Infection and Drug Resistance

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ORIGINAL RESEARCH

Association Between Biofilm Formation and Structure and Antibiotic Resistance in *H. pylori*

Xiaojuan Wu[1,2,*], Daoyan Wu[1,2,*], Guzhen Cui[1], Khui Hung Lee[3], Tingxiu Yang[1,2], Zhengrong Zhang[1,2], Qi Liu[4], Jinbao Zhang[1], Eng Guan Chua[3], Zhenghong Chen[1,2]

[1]Key Laboratory of Microbiology and Parasitology of Education Department of Guizhou & Joint Laboratory of Helicobacter Pylori and Intestinal Microecology of Affiliated Hospital of Guizhou Medical University, Guizhou Medical University, Guiyang, People's Republic of China; [2]Key Laboratory of Endemic and Ethnic Diseases of Ministry of Education, Guizhou Medical University, Guiyang, People's Republic of China; [3]Helicobacter Research Laboratory, the Marshall Centre for Infectious Disease Research and Training, School of Biomedical Sciences, University of Western Australia, Perth, Western Australia, Australia; [4]Department of Gastroenterology, Affiliated Hospital of Guizhou Medical University, Guiyang, People's Republic of China

*These authors contributed equally to this work

Correspondence: Zhenghong Chen, Department of Microbiology, School of Basic Medical Science, Guizhou Medical University, Guiyang, People's Republic of China, Tel + 86-13985006815, Email chenzhenghong@gmc.edu.cn

Background: Persistent infections caused by *Helicobacter pylori* (*H. pylori*), which are resistant to antibiotic treatment, pose a growing global public health concern. Biofilm formation is known to be associated with persistent infections due to its role in enhancing antimicrobial resistance and the tolerance of many pathogenic bacteria.

Objective: This study aims to evaluate the biofilm formation of clinical isolates of *H. pylori* and its impact on antibiotic eradication.

Methods: The thickness, morphology, and structure of biofilms derived from nine *H. pylori* strains were examined using confocal laser scanning microscopy, scanning electron microscopy, and transmission electron microscopy. Subsequently, the susceptibility of both planktonic and biofilm bacteria was assessed through the determination of minimum inhibitory concentration and minimum biofilm eradication concentration for amoxicillin, clarithromycin, levofloxacin, and tetracycline.

Results: The results revealed varying biofilm thicknesses and densities among the strains, characterised by the presence of numerous filaments intertwining and connecting bacterial cells. Additionally, several cases exhibited susceptibility based on MIC measurements but resistance according to MBEC measurements, with MBEC indicating a higher resistance rate. Pearson Correlation analysis demonstrated a positive correlation between biofilm thickness and MBEC results ($0 < r < 1$), notably significant for amoxicillin ($r = 0.801$, $P = 0.009$) and tetracycline ($r = 0.696$, $P = 0.037$).

Conclusion: Different strains of *H. pylori* exhibit variations in their capacity to release outer membrane vesicles (OMVs) and form biofilms. Biofilm formation can influence the effectiveness of amoxicillin and tetracycline in eradicating susceptible bacterial strains.

Keywords: *Helicobacter pylori*, antibiotic resistance, biofilm formation, biofilm-specific resistance

Introduction

Helicobacter pylori (H. pylori), a gastric bacterium, is one of the most common human pathogens, infecting approximately 50% of the global population. The persistence of *H. pylori* infection poses a significant risk factor for gastric diseases such as active and chronic gastritis, gastric and duodenal ulcers, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma.[1] The Kyoto global consensus report on *H. pylori* gastritis recommends the eradication of all *H. pylori* infections unless there are compelling medical reasons.[2] However, the rising antibiotic resistance rate has presented a growing challenge to *H. pylori* eradication efforts in recent years.[3] To enhance eradication efficacy, several international guidelines now recommend the use of personalised treatment based on drug susceptibility testing results.[4] Despite these efforts, instances of eradication failures persist and have been documented.[5,6]

Bacteria can acquire antibiotic resistance through various mechanisms, including reduced drug permeability, efflux pumps, alteration or bypassing of the drug target, production of antibiotic-modifying enzymes, and other physiological

Received: 28 March 2024
Accepted: 22 May 2024
Published: 20 June 2024

adaptations such as biofilm formation.[7] Biofilms represent communities of aggregated bacterial cells embedded in a self-produced extracellular matrix, which are composed of exopolysaccharides, minerals, proteins, and extracellular DNA. The composition and physical properties of biofilm extracellular matrices exhibit considerable diversity across different genera and within species.[8] Biofilm formation, once established, protects bacteria from antibiotics and immune defenses, resulting in persistent and chronic bacterial infections in the host.[9] This biofilm-mediated drug resistance or tolerance underlies the pathogenesis of persistent and chronic bacterial infections.[10] The development of chronic and refractory infections is a characteristic feature of *H. pylori* infection.[11] Previous studies have documented that *H. pylori* can establish biofilm either in vitro or in vivo, facilitating persistent bacterial colonisation within the gastric mucosa as well as reducing the effectiveness of antimicrobial treatments against this pathogen.[10–14]

To determine the antimicrobial susceptibility profiles, the agar or broth microdilution method, along with the Epsilometer test (E-test), is commonly used to examine the minimum inhibitory concentration (MIC) required to inhibit visible growth of planktonic bacterial cells.[15,16] However, MIC measurement, in most cases, does not accurately reflect the concentration needed to eradicate biofilm-associated infections, thereby affecting treatment efficacy.[17,18] Biofilm-associated bacteria exhibit significantly increased resistance to antimicrobial agents compared to their planktonic counterparts,[14,19] suggesting distinct mechanisms of resistance.[20] Microbial biofilms resist antibiotic treatment through two principal mechanisms: genetically-encoded antibiotic resistance and reversible phenotypes conferring drug tolerance. Antibiotic resistance is caused by mutation or inheritance of a resistance gene, efflux pumps more highly expressed in biofilms and so on. In contrast to antibiotic resistance, antibiotic tolerance is a transient and nonheritable phenotype defined by the physiological state of biofilm cell populations and biofilm-specific characteristics that limit drug diffusion and activity.[8,21] The minimum biofilm eradication concentration (MBEC) measurement may offer a more accurate antimicrobial resistance profile, aiding in the selection of appropriate antibiotics for treating biofilm-associated bacterial infections.[17,18]

In this study, we aimed to investigate the biofilm formation ability of different *H. pylori* clinical strains and its correlation with antibiotic resistance, to provide novel insights and possible explanations for refractory *H. pylori* infection.

