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Evaluation of *Helicobacter pylori in Vitro* Biofilm Formation by Scanning Electron Microscopy and Associated Genetic Determinants

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Abstract

Background: *Helicobacter pylori* is associated with gastroduodenal ulcer, chronic gastric ulcer, and gastric cancer and is a global public health problem. *H. pylori* develops biofilm on the surface of the human gastric mucosa. Biofilm reduces *H. pylori* eradication by increasing antimicrobial resistance. Detection of biofilm-associated genes by molecular methods may be a step in biofilm eradication strategy. **Materials and Methods:** E Test was applied for clarithromycin, metronidazole, levofloxacin, and tetracycline MIC studies of the strains. The clarithromycin MIC study was compared using the microbroth method. The crystal violet (CV) staining technique (measured at OD_{595}) was used to evaluate the biofilm formation ability of *H. pylori* clinical strains. Biofilm formation images of the strains and antibiofilm images formed by exposing the biofilm to clarithromycin were evaluated using scanning electron microscopy (SEM). After detection of three biofilm-associated genes by PCR, the evolutionary similarities of the strains were evaluated by Sanger sequencing. **Results:** This study investigates the relationship between antibiotic resistance, *in vitro* biofilm formation, and the presence of specific genetic determinants in three clinical isolates and one standard strain of *Helicobacter pylori*. Using antimicrobial susceptibility testing, crystal violet assays, scanning electron microscopy, and PCR, it was found that clinical isolates were highly resistant to clarithromycin and that all strains formed biofilms. The study successfully identified three biofilm-related genes (*lux*S, *rpo*D, *hom*D) across all strains and demonstrated that clarithromycin treatment reduced biofilm biomass in most but not all strains. Six of the nine target genes (*hypothetical protein K747_10375*, *hypothetical protein K747_09130*, *flagellar protein, alpha-(1,3)-fucosyltransferase*, *hypothetical protein K747_06625*, *cag pathogenicity island*

protein) were not detected. In the results obtained by the Sanger sequencing method, it was determined that there was phylogenetic similarity between the *H. pylori* standard strain ATCC 26695 and the three clinical *H. pylori* strains. **Conclusions:** The formation of biofilms by *H. pylori* can be genetically defined based on the capability of the strains to form a biofilm and the number of genes related to this ability.

Keywords

Helicobacter pylori, Biofilm Formation, Scanning Electron Microscopy, Biofilm Genes

1. Introduction

Helicobacter pylori is associated with such conditions as gastroduodenal ulcer, chronic gastric inflammation, and stomach cancer [1]. The International Cancer Research Agency classified the bacterium as a class I carcinogen [2]. *H. pylori*, which affects half of the world's population, is still a public health problem. The hostile acidity of the human stomach constitutes the natural niche of *H. pylori*, indicating the capacity for adaptation [3]. *H. pylori* strains that form biofilms on the surface of the gastric mucosa have been identified *in vivo* [4]. Biofilm is a complex polysaccharide structure that protects and maintains life in the presence of external stress [5]. Biofilms assist bacteria in spreading and persisting within the host. This is due to the fact that the matrix of the biofilm acts as a shield to protect bacteria from antibiotics and host defense mechanisms [6]. Studies have reported that strains that form high levels of biofilms are resistant to antibiotic exposure [7]. Nowadays, the prevalence of *H. pylori's* primary resistance to three antibiotics, namely clarithromycin (CLR), metronidazole (MTZ), and levofloxacin (LEV), is greater than 15 percent in all regions monitored by the World Health Organization (WHO). Because of the high antibiotic resistance, some regions of the world reported a decrease in the treatment rate of *H. pylori* infections after first-line therapy [8]. The formation of biofilms appears to be an important factor influencing susceptibility due to mutations in target genes that contribute to antibiotic resistance through resistance mechanisms [6]. Compared with planktonic bacteria, bacteria that can form biofilms are 10 to 1000 times more resistant to antibacterial agents [9]. Levels of biofilm formation vary among strains; this indicates a potentially complex mechanism involving a particular genotype or variant [10]. Biofilm is characterized by the embedding of cells in an exopolysaccharide matrix layer, exhibiting an altered phenotype in terms of growth rate and gene transcription [11]. Understanding the mechanism of biofilm formation is crucial for the development of *H. pylori* eradication approaches. There are strategies that allow the investigation of genetic mechanisms associated with a specific phenotype [12]. Many signals and gene products have been described that are associated with the early stages of biofilm formation and the three-dimensional structure of mature biofilms. *H. pylori* uses

a two-component chemotaxis signaling system to move in response to chemical cues from the environment. Autoinducer-2 (AI-2) is a quorum-sensing signal that is produced by the *Lux*S protein and accumulates in the bacterial environment in a density-dependent manner [13]. The *H. pylori* genome contains the sigma factor 70 (σ70) encoded by the HP0088 (*rpo*D) gene, which is the homolog of the Gram-negative vegetative sigma factor for the transcription of housekeeping genes in *H. pylori* and is required for general transcription in exponentially growing cells [14]. One family of *H. pylori* outer membrane proteins (OMPs) is the Hom family, a group of four proteins encoded by the genes *hom*A, *hom*B, *hom*C, and *hom*D. A common feature of *H. pylori* OMPs is the antiparallel β-sheets that form a β-barrel, a highly stable pore-like structure. The transmembrane domains of these proteins engage in interactions with host cell receptors. This suggests that OMPs may facilitate bacteria-cell and bacteria-host interactions [15]. Antimicrobial resistance in biofilms is complex and multifaceted. As the ecology and physiology of biofilms are better understood, it will be possible to identify more effective and specific antibiofilm compounds. The development of antimicrobial agents specifically targeting the biosynthesis of the glycocalyx or signaling molecules is necessary.

