclockwise (CCW) flagellar movement in liquid medium, typical of swimming motility. This might have interfered in motility on highly viscous or high-friction surface, demanding greater cell-cell interaction by means of rotational helicoidal flagella, characteristic of swarming agar culture, as compared to NT. HARSHEY, 2003; RAJAGOPALA et al., 2007; LAUGA and POWERS, 2009; NAKAMURA and MINAMINO, 2019).

Several studies have reported that antibiotics at concentrations below MIC can alter biofilm formation, morphology, cell wall hydrophobicity, sensitivity to oxidative stress, motility, bacterial growth, cytoplasmic pH regulation, and synthesis of repair proteins and heat shock proteins (functioning as chaperones) (BERNARDO et al., 2004; VALLEDOR and JORRIN, 2011; FERNANDEZ et al., 2012; DONOFRIO et al., 2014; DONOFRIO et al.,2015).

Spengler et al. (2004) demonstrated that subinhibitory concentrations (10% and 50% MIC) of the trifluoromethyl ketone derivative 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone inhibit swimming motility with CCW rotation of CLA-resistant *H. pylori* ATCC 9447 more efficiently than swimming motility of CLA-sensitive *H. pylori* 700392. Ciccaglione et al. (2019) showed that swarming motility is lower for *H. pylori* ATCC 43629 and 2A/12 treated with subinhibitory bovine lactoferrin concentrations as compared to non-treated strains. Yang et al. (2016) reported that subinhibitory allicin concentrations reduce swimming motility and biofilm formation by uropathogenic *Escherichia coli* strains (UPEC CFT073 and J96). Bahari et al. (2017) demonstrated significantly reduced swarming motility for *Pseudomonas aeruginosa* PAO1 cultivated in subinhibitory azithromycin, gentamycin, and curcumin concentrations (1/4 MIC and 1/16 MIC). Many studies have shown that subinhibitory concentrations of mupirocin and macrolides like erythromycin, clarithromycin, and azithromycin reduce flagellin expression, flagellum formation, and swarming motility of *P. aeruginosa* NGM111 and *Proteus mirabilis* NGM007 (MOLINARI; PAGLIA; SCHITO, 1992; KAWAMURA-SATO et al., 2000; HORII et al., 2003).

In vitro *H. pylori* Biofilm Formation

Results of the biofilm formation assays showed no biofilm formation in any of the tested antimicrobials at ½ MIC or in the case of NT after incubation for 1 day. After incubation for 3 days, *H. pylori* grown in AMX or TET at ½ MIC presented greater biofilm formation ability ($p < 0.0001$) than NT and was classified as strong biofilm former. After incubation for 6 days, *H. pylori* grown in any of the antimicrobials at ½ MIC presented strong biofilm formation as compared to NT, without statistical significance (Figure 6).

We quantified bacterial biomass during biofilm formation by crystal violet absorbance values. Crystal violet can bind to negatively charged proteoglycans and to polysaccharides present in extracellular polymeric compounds of biofilms (HESS; HENRY-STANLEY; WELLS, 2015; SERVETAS et al., 2016). Thus, the larger the amount of biomass, the greater the coloration and the absorbance value (STIEFEL et al., 2016; WILSON et al., 2017). Nevertheless, numerous studies have demonstrated that FBS concentration and incubation time are important for *H. pylori* biofilm formation assays—these factors may influence bacterial adhesion to surfaces, negatively impacting biofilm development. Other factors such as standardized washing steps along the experiment can also impact the assays—they are crucial for reproducibility, thereby avoiding biofilm biomass under- or overestimation. (HESS; HENRY-STANLEY; WELLS, 2015; STIEFEL et al., 2016; WILSON et al., 2017; AZEREDO et al., 2017).

After standardization, our results evidenced strong biofilm production by *H. pylori* grown in AMX or TET at ½ MIC ($p < 0.0001$) for 3 days as compared to NT. Cole et al. (2004) and Yonezawa et al. (2013) reported that H. pylori requires longer incubation (≥ 3 days) for biofilm formation. Studies have shown that H. pylori can form biofilm in the

presence of environmental variations or subinhibitory concentrations of antimicrobials, an important survival strategy (PATTIYATHANEE; VILAICHONE; CHAICHANAWONGSAROJ, 2009; CAMMAROTA et al., 2012; CELLINI, 2014).