Materials and Methods

Bacterial Strains

The *H. pylori* strain 26,695 and eight clinical isolates were employed in this study. The clinical isolates were obtained from patient gastric biopsy specimens in our previous study, with HPG1~HPG4 isolated from patients with chronic gastritis, HPG5~HPG7 isolated from patients with gastric ulcer, and HPG8 isolated from a patient with gastric cancer.[22] The *H. pylori* strain 26,695 was provided by the Institute Pathogenic Biology of Qilu Medical College in Shandong University, China.

The *H. pylori* strains were cultured routinely on BHI Agar (OXOID, UK) plates supplemented with 7% (v/v) defibrinated sheep blood and incubated in a 10% CO_2 environment at 37°C. For long-term storage, the strains were stored at −80°C in Brain Heart Infusion broth (BHI, OXOID, UK) containing 20% (v/v) glycerol and 10% (v/v) fetal bovine serum (FBS, Solarbio, China).

Construction of Biofilm

The colony biofilm assay was conducted in this study with minor modifications.[23] Briefly, the *H. pylori* strains were cultured on BHI Agar plates supplemented with 7% (vol/vol) defibrinated sheep blood. Following a 3-day incubation period at 37°C in a 10% CO_2 environment, the *H. pylori* growth on the agar plate was harvested and resuspended in 1 mL of BHI broth medium. The initial concentration of the bacterial suspension was adjusted to an optical density (OD_{600}) of 0.2. To ensure the stable growth of *H. pylori* at the interface, three sterile and dry 1 cm^2 nitrocellulose (NC) membrane (Millipore, USA) were placed on a fresh agar plate (diameter is 60 mm). Subsequently, 20 μL of bacterial suspension was inoculated onto each NC membrane and incubated at 37°C in a 10% CO_2 environment for additional four days. Three NC membranes with biofilms were prepared for confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM),

transmission electron microscopy (TEM), and determination of minimum biofilm eradication concentration (MBEC), respectively. As a negative control, sterile BHI broth medium was inoculated onto the membrane.

Confocal Laser Scanning Microscopy (CLSM)

The NC was washed three times with phosphate buffered saline (PBS) to eliminate planktonic bacterial cells. Subsequently, the biofilm on the NC membrane was stained with a fluorescent dye from LIVE/DEAD Baclight^{TM} Bacterial Viability Kits (Molecular Probes, Invitrogen, USA) in a 12-well plate for 20 minutes at 25°C under lightproof condition. Subsequently, after three gentle PBS washes to remove any excess dye, the NC membrane was affixed onto a slide, covered with a coverslip, and analysed using CLSM (Olympus FV1000, Japan). A 100× oil microscope objective was utilised to capture image stacks encompassing the entire thickness of the biofilm, scanning from its superficial layer to its bottommost layer. Photographs were taken at intervals of 0.5–1μm. The thickness of bacterial biofilm in that specific area was calculated by multiplying the number of photographs acquired within one visual field by their interval value. Subsequently, these resultant images were processed into three-dimensional representations using the FV10-ASW software. For the purpose of image observation using a 100× oil immersion microscope, two locations were randomly selected within each biofilm (one at the periphery and one in the central region), with three distinct biofilms per strain being examined.

Scanning Electron Microscopy (SEM)

Biofilm-growing bacteria and planktonic bacteria of *H. pylori* were observed using scanning electron microscopy (SEM). The samples for SEM analysis were prepared according to the following procedures. For the biofilm-forming bacteria, the NC membrane with the biofilm was gently washed three times with PBS to remove any planktonic bacteria. Subsequently, the biofilms were fixed in 2.5% (v/v) glutaraldehyde at 4°C overnight. As for the planktonic bacteria, following inoculation onto fresh agar plates and cultivation at 37°C in a 10% CO_2 environment for 3 days, *H. pylori* cells were collected in PBS solution. The collected planktonic bacteria were then fixed with 2.5% (v/v) glutaraldehyde at 4°C overnight after centrifugation of the bacterial suspension (3000 rpm, 5 min). Following fixation, the samples underwent three washes with cacodylate buffer and dehydration through a series of graded ethanol solutions (25%, 50%, 75%, 95%, and 100% respectively). Subsequently, freeze-drying was performed on these samples before sputter coating them with gold for observation under SEM [ZEISS Sigma 300 (Biofilm), ZEISS Gemini SEM 300 (Planktonic), Germany].

Transmission Electron Microscope (TEM)

Samples from biofilm or planktonic cultures were placed in 2.5% (v/v) glutaraldehyde at 4°C overnight, and then washed three times with PBS and fixed with 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 2 hours at 4°C. After two washes with nanopure water (5 min), the samples underwent ethanol dehydration series: 25% (5 min), 50% (5 min), 75% (5 min), 95% (10 min), and 100% (5 min, 5 min, and 10 min). Subsequently, the samples were embedded in Epon 813 embedding solution for thin sectioning. The sections were stained with uranyl acetate and lead citrate before observation with a Hitachi H-7650 electron microscope.