This study aimed to define the relationship between antibiotic resistance and biofilm formation ability among three clinical *H. pylori* strains and the standard *H. pylori* ATCC 26695 strain, to determine the biofilm formation potential of the strains by scanning electron microscopy, to determine the morphological similarities and differences between the strains, to evaluate the antibiofilm effect of clarithromycin, to identify 9 biofilm-associated genes (*lux*S, *rpo*D, *hom*D, *hypothetical protein K747_09130, flagellar protein, hypothetical protein K747_10375*, *alpha-(1,3)-fucosyltransferase*, *hypothetical protein K747_06625*, *cag pathogenicity island protein*) by PCR, and to investigate the phylogenetic similarities of the strains. The findings here will allow us to evaluate the potential usability of a genomic approach by correlating *H. pylori* biofilm formation with the identified genes.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Three clinical isolates and *H. pylori* ATCC 26695 were used in the study. *H. pylori* ATCC 26695 was purchased from the American Type Culture Collection (Rockville, MD, USA) and utilized exclusively for this investigation. According to the instructions in the catalogue, *H. pylori* ATCC 26695 was reviewed and subcultured, and aliquots were kept at −80˚C for use in later studies. Clinical isolates were retrospectively obtained from the patients' antrum and corpus gastric biopsies. Rapid urease test (RUT) and histopathology were also performed. Three clinical isolates in stock culture medium stored in aliquots in BHI medium supplemented with 20% glycerol at −80˚C were brought to room temperature and cultured on Columbia Blood agar (Oxoid) medium containing 7% defibrinated Horse Blood (SR0050C, Thermo scientific**,** Oxoid) with *H. pylori* Selective

Supplement (DENT, Oxoid) for three days at 37˚C in microaerophilic conditions using the GasPak Campy Container System (Becton Dickinson and Company) in an anaerobic container (Oxoid). Subcultures were made from the colonies that grew as a result of incubation. Motility characteristics of *H. pylori* colonies grown in culture were evaluated, and catalase, urease, and oxidase tests were performed.

2.2. Evaluation of Antimicrobial Susceptibility Testing with the E-Test Method

Antibacterial susceptibility testing was performed using the E-test to determine the minimal inhibitory concentration (MIC) values of three *H. pylori* clinical strains and the *H. pylori* standard strain ATCC 26695 against metronidazole, clarithromycin, tetracycline, and levofloxacin.

Mueller-Hinton agar supplemented with 7% defibrinated horse blood was used for this purpose. Bacterial suspensions with a standard turbidity of 3.0 McFarland (~6×10^8 CFU/ml) were obtained using the Densimat device (Biomerieux SA, France) and spread on Mueller–Hinton agar supplemented with 7% defibrinated horse blood. Once the agar surface had dried, the E-test (Biomerieux Test Strips, France) strips for the selected antibiotics were placed on the agar. The plates were incubated for three days at 37˚C in a microaerobic environment. The MIC values (mg/L) of clarithromycin, levofloxacin, metronidazole, and tetracycline were ≤0.25, ≤1, ≤8, and ≤1, respectively. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria were used to evaluate the results [16].

2.3. Assessment of Clarithromycin Minimum Inhibitory Concentration (MIC) by Microbroth Method

The antibiotic susceptibility profile of the three clinical strains *H. pylori* and *H. pylori* standard strain ATCC 26695 was determined by the microbroth dilution method against clarithromycin.

A stock solution of 100 µg/mL (v/v) was prepared by adding 9 mL of phosphate buffered saline (PBS) to 1 mL of clarithromycin solution. The stock solution was sterilized using a 0.22 µm sterile syringe filter and made ready for use.

A bacterial suspension equivalent to a 3.0 McFarland standard was prepared from a three-day subculture on Columbia Blood Agar (Oxoid) medium containing 7% defibrinated horse blood and *H. pylori* Selective Supplement (DENT, Oxoid). A 100 µl volume of Mueller-Hinton broth supplemented with 10% FBS and a 25 µl volume of the bacterial suspension were added into every well of a 96-well microplate. Finally, 100 µl of clarithromycin was added to the initial well, and two-fold serial dilutions were made to the other wells. The last well in the plate was evaluated for medium contamination control, and the previous well was evaluated for bacterial growth in the medium. The plates were incubated at 37˚C for three days under microaerophilic conditions. Then, the plates were examined for growth inhibition. The MIC was defined as the lowest concentration of antibiotic that completely inhibited visible bacterial growth. The study was conducted three times at different

time points.

2.4. Biofilm Formation and Its Quantification

Bacteria were cultured on Columbia Blood Agar (Oxoid) medium containing 7% defibrinated horse blood and *H. pylori* Selective Supplement (DENT, Oxoid). Then the bacterial growth in the culture was collected in 1 ml of Brucella broth (BB, Biolife, Italiana). Bacterial suspensions with a standard turbidity of 3.0 McFarland (~6 × 10^8 CFU/ml) were obtained using the Densimat device (Biomerieux SA, France). 20 µl of *H. pylori* suspension and 180 µl of Brucella Broth supplemented with 10% fetal bovine serum (Capricorn Scientific, USA) were added into the wells of a 96-well flat-bottom sterile polystyrene microtiter plate (Greiner Bio-one Austria). Biofilm formation was evaluated by incubation in an anaerobic jar in a microaerophilic environment for three days at 37˚C. The planktonic cell suspension was removed by washing the microplate three times with sterile phosphate-buffered saline (PBS, pH 7.3). The microplate was then left to air-dry for a period of one hour. Cells attached to the microplate were fixed for 15 minutes by adding 200 µl methanol (Merck, Germany) to each well. The wells were emptied and dried for an hour. Each well was stained for 5 minutes with 200 µl of 1% (w/v) crystal violet (CV) (Merck, Germany) and emptied. The wells were washed two times with PBS and air-dried for 15 minutes. Then 200 µl ethanol-acetic acid (ethanol: acetic acid = 95:5) (Merck, Germany) was added to the wells stained with crystal violet and waited for 1 minute. The amount of biofilm was measured with a microtiter plate reader (Biotek-Synergy Ht, USA) at a wavelength of 595 nm. These experiments were conducted in triplicate. In this study, measurement of wells containing only medium was used as a negative control. The formation of biofilms was evaluated according to three categories: a negative biofilm-former OD_t < control ODc, a weak biofilm-former ODt ≥ the control ODc, and a strong biofilm-former ODt ≥ 2 times the control ODc [17].