Attaran and Falsafi (2017) reported that *H. pylori* clinical isolates have increased biofilm formation in the presence of mucin and subinhibitory AMX concentrations. According to Bessa et al. (2013), subinhibitory AMX and CLA concentrations increase *H. pylori* biofilm formation and decrease LuxS gene expression. Pattiyathanee et al. (2009) demonstrated that subinhibitory curcumin concentrations inhibit *H. pylori* 43504 biofilm formation after incubation for 5 days, but they verified that biofilm formation is re-established after incubation for 10 days. Chadha (2021) reported that AMX at ½ MIC inhibits *E. coli* growth and swimming motility and induces biofilm formation. Subinhibitory TET concentrations increase the biofilm formation ability of five *Enterococcus faecalis* clinical isolates and the respective control strain (BERNARDI et al., 2021).

Knowing how subinhibitory concentrations of antimicrobials affect *H. pylori* motility and biofilm formation can aid the development of more efficient treatment regimens and the application of other strategies for disease eradication.

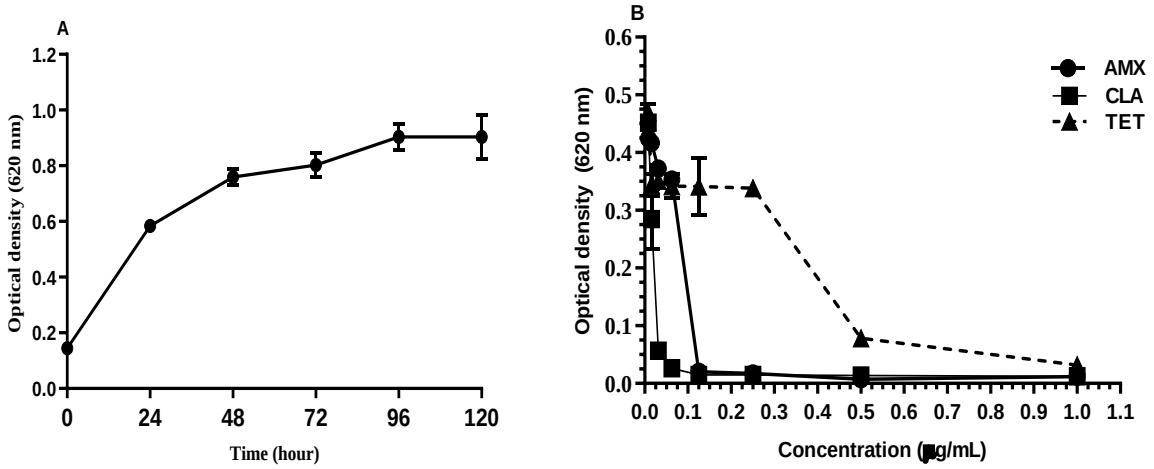

Figure 1. *H. pylori* growth curve (A) and dose-response effect of AMX, CLA, and TET concentrations on H. pylori growth after incubation for 72 (B).