The Minimum Inhibitory Concentration (MIC)

To assess the susceptibility of planktonic bacteria to four antibiotics (amoxicillin, clarithromycin, tetracycline, and levofloxacin), we employed the agar dilution method as previously described by Osato,[24] with slight modifications for determining the MIC. Briefly, 2 mL of Mueller Hinton (MH, Hangzhou Binhe Reagent Co. LTD, China) agar supplemented with 7% (v/v) defibrinated sheep blood and serial dilutions of antibiotics (32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.031, and 0.003 mg/L) were added into each well of a 24-well plate prior to solidification. *H. pylori* cultures grown on agar plates for 3 days were collected in 1 mL of sterile PBS, and the bacterial suspension was adjusted to an OD_{600} of 0.8. Subsequently, 10 μL of the bacterial suspension were inoculated onto the agar of each well. All the plates were incubated at 37°C for 3 days in a 10% CO2 environment, and MIC values were determined. Clinical breakpoints (v13.1) specified in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines

were applied to define antibiotic resistance as follows: MIC >0.125 mg/L for amoxicillin, >0.25 mg/L for clarithromycin, and >1mg/L for both tetracycline and levofloxacin.

Determination of the Minimum Biofilm Eradication Concentration (MBEC)

The survival of bacteria in biofilm following antibiotic exposure was quantified using the modified MBEC method, as previously described.[16,17] Briefly, NC membranes with biofilms were gently washed three times with sterile PBS to eliminate any planktonic bacteria. Subsequently, the membranes were placed into 2 mL MH broth supplemented with 10% FBS and serial two-fold dilutions of antibiotics (0.5 mg/L to 256 mg/L). The cultures were incubated at 37°C with shaking at 120 rpm in a 10% CO_2 environment. Following a 24-hour incubation period, the NC membranes with biofilms from the broth medium were gently washed three times with sterile PBS. The attached biofilm on the NC membrane was carefully scraped off using a sterile inoculation loop and collected in sterile PBS. The bacterial suspension was adjusted to an OD_{600} of 0.8, and then inoculated onto agar plates without antibiotics in the wells of a 24-well plate using a volume of 10 μL per well. The plates were cultured at 37°C under a 10% CO_2 atmosphere for a duration of 72–96 hours. As controls, all strains that did not undergo antibiotic treatment were also inoculated onto agar plates without antibiotics.

Statistical Analysis

The data were presented as the mean ± standard error of the mean (SEM). Differences in biofilm thickness among strains were assessed using one-way ANOVA, with *P* values adjusted for multiple comparisons using Tukey's test. An adjusted $P < 0.05$ was considered statistically significant. The impact of biofilm on antibiotic susceptibility of *H. pylori* strains was evaluated using the Mann Whitney *U*-test and Fisher's exact test. Correlation analysis was performed using Pearson's correlation model. Data analysis was conducted with GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

Results

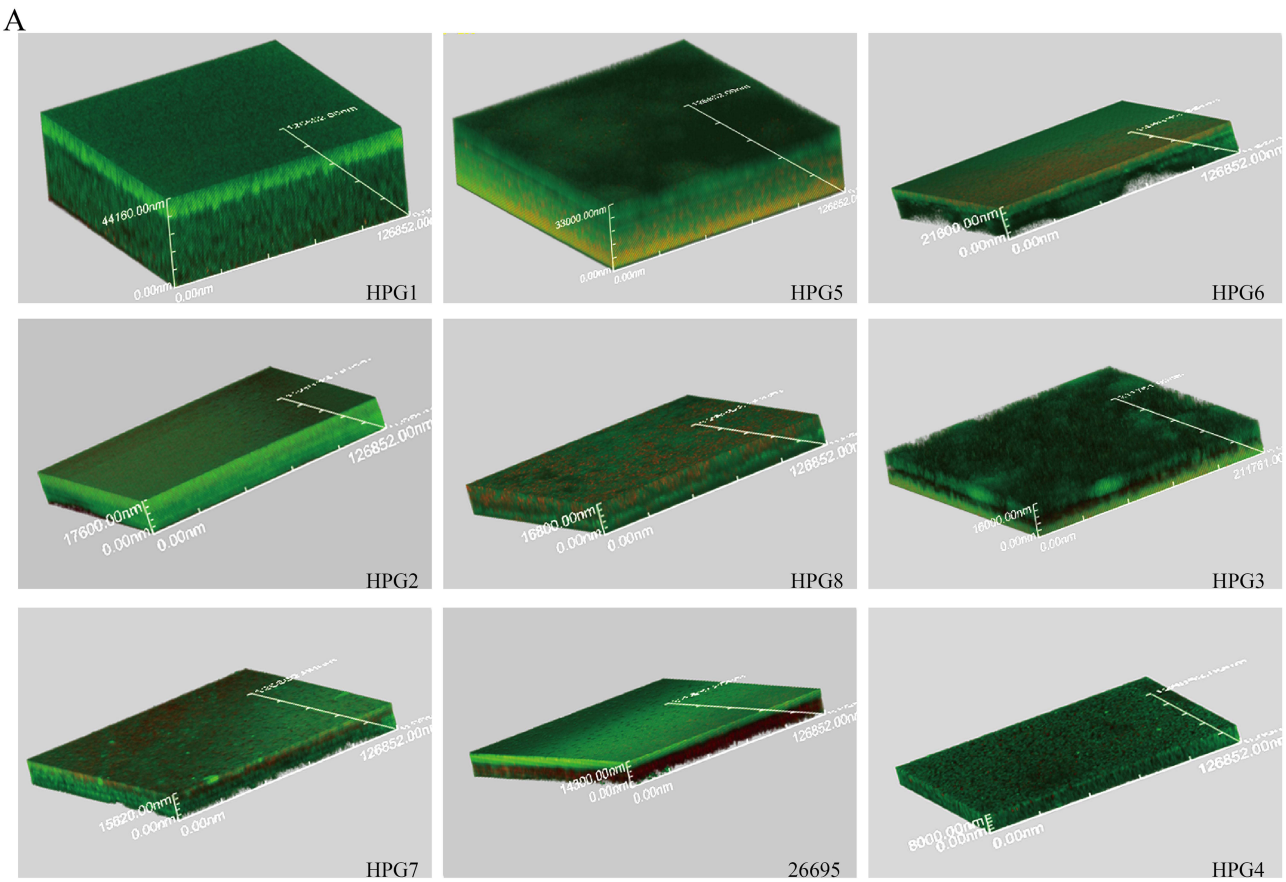
Measurement of Biofilm Thickness by CLSM