2.5. Scanning Electron Microscopy (SEM)

2.5.1. Detection of the Presence of *H. pylori* Biofilm Formation in the Microplate Environment

Subcultures of the strains were performed on Columbia Blood Agar medium containing 7% defibrinated horse blood and a *H. pylori* selective supplement. The resulting cultures were collected in 3 ml of Brucella broth, and the bacterial density was adjusted to 3.0 McFarland (6 × 10^8 CFU/ml). Twenty-four flat-bottom sterile microplates (Biolife, Italy) were used to assess the formation of *H. pylori* biofilms. Round sterile coverslips (Isolab) with a diameter of 12 × 12 mm were placed in the microplate wells. 925 µl Brucella broth consisting of 10% fetal bovine serum and 25 µl *H. pylori* bacterial suspension were transferred to microplate wells. Microplates were incubated for four days at 37˚C in a microaerophilic environment using the GasPak Campy Container System (Becton Dickinson and Company). After incubation, the planktonic cell suspension in the well was removed with a pipette. Bacteria were fixed by adding

2.5% glutaraldehyde and 4% paraformaldehyde (PFA) in Sorenson buffer to the wells.

The dehydration was carried out with an alcohol series (50%, 70%, 95%, 100%) the day before the imaging [16]. The final critical drying was completed overnight with HMDS (Hexamethyldisilazane). On the day of imaging, coverslips were placed on aluminum stubs with double-sided carbon tape, and a 10 nm Au/Pd sputter coating was applied. The ready-for-imaging coverslips were viewed at 5,00x and 20,00x magnifications with the SE2 detector on a Zeiss Sigma 500 FESEM. The study was carried out by the Izmir Biomedicine and Genome Center Electron Microscopy Research Support Unit.

2.5.2. Plate Study Investigating the Clarithromycin Antibiofilm Effect

Preparation and dilution of clarithromycin stock solution

Clarithromycin solution was prepared by dissolving 25 mg clarithromycin (Sigma-Aldrich) in 25 mL ethanol (v/v). A stock solution of 100 µg/mL (v/v) was obtained by adding 9 mL of clarithromycin solution to 1 mL of phosphate-buffered saline (PBS). The stock solution was sterilized with a 0.22 µm sterile syringe filter, and 2-fold serial dilutions were made with brucella broth in sterile glass tubes. The final concentration was left at 1.57 µg/mL (v/v).

For the study, previously sterilized round 18 mm coverslips were placed in the wells of the 12-well plate under aseptic conditions. A 4-day biofilm study was performed as mentioned above, with a final volume of 950 µl in the wells. At the end of the 4th day, 950 µl of 1.57 µg/ml clarithromycin was added to each well of a plate of each strain, and the plates were incubated in a microaerophilic environment at 37˚C in an anaerobic jar for 24 hours. Then, the SEM application steps were applied as mentioned above.

2.6. Molecular Analysis

2.6.1. DNA Extraction

Bacterial suspension was prepared from colonies of the *H. pylori* ATCC 26695 standard strain and three *H. pylori* clinical strains grown on Columbia Blood Agar medium containing 7% horse blood supplemented with *H. pylori* Selective Supplement DENT (Oxoid) in a sterile tube containing 1 ml of Phosphate Buffered Saline (PBS, Biochrom AG) using the Automatized Densimat (Biomerieux, France) device at a turbidity of McFarland 3 (6×10^8 CFU/mL), and a commercial microbial DNA isolation kit (DNeasy UltraClean Microbial Kit, Hibrigen, Kocaeli, Türkiye) was employed for the purpose of obtaining the DNA of the strains. Extraction procedures were conducted in accordance with the manufacturer's instructions.

2.6.2. Polymerase Chain Reaction (PCR)

Identifying genes related to biofilm formation

Nine genes related to the biofilm formation ability in *H. pylori* standard strain 26695 and three clinical strains were investigated. The PCR method was used to

define the existence of 9 gene regions (*lux*S, *rpo*D, *hom*D, *hypothetical protein K747_10375, flagellar protein, alpha-(1,3)-fucosyltransferase, hypothetical protein K747_06625, hypothetical protein K747_09130, cag pathogenicity island protein).* Amplified products were resolved by gel electrophoresis on 1% agarose (Seakem® LE Agarose, Lot no. 50004, Rockland, USA) and then, gel images were taken with the Gen Box SDR Bio-Imaging System. Bands with sizes of 1409 bp for *hom*D, 179 bp for *lux*S and 119 bp *rpo*D were considered as positive results (Table 1).

2.6.3. Sanger Sequencing

In the polymerase chain reaction (PCR) product purification step, the HighPrep™ PCR Clean-up System (MAGBIO, Catalogue No: AC-60050) kit was employed for the purification of amplicons derived from the *hom*D, *lux*S, and *rpo*D target gene regions. For Sanger sequencing samples after the PCR product purification step (Table 1), the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the ABI 3730XL Sanger sequencer (Applied Biosystems, Foster City, CA) were employed for this purpose. The master mix content prepared for Sanger sequencing is given in Table 2. After aligning the sequence data, phylogenetic trees were constructed using the UPGMA method.

Table 1. Primers used for amplification of *H. pylori* biofilm genes.

Gene	Primer sequence (5'-3')	Product size (bp)	PCR condition
outer membrane protein (*Hom*D)	F: GACGCTCAAGGCAAGGTAG R: AACACATCCATTCCCCCACC	1409	95˚C, 2 min; 40x (95˚C, 30 s; 57˚C, 30 s; 72˚C, 1 min); 72˚C, 7 min
*rpo*D	F: TCATCGTCATCATCAAAGCTC R: TTAGACGGGATTTGCTCGGTG	119	95˚C, 2 min; 40x (95˚C, 30 s; 58˚C, 1 min; 72˚C, 1 min); 72˚C, 5 min
*lux*S	F: GCGTTAATGGGGATTTGATTG R: TGTGAGATAAAATCCCGTTTGG	179	95˚C, 2 min; 40x (95˚C, 30 s; 55˚C, 1 min; 72˚C, 1 min); 72˚C, 5 min
hypothetical protein K747_10375	F: CATCTCGCGTGATGGGGT R: TCTTCTTGCTTTTTGGCGAT	426	
hypothetical protein K747_09130	F: GAGTGGGATAGAGTTAGAAC R: GTATTAGCCGCTGCTTC	777	
flagellar protein	F: GTGAGTTGTGCATACGCT R: AGGCCACTGAGTTTTTAGGT	388	
alpha-(1,3)-fucosyltransferase	F: TCCAGCCCTTACTAGACGCT R: AGCTCCAAAAGAGGGGTAGC	1279	
hypothetical protein K747_06625	F: GGCTCACCACTATACCGCTT R: TGACCGGCTCTTTTGTGTCA	1089	
cag pathogenicity island protein	F: AACGCTCCATCAAGAGCCAA R: CCCGCTCTTGCTTCCTTACT	1332	

(F) = Forward primer, (R) = Reverse primer, (bp) = Base pair.