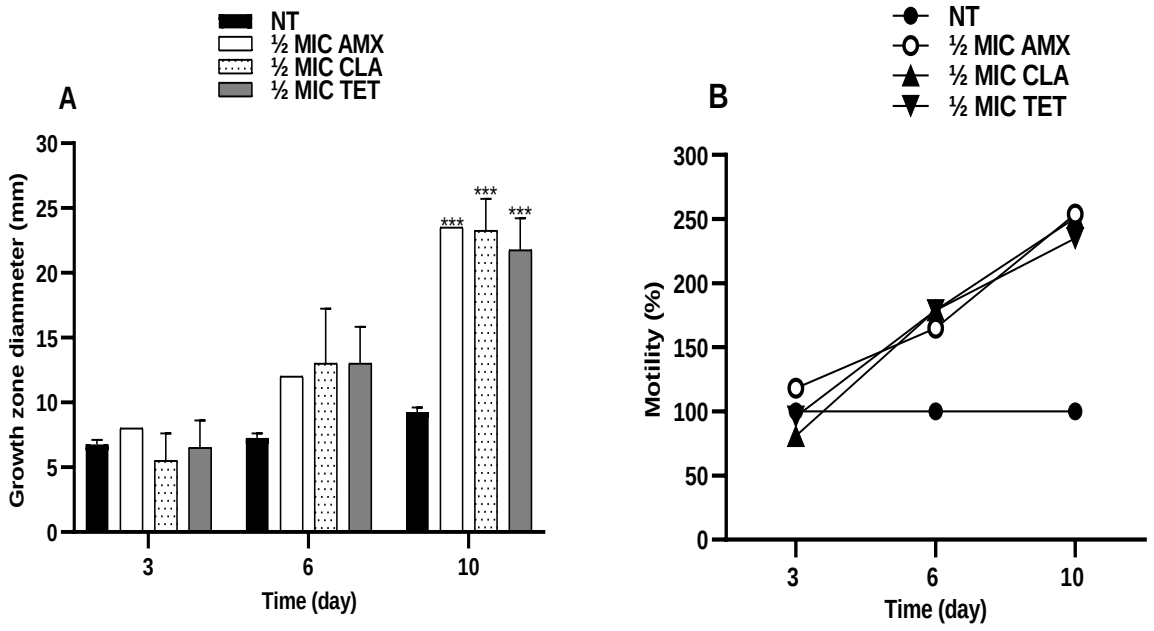

Figure 2. Swimming motility assay in terms of growth diameter (mm) (A) and percentage (%) (B). Intra-group statistical difference in relation to the non-treated strain ** $p < 0.01$; *** $p < 0.001$.

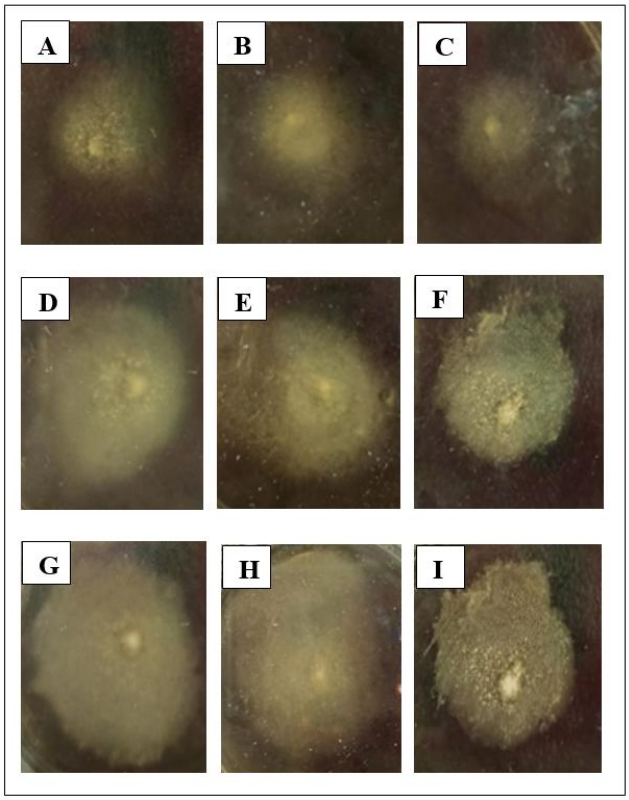

Figure 3. Representative swimming motility plates for *H. pylori* grown in AMX, CLA, or TET at ½ MIC after incubation for 3, 6, and 10 days. The swimming motility diameter and area were measured and normalized to the non-treated strain.