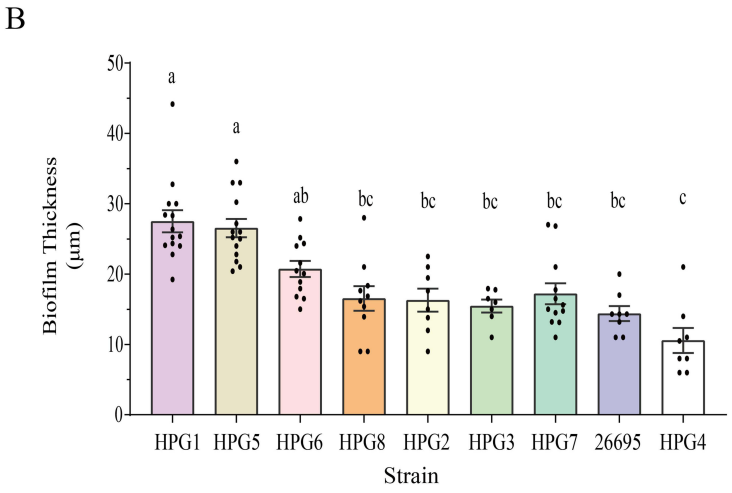
To evaluate the biofilm formation ability of all strains, we utilized CLSM to measure the thickness of biofilms stained with the LIVE/DEAD BacLight™ Bacterial Viability Kit (Figure 1A). As summarised in Table 1, the biofilm thickness values of the nine *H. pylori* strains ranged from 9.58 ± 1.16 to 31.5 ± 2.72 μm, and were normally distributed ($P > 0.05$) according to Shapiro–Wilk test results. As expected, no fluorescence signal indicative of the presence of biofilm was detected in the negative control. We further performed one-way ANOVA to compare the differences in biofilm thickness levels between strains. Both HPG1 and HGP5 strains demonstrated the strongest biofilm formation, exhibiting significantly thicker biofilms compared to the other strains whilst HPG4 displayed the lowest level of biofilm formation (Figure 1B).

Biofilms Observed by SEM and TEM

Based on the biofilm thickness results, three strains of *H. pylori* - HPG5, HPG6 and 26,695- strong, intermediate and poor biofilm formation capacities, respectively, were selected for further SEM and TEM analysis. In our SEM analysis, it was observed that the biofilms of both HPG5 and HPG6, but not that of 26,695, comprised intricate networks of thin filament bundles formed around spiral- or coccoid-shaped bacterial cells (Figure 2). Notably, strains HPG6 and 26,695 exhibited a higher prevalence of coccoid morphology (diameter: 0.5–1.5 μm) whilst spiral-shaped bacteria (length: 1.5–4μm; width: 0.5–1μm) were predominantly observed in HPG5 strain. The SEM analysis of planktonic HPG5, HPG6 and 26,695 *H. pylori* cells revealed typical spiral-shaped bacterial morphology with minimal, if any, presence of biofilm filaments.

Subsequently, TEM was employed to examine the biofilm and planktonic bacteria of strains HPG5, HPG6, and *H. pylori* 26,695. The presence of small spherical outer membrane vesicles (OMVs) was observed in the biofilms of strains HPG6 and HPG5 (Figure 3). In contrast, OMVs were infrequently detected in the planktonic extracellular milieu as well as the biofilm of *H. pylori* 26,695.





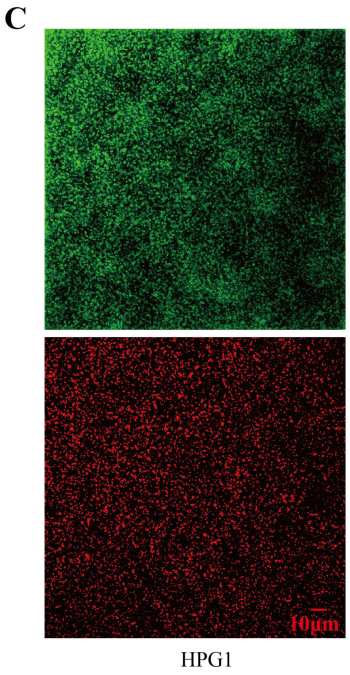


Figure 1 Biofilm formation capability exhibited by nine strains of *H. pylori*. (**A**) Confocal laser scanning microscopy (CLSM) images depicting the biofilms formed by the nine *H. pylori* strains. (**B**) A comparative analysis of biofilm thickness among the strains was conducted using data acquired from the experiment described in panel A. The data presented represent the means ± standard errors of three independent experiments (n = 6). Differences between strains were determined using one- way ANOVA, with different letters indicating a significant difference between groups at $P < 0.05$ and identical letters indicating no significant difference at $P > 0.05$. (**C**) CLSM images of strain HPG1 biofilm-associated cell stained using the LIVE/DEAD BacLight™ Bacterial Viability Kit (including SYTO 9, a green fluorescent membrane-permeable dye for labeling all bacteria by staining nucleic acid, and PI, a red fluorescent membrane-impermeable dye for labeling only bacteria with damaged membranes).

https://doi.org/10.2147/IDR.S468126 DovePress

Table 1 Biofilm Thickness of 9 *H. Pylori* Strains

Strain	$\overline{X}$ ± SEM (μm) (n = 6)	Shapiro–Wilk test *P* value
HPG1	31.50 ± 2.72	0.088
HPG2	17.93 ± 1.60	0.869
HPG3	15.07 ± 0.98	0.780
HPG4	9.58 ± 1.16	0.805
HPG5	30.90 ± 1.56	0.688
HPG6	22.46 ± 1.58	0.965
HPG7	16.14 ± 1.15	0.415
HPG8	19.18 ± 1.94	0.070
26,695	13.65 ± 0.94	0.198

Note: $\overline{X}$ ± *SEM*, the mean ± standard error of the mean.