Table 2. Sanger sequencing master mix content.

Components	volume (µl)
BigDye	1 µl
27F/1100R primer	0.32 µl
5X Buffer	2 µl
ddH_2O	2,68 µl
PCR product	4 µl
Total Volume	10 µl

F: Forward R: Reverse.

3. Results

Three *H. pylori* isolates obtained by culture and the *H. pylori* standard strain 26695 were tested for antibacterial susceptibility. In accordance with the EUCAST interpretive criteria, the resistance rate of the *H. pylori* isolates to the tested antibiotics was as follows (Figure 1): Clarithromycin 75% (n = 3) (clinical strain 1: 256 µg/ml, clinical strain 2: 256 µg/ml, clinical strain 3: 256 µg/ml; *H. pylori* ATCC 26695: ≤0.25 µg/ml), metronidazole 50% (n = 2) (clinical strain 1: 0.016 µg/ml, clinical strain 2: 256 µg/ml, clinical strain 3: 256 µg/ml; *H. pylori* ATCC 26695: 0.016 µg/ml), levofloxacin 50% (n = 2) (clinical strain 1: 0.12 µg/ml, clinical strain 2: 256 µg/ml, clinical strain 3: 256 µg/ml; *H. pylori* ATCC 26695: 0.016 µg/ml) were resistant, and all 4 isolates (clinical strain 1: 0.016 µg/ml, clinical strain 2: 0.016 µg/ml, clinical strain 3: 0.016 µg/ml; *H. pylori* ATCC 26695: 0.016 µg/ml) (100%) were susceptible to tetracycline. Three clinical strains of *H. pylori* were resistant and the *H. pylori* standard strain ATCC 26695 was susceptible to clarithromycin in the micro-broth dilution method (clinical strain 1: 100 µg/ml, clinical strain 2: 100 µg/ml, clinical strain 3: 100 µg/ml; *H. pylori* ATCC 26695: 0.78 µg/ml).

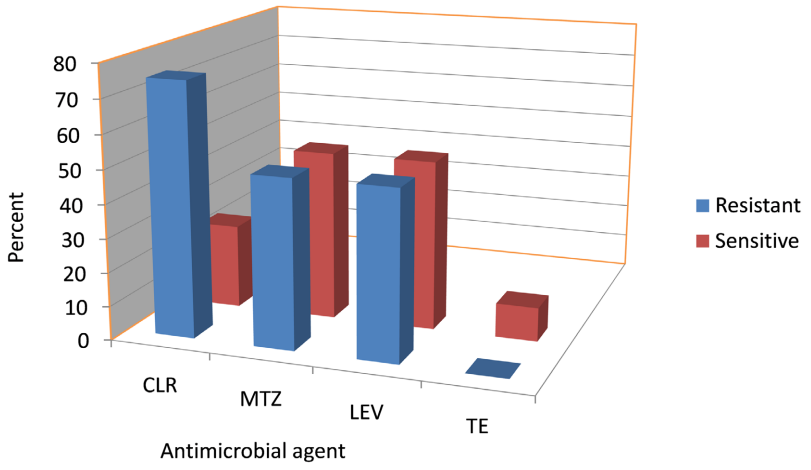

Figure 1. Antibiotic susceptibility percentages for 3 *H. pylori* clinical isolates and the *H. pylori* standard strain 26695. CLR: Clarithromycin, MTZ: Metronidazole, LEV: Levofloxacin, TE: Tetracycline.

The biofilm-forming capacity of *H. pylori* strains in culture media was determined in a microplate-based assay using flat-bottomed polystyrene microtitre plates (Greiner bio-one Austria) with CV staining. The results were evaluated by spectrophotometric measurement (OD_{595}). Three *H. pylori* clinical strains and one *H. pylori* standard strain 26695 (n = 4) were used in the study.

The results obtained from the experiments were as follows: BB + 10% FBS 75% (n = 3) strong biofilm producer, 25% (n = 1) weak biofilm producer (Figure 2, Table 3).

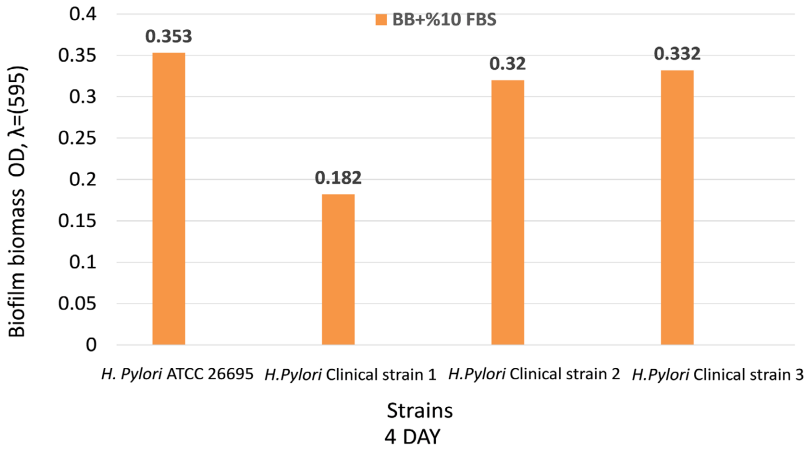

Figure 2. Four-day biofilm evaluation of strains in culture medium containing BB + 10% FBS.

Table 3. Average OD_T of *H. pylori* Biofilm Formation in BB + 10% FBS culture media.

	*OD_T of BB + 10% FBS	Number and Percentage for Biofilm Formation on Culture Media
**OD_C	0.142	weak biofilm (n = 1) (25%) strong biofilm (n = 3) (75%)
H. pylori ATCC 26695	0.353	strong
H. pylori clinical strain 1	0.182	weak
H. pylori clinical strain 2	0.320	strong
H. pylori clinical strain 3	0.332	strong

*OD_T = Optical Density of the isolates **OD_C = Optical Density of the controls (BB + 10% FBS).

Four-day biofilm formation images of four strains (first clinical strain, second clinical strain, third clinical strain, *H. pylori* ATCC 26695) were evaluated. Biofilm formation was observed in all strains. In the images acquired with scanning electron

microscopy (SEM), ATCC 26695 and clinical strain 3 showed coccoid morphology, and clinical strain 1 and clinical strain 2 showed bacillus morphology, within the biofilm consisting of cells with coccoid and bacillus morphology.