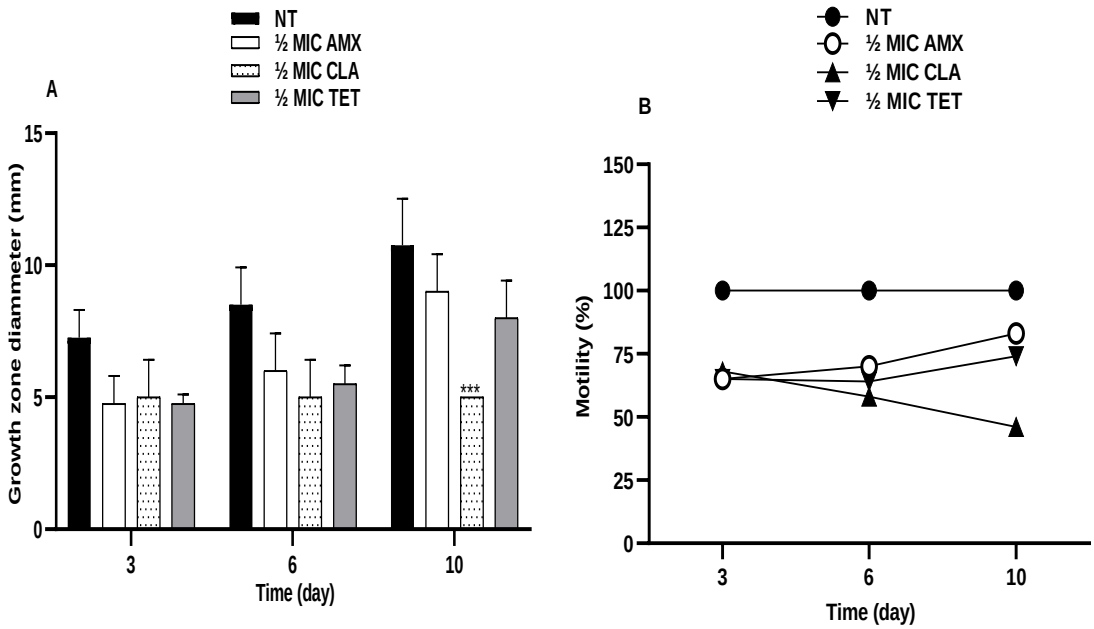

Figure 4. Swarming motility assay in growth diameter (mm) (A) and percentage (%) (B). Intra-group statistical difference in relation to the nontreated strain ** $p < 0.01$; *** $p < 0.001$.

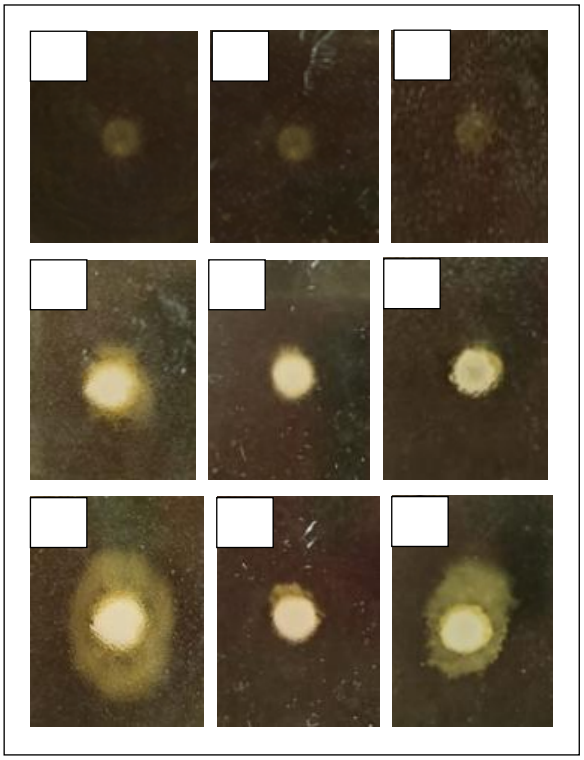

Figure 5. Representative Swarming motility plates for *H. pylori* grown in AMX, CLA, or TET at ½ MIC after incubation for 3, 6, and 10 days.

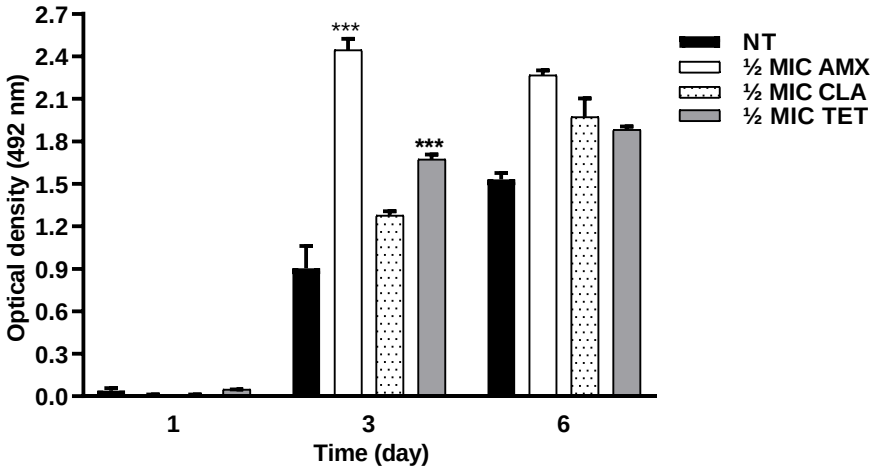

Figure 6. Quantitative measurement of biofilm formation by *H. pylori* grown in AMX, CLA, or TET at ½ MIC TET visualized by crystal violet staining after incubation for 1, 3, and 6 days.

Conclusion

Our results suggest that subinhibitory AMX, CLA, and TET concentrations favor *H. pylori* swimming motility and biofilm formation after incubation for 3 days. This may have clinical consequences and make microorganism eradication difficult. New studies based on the treatment regimen advocated by the V Maastricht Consensus report and the IV Brazilian Consensus report on *H. pylori* are underway to evaluate the expression of genes and virulence factors involved in the motility and biofilm formation by *H. pylori* 43504 grown in the presence of subinhibitory concentrations of antimicrobials.

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