Antibiotic Resistance and Tolerance of Biofilm Bacteria and Planktonic Bacteria

The MIC and MBEC results, indicating the susceptibility levels of planktonic and biofilm-associated bacterial cells to antibiotics, respectively, are presented in Table 2. Generally, the MBEC values were higher than the MIC values for amoxicillin, levofloxacin, and tetracycline ($P < 0.05$), except for clarithromycin ($P = 0.071$) (Figure 4). As shown in Table 2, resistance to antibiotic treatment determined by MBEC was significantly higher than those determined by MIC for three different antibiotics including amoxicillin, levofloxacin and tetracycline (*P values* = 0.029, 0.029 and 0.009, respectively).

The Correlation Between Biofilm Formation Ability and Antibiotic Resistance and Tolerance

To elucidate the relationship between biofilm formation capacity and antibiotic resistance and tolerance in *H. pylori*, we performed a Pearson correlation analysis on the MBEC and biofilm thickness values acquired from all nine *H. pylori*

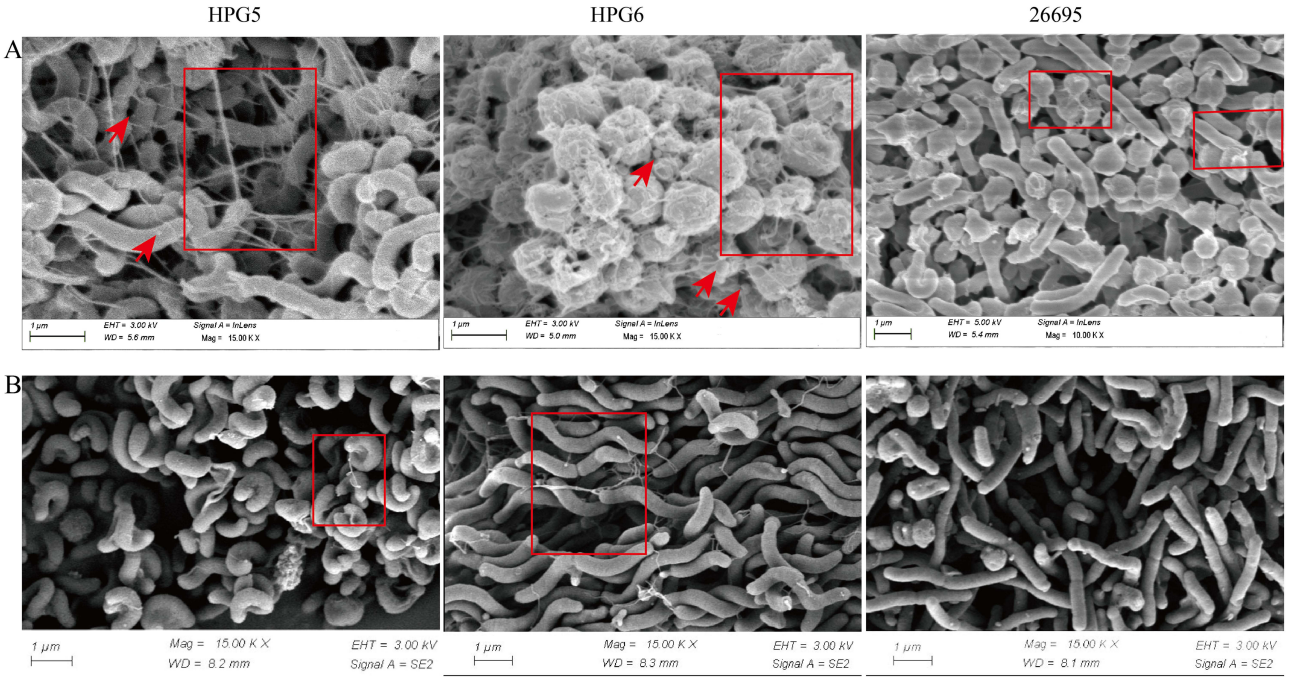


Figure 2 Scanning electron microscopy images of *H. pylori* biofilms and planktonic cell morphology at magnification of 15000x, except for the 26,695 biofilm, which was captured at 10000x magnification. (**A**) The SEM images of HPG5, HPG6 and 26,695 *H. pylori* biofilms. (**B**) The SEM images of planktonic HPG5, HPG6 and 26,695 *H. pylori* bacterial cells. The filament bundles within the biofilm are marked by red boxes. The scale bar measures 1μm.