It is thought that the metabolic status of the strains may result in the ability to form biofilms and morphological changes (Figure 3).

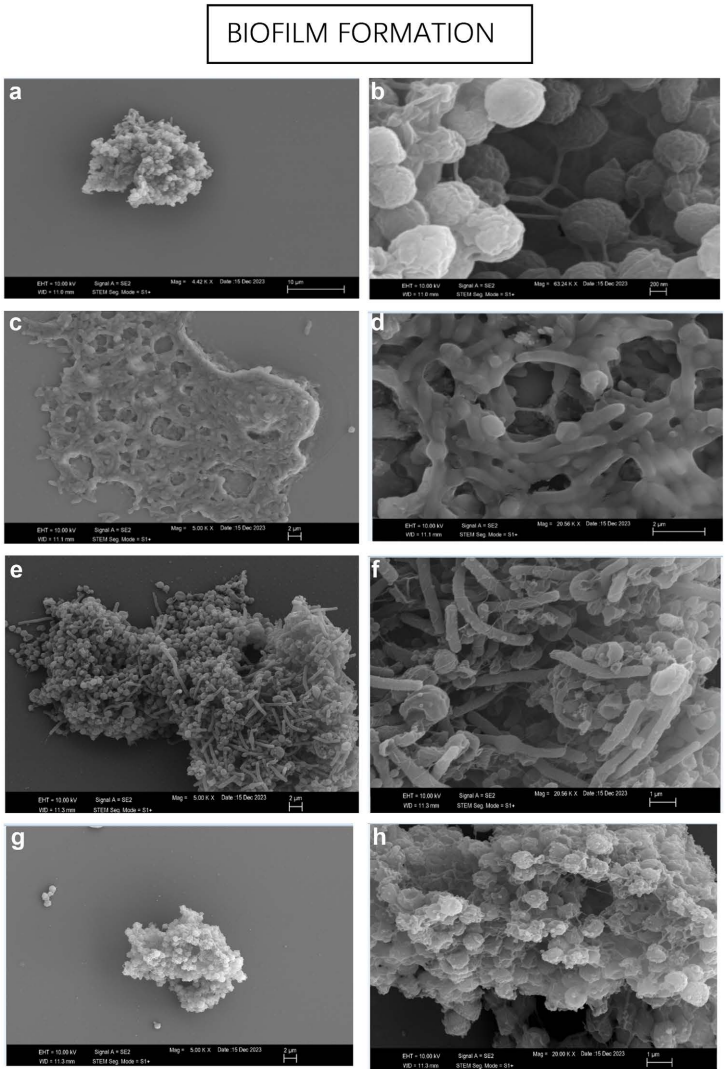

Figure 3. SEM images of biofilms *H. pylori* 26695 strain (a, b), clinical strain 1 (c, d), clinical strain 2 (e, f), clinical strain 3 (g, h). The 4-day-old biofilm of each strain on the coverslip was investigated using SEM. Photos ×500; ×20.00; taken at magnification. Scale bar (1 - 2 μm) shown at the bottom of each electron micrograph.

More examination of the biofilms with SEM revealed *H. pylori* cells, composed of coccoid, spiral, and filamentous forms stacked in a few strata, firmly packed and surrounded by an extracellular matrix. In the biofilms of clinical strain 2 and clinical strain 3, the presence of tiny spherical outer membrane vesicles (OMVs) was detected. *H. pylori* biofilm formation was a complex process involving physiological changes resulting in morphological changes such as coccoid, spiral,

and filamentous forms. According to the obtained images, not all mature biofilm cells develop uniformly. The morphological differences observed in *H. pylori* biofilms were specific to the *H. pylori* species and varied depending on natural metabolic activity.

In the images obtained by exposing four strains (1st Clinical strain, 2nd Clinical strain, 3rd Clinical strain, *H. pylori* ATCC 26695) to clarithromycin, no change was observed in the biofilm biomass of the 2nd Clinical strain, while a significant deterioration was observed in the biofilm biomass of the other three strains (1st Clinical strain, 3rd Clinical strain, *H. pylori* ATCC 26695) (Figure 4).

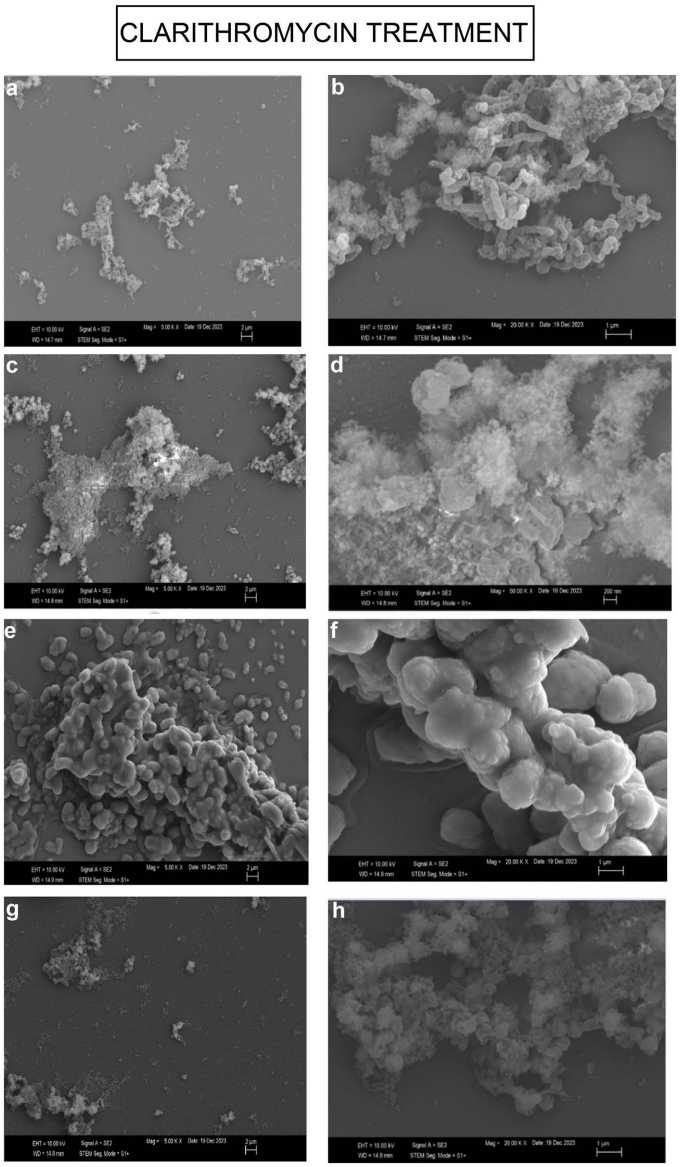

Figure 4. Scanning electron microscope (SEM) images of biofilms of *H. pylori* 26695 and 3 clinical strains exposed to 0.78 μg/mL clarithromycin. SEM images of biofilms of *H. pylori* strain 26695 (a, b), clinical strain 1 (c, d), clinical strain 2 (e, f), clinical strain 3 (g, h). Photographs were taken at magnifications of ×500; ×20,000. Scale bars (1 - 2 μm) are shown below each electron microscope image.

Three biofilm-associated genes (*lux*S, *rpo*D, *and hom*D) were successfully identified in the study (Figure 5). Biofilm based on both phenotypic and genotypic features was evaluated as biofilm positive/gene positive (n = 4, 100%).

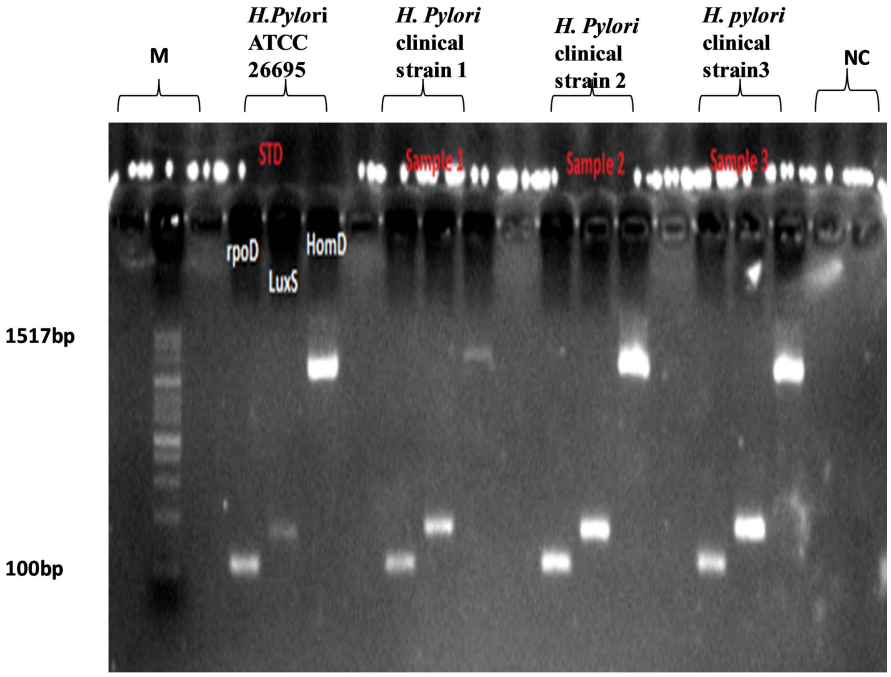

Figure 5. Agarose gel electrophoresis of PCR products obtained using primer pairs *lux*S (179 bp), *rpo*D (119 bp), and *hom*D (1409 bp). NC: Negative control (distilled water), PC: Positive control (STD; *H. pylori* ATCC 26695), Representative positive samples (1, 2, 3): *H. pylori clinical strains* in this study. M: Marker, 100bp DNA Ladder H3 RTU, GeneDireX.

According to the findings obtained from Sanger sequencing, it was decided that there was an important amount of differentiation in the DNA of sample number 2 for the *hom*D gene region in the phylogenetic tree created. Additionally, many single nucleotide differences were detected in samples 1 and 3 compared to the reference DNA (*H. pylori* ATCC 26695) (Figure 6).

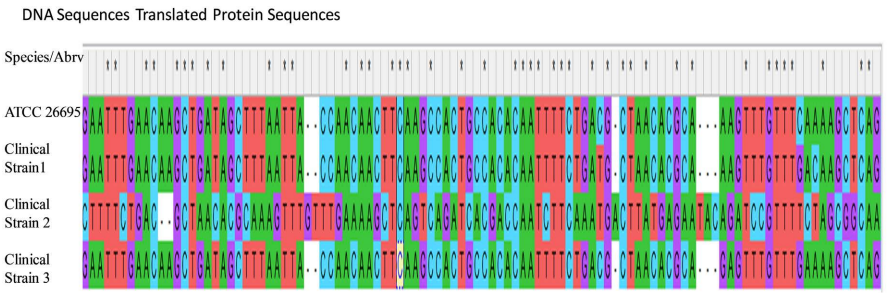

Figure 6. Comparison of *hom*D gene sequences of *H. pylori* clinical strain 1, *H. pylori* clinical strain 2, *H. pylori* clinical strain 3 with the *H. pylori* ATCC 26695 reference gene.

For the *lux*S gene region, it was shown that there was a limited level of differentiation due to one nucleotide alteration in the DNA of sample number 3

(Figure 7).

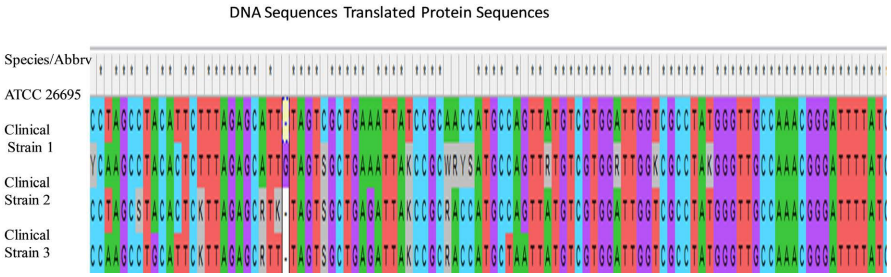

Figure 7. Comparison of the *lux*S gene sequences of *H. pylori* clinical strain 1, *H. pylori* clinical strain 2, *H. pylori* clinical strain 3 with the *H. pylori* ATCC 26695 reference gene.