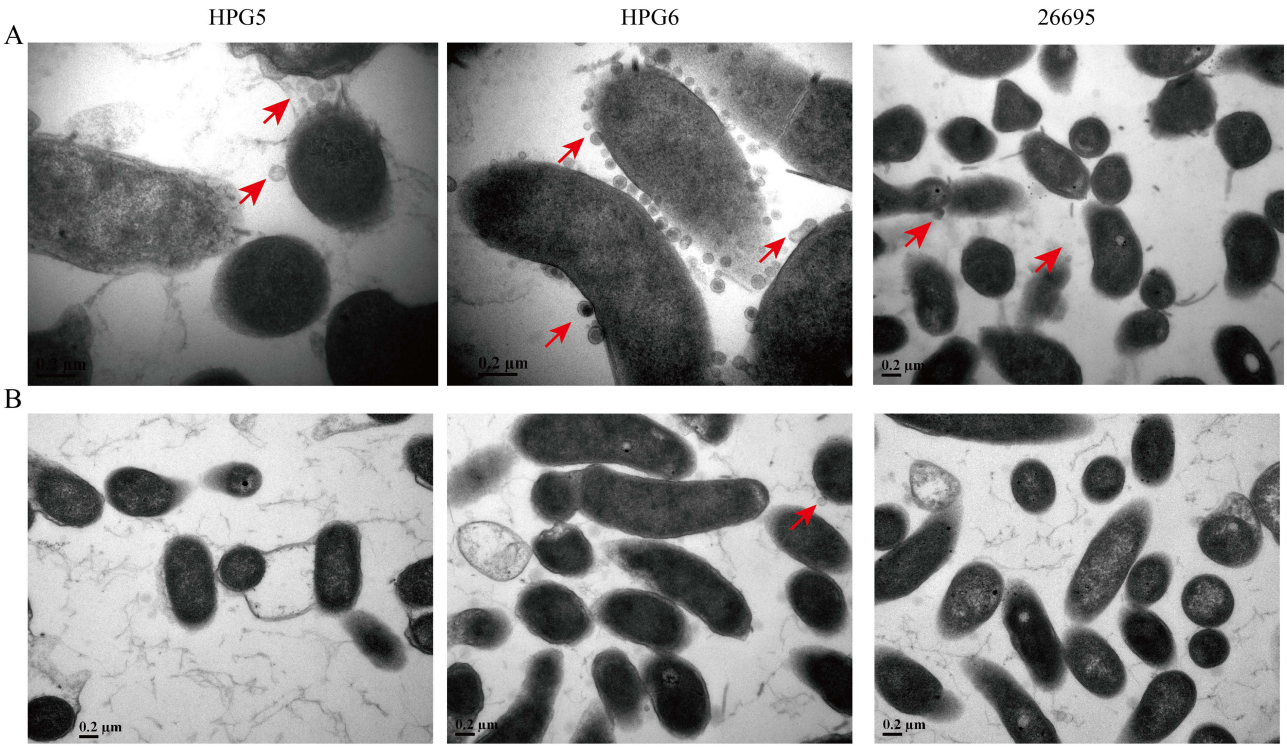


Figure 3 Transmission electron microscope images of *H. pylori* biofilms and planktonic cell morphology at 80000x magnification. (**A**) The TEM images of HPG5, HPG6 and 26,695 *H. pylori* biofilms. (**B**) The TEM images of planktonic HPG5, HPG6 and 26,695 *H. pylori* bacterial cells. Outer membrane vesicles (OMVs) are indicated with red arrows. Compared with planktonic, OMVs were more abundant in the biofilm of same strain. The scale bar indicates 0.2μm.

strains. As depicted in Figure 5, strong and significant positive associations between biofilm thickness and MBEC values were revealed for amoxicillin (r=0.801, P=0.009) and tetracycline (r=0.696, P=0.037).

Discussion

The primary distinction between biofilm bacteria and planktonic bacteria lies in the tight packing and encapsulation of bacteria within the extracellular polymeric substance (EPS) they secrete.[8] This matrix effectively modifies the surface properties of the

Table 2 MIC and MBEC Measurements of Nine *H. Pylori* Strains

Antibiotic / Strain	AMO (mg/L)		CLA (mg/L)		TET (mg/L)		LEV (mg/L)	
	MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC
HPG1	0.5	64	16	100	0.5	128	8	32
HPG2	0.125	1	0.125	0.25	1	4	0.5	2
HPG3	0.5	16	0.125	0.25	2	32	2	16
HPG4	0.5	16	0.125	1	4	32	4	16
HPG5	0.125	64	16	128	1	128	1	64
HPG6	0.125	64	0.0625	2	1	8	0.5	32
HPG7	0.25	16	16	100	2	32	8	128
HPG8	0.003	0.5	8	128	0.5	128	0.5	4
26,695	0.0625	2	0.0625	2	0.5	4	0.5	4
Resistance rate	4/9	9/9	4/9	7/9	3/9	9/9	4/9	9/9
P value	0.029		0.335		0.009		0.029	

Note: The Fisher exact test was employed to access the difference between the resistance rates based on MIC and MBEC values.
Abbreviations: AMO, amoxicillin; CLA, clarithromycin; TET, tetracycline; LEV, levofloxacin.