It was shown that there was differentiation in the DNA of sample number 2 for the *rpo*D gene region (Figure 8).

Figure 8. Comparison of *rpo*D gene sequences of *H. pylori* clinical strain 1, *H. pylori* clinical strain 2, *H. pylori* clinical strain 3 with the *H. pylori* ATCC 26695 reference gene.

Following the results, it was decided that there was a limited degree of differentiation in the phylogenetic tree constructed for the *rpo*D gene region due to one nucleotide alteration in the DNA of sample number 3 (Figure 9).

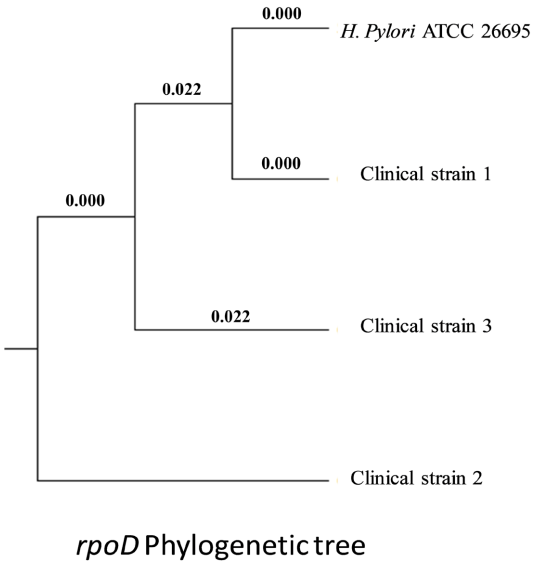

Figure 9. *rpo*D phylogenetic tree.

For the *hom*D gene region, an important quantity of variation was found in the DNA of sample number 2. In addition, many single nucleotide variations were detected in samples 1 and 3 compared to the reference DNA (*H. pylori* ATCC 26695 reference gene) (Figure 10).

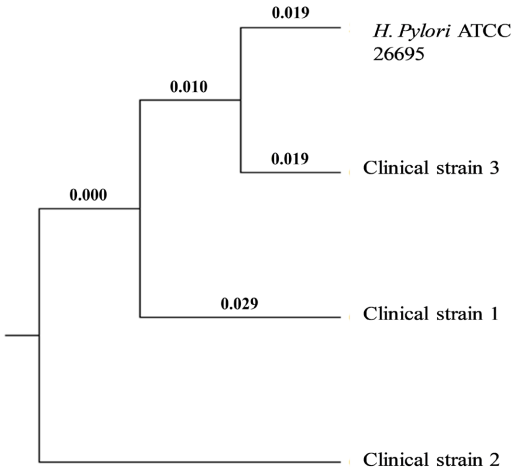

Figure 10. *hom*D phylogenetic tree.

According to the results, it was determined that there was a limited degree of differentiation in the phylogenetic tree constructed for the *lux*S gene region due to one nucleotide alteration in the DNA of sample number 3 (Figure 11).

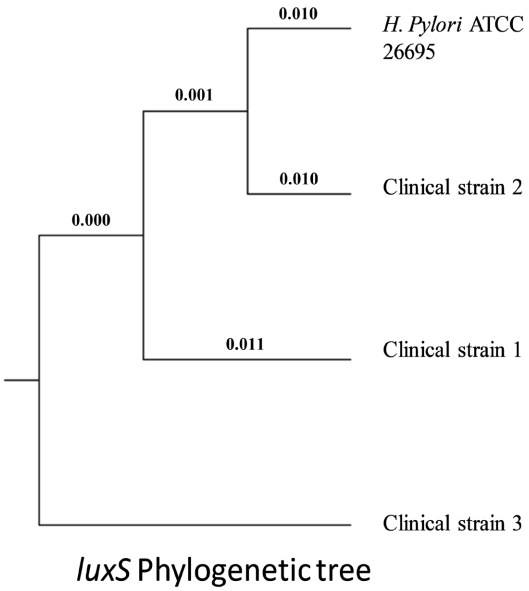

Figure 11. *lux* S phylogenetic tree.

4. Discussion

The main difference between planktonic bacteria and biofilm bacteria is that the

bacteria are tightly packed and encapsulated in the extracellular polymeric substance (EPS) that they excrete.

The matrix effectually changes the characteristics of the bacteria, facilitates first adherence to the dynamic surface, and prevents the aggregation of extreme bacteria [18]. Previous studies have shown that *H. pylori* is capable of forming biofilms both *in vivo* and *in vitro* [19].

Therefore, the biofilm formation capability in *H. pylori* was evaluated in this study using scanning electron microscopy [20]. All three clinical strains of *H. pylori* and the ATCC 26695 strain of *H. pylori* tested in this study showed that they were able to form biofilms *in vitro*, varying from strong to weak. The observations of spiral-shaped, coccoid, and filamentous *H. pylori* cells by SEM biofilm examination contradicted Cole *et al.*'s observations, which showed that mature *H. pylori* biofilms composed of coccoid cells from 3 to 5 days were formed [21]. Another study found that the formation of *H. pylori* biofilms was a complex process that involves various physiological transformations and causes morphological changes such as coccoid, filament, and spiral forms [22]. In this work, it was predicted that morphological differences in *H. pylori* resulted in different metabolic activities and rates between strains because of the nature of the bacteria. By SEM analysis, dense bundles of thin filaments forming bacterial flagella were observed surrounding bacterial cells in mature *H. pylori* biofilms, except for clinical strain 1. Findings have shown that flagella are important for keeping cells together as well as for bacterial mobility [23]. In *H. pylori*, it was shown that the flagella assembly genes in *H. pylori* cells that form biofilms were significantly regulated, while the flagella mutant strain was shown to reduce biofilm biomass [19].

These evaluations show that the flagella system plays a crucial part in the formation of the *H. pylori* biofilm. OMVs are crucial components of the extracellular matrix produced by Gram-negative bacteria. The biological role of these vesicles has not been fully elucidated, but they are described to be involved in toxin delivery, protein and DNA transfer, and signalling between bacteria. Biochemical characterization of vesicles isolated from pathogenic species has identified virulence-associated factors such as toxins, invasins and host-effector molecules. *H. pylori* has the ability to continuously secrete OMVs. *H. pylori* OMVs have been associated with gastric disease [24] The presence of OMVs was detected in clinical strain 2 and clinical strain 3, two strong biofilm-forming strains.