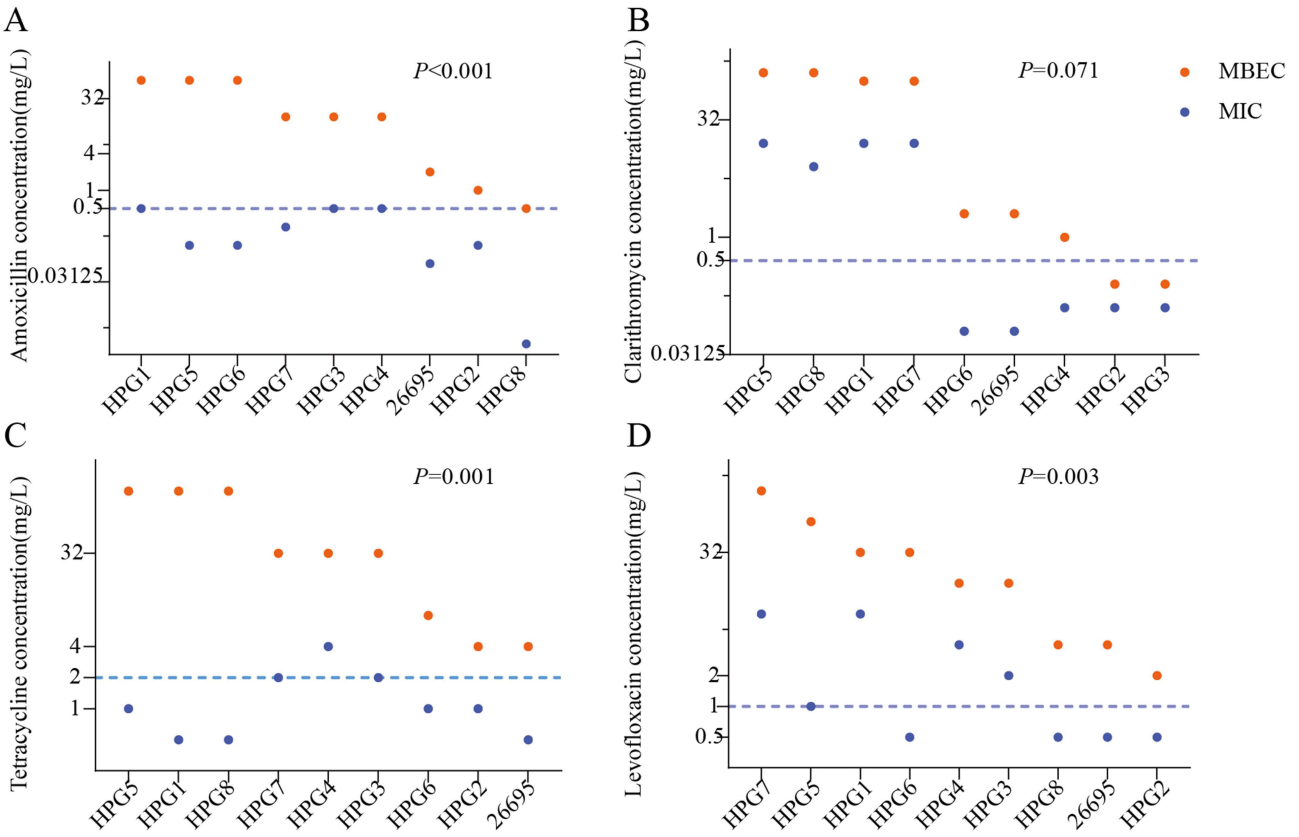


Figure 4 The MBEC and MIC values of nine *H. pylori* strains. (**A**) Amoxicillin; (**B**) Clarithromycin; (**C**) Tetracycline; (**D**) Levofloxacin. The MBEC values were higher than the MIC counterparts, with the blue dashed line denoting the clinical breakpoint for each antibiotic. Mann–Whitney test analysis revealed significant differences ($P < 0.05$) between MIC and MBEC values across all antibiotics, except for clarithromycin ($P = 0.071$).

bacteria, facilitating initial adhesion to active surfaces while preventing excessive bacterial aggregation.[8] Previous studies have demonstrated the ability of *H. pylori* to form biofilms both in vitro and in vivo.[11–13] Therefore, we assessed the biofilm-forming capacity of *H. pylori* by evaluating their thickness using confocal laser microscopy. Consistent with these previous findings,[25,26] all nine *H. pylori* strains tested in this study were capable to form biofilm in vitro, displaying varying degrees of biofilm formation ranging from weak to strong. Further examination of the biofilms under SEM revealed *H. pylori* cells, either spiral or coccoid stacked in several layers, tightly packed and surrounded by extracellular matrix. The observed morphology did not appear to correlate with the biofilm formation capacity of a particular strain. The observation of spiral-shaped cells in our *H. pylori* biofilms is inconsistent with the finding of Cole et al,[27] who reported that mature *H. pylori* biofilms of 3 to 5-days old primarily consist of coccoid cells. However, another previous study suggested that *H. pylori* biofilm formation is a complex process accompanied by various physiological changes, leading to diverse morphological transformations, including spiral, coccoid, and filamentous forms.[28] Ultimately, all cells within the matured biofilm transition into a coccoid form.[28] The morphological variations within *H. pylori* biofilms in this study may be attributed to the quasispecies nature of *H. pylori*, resulting in inherently distinct metabolic rates and activities among strains.

Through SEM analysis, we observed dense, thin filament bundles formed by bacterial flagella around bacteria cell in mature *H. pylori* biofilms, with the exception of strain 26,695. It is likely that in our study, a naturally occurring non-flagellated variant of strain 26,695 was used, thus accounting for the absence of flagellar filament in its biofilm.[29] Our findings suggest that flagella play additional roles beyond bacterial cell motility, contributing to biofilm integrity by holding *H. pylori* cells together. Furthermore, in a previous study, extensive flagella have been observed within *E. coli* biofilm, where flagellar rotation was crucial for tethering cells together.[30] In *H. pylori*, flagellar assembly genes were shown to be significantly upregulated in biofilm-forming *H. pylori* cells, while a flagellated mutant strain displayed