The adhesion of bacteria to host cells is mediated by adhesins on the bacterial cell surface. Previous studies have shown various adhesins in OMVs released with *H. pylori*; these are binding adhesin (*Bab*A), adhesion-related lipoprotein (*Alp*A), sialic acid-binding adhesin (*Sab*A) and external inflammatory protein A (*Oip*A). These adhesins allow OMVs to bind to human gastrointestinal epithelium, causing inflammatory reactions and stimulating bacterial adhesion [25]. *H. pylori*'s ability to form biofilms on the surface of the human stomach was shown to play a crucial role in antibiotic resistance [26]. In determining antimicrobial susceptibility, the Epsilometer test (E-test) is used in conjunction with the broth

microdilution or agar method to determine the minimum inhibitory concentration (MIC). However, in most cases, MIC measurements do not correctly reflect the concentrations required to eliminate biofilm infections, which affects the potency of the treatment [27]. *H. pylori* ATCC 26695 in the current study had low MIC values despite its strong biofilm-forming ability. In this work, it is important to accept the limitations imposed by the size of small samples when assessing the capacity to form biofilms using antimicrobial agents.

H. pylori-associated diseases cause significant health problems worldwide. As a result of the increase in antimicrobial resistance, *H. pylori* has become one of the most successful human pathogens, evading host immune responses.

Current antibiotic-based therapy against *H. pylori* infection, the main cause of gastric adenocarcinoma, causes dysbiosis and significant treatment failures due to decreasing eradication rates [28] [29]. The capacity of *H. pylori* to form biofilms results in resistance to treatment [30].

In the antibiofilm study of clarithromycin, a strong antibiofilm effect was observed in other strains except clinical strain 2. Apart from our current study, no correlation was obtained with the study of Fauzia *et al.*, which was the only study that determined the relationship between antibiotic resistance of different *H. pylori* strains and biofilm [17]. It is noteworthy that the need to increase research on *H. pylori* biofilm is urgent as an important strategy to prevent the spread of antibiotic resistance [31]. The formation of biofilms is a mechanism that involves complex interactions and regulatory processes among bacteria. In vitro studies of biofilm formation in *H. pylori* revealed the generation of a substantial biomass after 72 hours [32]. Bacterial-bacterial interactions, motility and adhesion, which are crucial in the initiation of biofilm formation, are genetically different in good and poor biofilm-forming strains. In the bacterial signalling system, a molecule called autoinducer (AI) plays a crucial role in the formation of bacterial biofilms [33]. *H. pylori* expresses the *lux*S gene, which produces the autoinducer 2 (AI-2) quorum sensing molecule. Cole and colleagues reported the relationship between the *lux*S gene and the formation of biofilms in *H. pylori* [21]. In a study by Rader *et al.*, *lux*S mutants showed low mobility and highlighted the importance of AI-2 quorum sensing molecules as regulators of *H. pylori* flagella-related genes [34]. Jolaiya *et al.* and De la Cruz *et al.* in their studies showed that mutation in the *rpoD* gene caused *H. pylori* to lose its ability to grow [35] [36].

When strains were assessed under the same conditions, biofilm formation was shown to be significantly higher in some strains than in poor biofilm-forming strains, indicating diversity in biofilm formation. The identification of a specific gene region in good biofilm-forming *H. pylori* strains suggests that the biofilm formation phenotype was influenced by more than one genetic factor. Using comparative genomic approaches, the data suggest that the formation of biofilms in *H. pylori* might be affected by related genes [37].

In the study, three genes (*lux*S, *rpo*D, *hom*D) out of nine biofilm-associated target genes were identified in all strains, while six (*hypothetical protein*

K747_10375, *hypothetical protein* K747_09130, *flagellar protein*, *alpha-(1,3)-fucosyltransferase*, *hypothetical protein* K747_06625, *cag pathogenicity island protein*) were not detected. This is a significant negative finding that warrants exploration, including potential reasons such as strain-specific genetic variability, primer design limitations, or true absence in these isolates and its implications. Wong *et al*., by comparing the annotated genes of poor, moderate and good biofilm-forming *H. pylori* strains, reported that 3 hypothetical genes (*hypothetical protein* K747_06625*, hypothetical protein* K747_09130 *and hypothetical protein* K747_10375) and 4 functional genes (*flagellum protein, outer membrane protein (hom*D*), cag pathogenicity island protein and alpha-(1,3)-fucosyltransferase*) are associated with the ability to form biofilms [38]. Moadelighomi *et al*. conducted a qRT-PCR analysis of the effect of eugenol on the formation of the biofilm and the expression levels of *H. pylori rpo*D and *lux*S genes and showed that *rpo*D and *lux*S genes played an essential role in the formation of the biofilm [14]. Fauzia *et al*. investigated the nucleotide polymorphisms (SNPs) related to the phenotype of *H. pylori* biofilm formation and determined the presence of *alp*A, *alp*B, *glu*P, *csd*4, *cag*E, *csd*5, *hom*D, *fut*B genes in biofilm formation [17].

The findings, compared with the results of previous studies, show a significant correlation between the presence of genes involved in biofilm formation and the observed results. Despite the lack of biofilm genes examined, biofilm production capacity suggests that other genetic determinants participate in matrix formation in *H. pylori*. It is also important to note that biofilm formation in the complex gastric mucosal environment may involve additional factors not addressed in this study.

5. Conclusions

The data obtained in this study demonstrate that clarithromycin is effective in inhibiting the growth and biofilm formation of *H. pylori in vitro*. This suggests that various environmental factors contribute to clarithromycin resistance. Furthermore, suppression of the *rpo*D, *hom*D, and *lux*S genes, which are associated with biofilm formation in *H. pylori*, may negatively impact biofilm formation in this bacterium and increase bacterial susceptibility to various environmental conditions.

Weak and strong biofilm-forming strains may differ genetically in terms of motility, bacterial-bacterial interactions, and adhesion. The results obtained from this study indicate that biofilm formation in *H. pylori* can be genetically defined and may be affected by multiple genes. Although the antibiofilm effect of clarithromycin was observed in the study, antimicrobial resistance in biofilms is complex and multifaceted. The development of antimicrobial agents targeting the biosynthesis of glycocalyx and signaling molecules is necessary.

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Conflicts of Interest

The author reports no relevant conflicts of interest for this article.

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