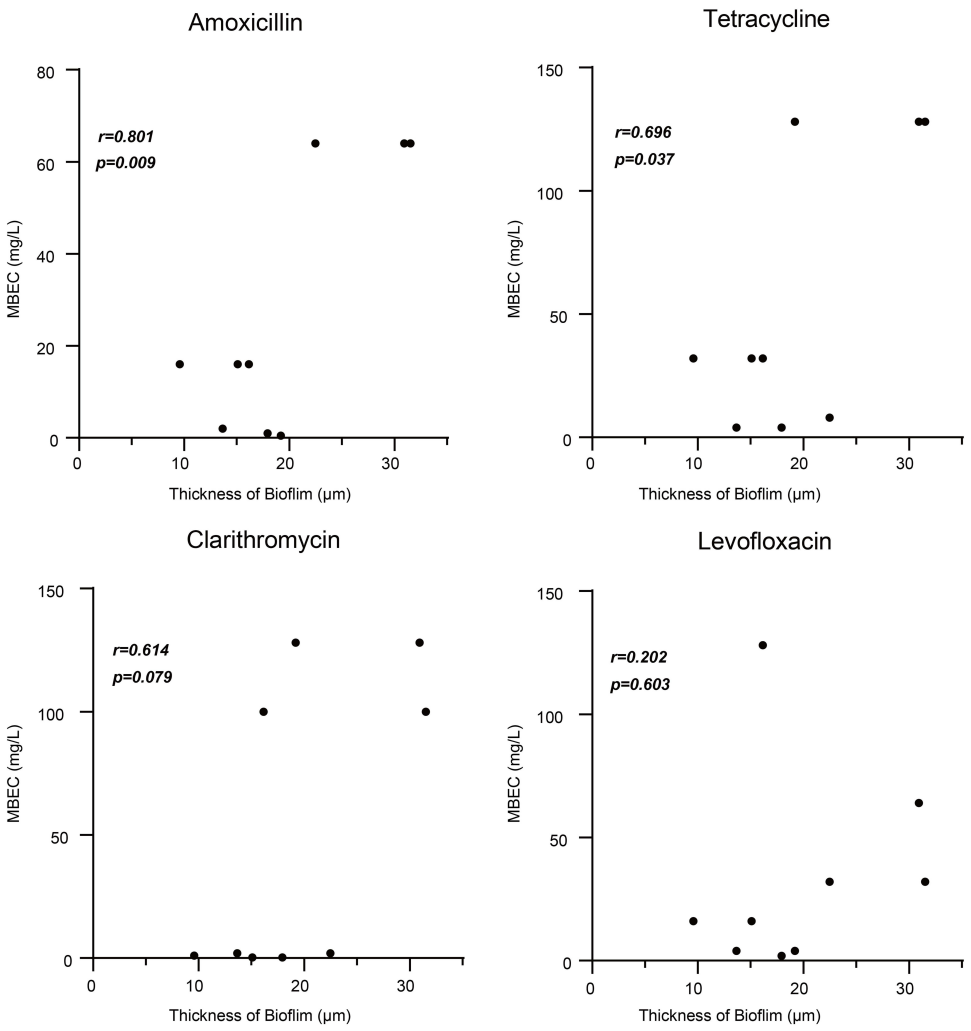


Figure 5 Scatter plot of the correlation between MBEC and biofilm Thickness. The plot demonstrates a positive correlation between in the results of four antibiotics and biofilm thickness ($0 < r < 1$), as determined by the Pearson correlation analysis. $P < 0.05$ indicates a significant correlation.

significantly less biofilm biomass.[11] Together, it is evident that the flagellar system plays a crucial role in *H. pylori* biofilm formation.

OMVs have been recognised as an important component of the extracellular matrix in bacterial biofilms formed by gram-negative bacteria.[31] Similar to many other bacterial species, *H. pylori* is capable of secreting OMVs in a continuous manner.[32] Through TEM, we observed OMVs accumulating within the extracellular matrix of *H. pylori* biofilms compared to their planktonic counterparts. Notably, both strong biofilm-producing strains, HPG5 and HPG6, exhibited a higher density of OMVs in their biofilms compared to that of the weak biofilm-forming strain 26,995. Bacterial adherence to host cells is mediated by adhesins on the bacterial cell surface.[32] Previous studies have identified several adhesin in OMVs released by *H. pylori*, including binding adhesin (BabA), adherence associated lipoprotein (AlpA), sialic acid binding adhesin (SabA), outer inflammatory protein A (OipA).[32–34] These adhesins facilitate the binding of the OMVs to human gastric epithelium, initiating a cascade of inflammatory responses and promoting bacterial adhesion and subsequent invasion. Consequently, the findings demonstrate the essential role of OMVs as constituents of the extracellular matrix of *H. pylori* biofilms. However, the specific components within the OMV contributing to *H. pylori* biofilm formation remain unclear, necessitating further investigations.

Eradicating *H. pylori* infection is an effective measure to prevent metachronous gastric cancer and preneoplastic gastric lesions.[2,4] However, the susceptibility of *H. pylori* to antibiotics directly influences the efficacy of eradication therapy, with

antibiotic resistance emerging as a primary factor contributing to the ineffectiveness of antibacterial intervention. Additionally, a previous trial reported the ability of *H. pylori* to form biofilm on human gastric mucosa biofilm, with these biofilms persisting in patients who failed their treatment, indicating the crucial role of biofilm formation in *H. pylori* resistance to antibiotics.[35] Biofilm-associated bacteria resist antibiotic treatment through two primary mechanisms: antibiotic resistance and tolerance.[8] Brauner et al proposed a framework for classifying bacterial strain drug responses based on the measurement of the MIC and a defined quantitative indicator of tolerance, the minimum duration for killing (MDK).[20] The MIC serves as a key criterion for determining resistance, with only an increase in MIC indicating antibiotic resistance. Conversely, "tolerance" describes the ability, whether inherited or not, of microorganisms to survive transient exposure to high concentrations of an antibiotic without a change in the MIC, often achieved by slowing down an essential bacterial process. In our antimicrobial susceptibility testing assays, significantly higher MBEC values were obtained compared to MIC, surpassing the MIC clinical breakpoints for amoxicillin, levofloxacin and tetracycline. Moreover, some clinical strains in the present study had high values of MICs, even exceeded the clinical breakpoint for certain antibiotics despite their weak biofilm formation ability. Furthermore, a strong association between biofilm thickness and MBEC measurements was observed for amoxicillin and tetracycline, respectively. Together, our results suggest that the conventional MIC testing approach may not provide accurate *H. pylori* antimicrobial susceptibility profiles, and resistance and tolerance to antibiotics may be influenced by a strain's biofilm formation capacity. Biofilm-associated drug resistance and tolerance play a major role in the pathogenesis of many subacute and chronic bacterial diseases, contributing to their resistance to antibiotic treatment. Therefore, for patients with difficult-to-treat *H. pylori* infection, it may be essential to test antimicrobial susceptibility not only for planktonic bacteria but also for biofilm-associated *H. pylori* cells, enabling identification of the true resistance profile, and thereby improving treatment success rate through appropriate antibiotic combinations.

Although this study demonstrated the potential importance of assessing biofilm formation capacity as well as performing the MBEC assay for strains isolated from patients with multiple treatment failures, it is essential to acknowledge the limitations imposed by the relatively small sample size. Therefore, larger-scale studies and additional clinical trials will be necessary.

Conclusion

The present study demonstrates that *H. pylori* isolates exhibit varying levels of biofilm, and the MBEC of antibiotics against certain strains surpasses the MIC for planktonic bacteria within the same strain. Consequently, the formation of biofilms can significantly impact the efficacy of amoxicillin and tetracycline in eradicating susceptible bacterial strains.

Ethics Approval

This study received approval from the Human Medical Ethics Committee of Guizhou Medical University (Ethics review approval number: 2017-52), and all procedures, including the protocol for obtaining informed consent, were conducted in adherence to the principles of the Declaration of Helsinki.

Acknowledgments

The present work was funded by the National Natural Science Foundation of China (No.82260402 and No.82160390), Basic Research Program of Guizhou Science and Technology Plan (No. ZK [2022] 341 and No. ZK [2022] 368), Excellent Young Talents Plan, Youth Science and Technology Talent Growth Project (2022)112, PhD Start-up Funding of Guizhou Medical University 22QNRC02 and [2020]068), Foundation of Key Laboratory of Microbiology and Parasitology of Education Department, Guizhou (QJJ [2022] 019) and the 111 Project (D20009).

Disclosure

The authors declare no conflicts of interest in this work.

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