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Genotypes and Phylogenetic Analysis of *Helicobacter pylori* Clinical Bacterial Isolates

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Abstract: *Helicobacter pylori* is a human pathogen bacterium associated with gastritis, peptic ulcer, and gastric cancer. It can be identified through the 16S rRNA gene and characterized through *cagA* and *vacA* virulence genes. Clinical cultures of *H. pylori* were isolated and identified from human stomach biopsies. The isolates were characterized according to their colonial and microscopic morphology, and molecular genotyping was conducted to determine the bacterial virulence. A phylogenetic analysis of the 16S rRNA gene sequencing was performed. In addition, multilocus sequence typing analysis was performed to determine the phylogeographic nature of the isolated strains. Three bacterial isolates were selected from 22 gastric biopsies, identified as *H. pylori* through colonial morphology, Gram staining, urease, catalase, and oxidase tests and identification of the *ureC* gene through end-point PCR. Amplification of 16S rRNA, *urea*, and *tonB* genes was performed, as well. Differences between the *cagA* and *vacA* genotypes were determined among the isolates. The phylogenetic analysis confirmed the identity of the three isolates as the specie *Helicobacter pylori*. Different genotypes were obtained for each *H. pylori* strain, and all the clinical isolates showed the *vacA* s2/m2 genotype, indicating an absence of the VacA cytotoxin. Only HCGDL-MR01 is a *cagA* gene carrier with a greater risk to develop a serious disease, such as stomach cancer and peptic ulcer. The multilocus sequence typing placed all the strains within the hpEurope population structure.

Keywords: *H. pylori*; virulence factor; 16S rRNA; gastritis

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1. Introduction

Helicobacter pylori—a human pathogen bacterium distributed around the world—was isolated by Marshall and Warren for the first time in 1983, demonstrating its relationship with active gastritis [1], duodenal gastric ulcer [2], gastric cancer [1], and mucosa-associated lymphoid tissue (MALT) lymphoma [3]. *H. pylori* was classified as carcinogen Class 1 in 1994 by The International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) [3]. This bacterium is a Gram-negative [2], spiral-shaped [1], microaerophilic bacterium. Its dimensions are ~0.5 µm width and ~2.5 µm length, and it presents from two to six polar flagella [4]. One of the main colonization mechanisms of *H. pylori* is the production and release of urease, which allows hydrolyzation of urea in ammonium and CO_2 to generate an alkaline microenvironment, allowing its survival in the stomach acid environment [5]. *H. pylori* should be cultured in nutrient-rich agar supplemented with horse or sheep blood with low oxygen saturation and 5–10% CO_2 atmosphere. Growth is generally observed from two to five days. The colonies are described as small, round, and translucid in agar.

Identification of *H. pylori* can be performed through urease production, catalase tests, and microscopy by observing its spiral or curve morphology and high motility [6], as well as by identifying specific genes, such as the *ureC* gene, which codifies for phosphoglucosamine mutase, essential for cell growth [7], through the polymerase chain reaction (PCR) technique. Molecular characterization can be complemented by also performing amplification for other genes related to pathogenicity and virulence, e.g., *ureA*, *cagA*, and *vacA*. On the other hand, the *tonB* gene, which encodes FrpB4, a TonB-dependent transporter that carries nickel through the external membrane since this metal acts as cofactor for the urease [8], can also be amplified.

Vacuolating cytotoxin VacA is codified by the *vacA* gene and presents variable signal (*s*), middle (*m*), and intermediate (*i*) regions. Each variable region presents allelic variants implied in the risk of developing gastric diseases. In addition, VacA may contribute to *H. pylori* colonization in the stomach. The VacA cytotoxin is secreted through a V-type autotransporter secretion system, which induces structural and functional changes in epithelial cells and, notably, the largest intracellular vacuole formation in vitro. The vacA+ phenotype also causes mitochondrial disfunction, conducing to epithelial gastric cells apoptosis through modulation of transduction pathways associated with autophagia, inhibiting T cell proliferation and cytokine production [5,9,10]. The CagA cytotoxin—codified by the *cagA* gene—is another important *H. pylori* pathogenicity virulence factor. This gene is found within the DNA 40 kb (kilobase) segment, known as the cytotoxin-associated gene A pathogenicity island (cagPAI), consisting of up to 32 open reading frames (ORFs). It also codifies for the Type IV secretion system (T4SS) components, which form a syringe-like structure to deliver CagA into gastric epithelial cells. Strains are usually classified as *cagA*+ and *cagA*−. The *cagA*+ genotype is considered the most virulent of both, and a higher risk of gastric cancer or peptic ulcer disease development has been found in individuals infected with strains harboring cagPAI compared with individuals infected with cagPAI-negative *H. pylori* strains. Its presence has been also associated with gastric mucosal atrophy and duodenal ulcer. It is subsequently phosphorylated by the host kinases in specific sites, such as EPIYA motifs. In this way, it interacts with several signaling pathways in the cytoskeleton, altering the host cellular morphology and motility. Once CagA is tyrosine-phosphorylated by kinases, it joins and activates SHP-2 tyrosine phosphatase—an oncoprotein whose mutation is associated with malignant processes [11,12].

On the other hand, 16S rRNA gene sequencing is widely employed for bacterial phylogenetic studies, since it is found in all bacteria, allowing the establishment of relationships among isolates, determination of their phylogenetical location at the genus level, and relationship with close genera, e.g., *Campylobacter* spp. [13]. The genetic variability in the core genome, such as the constitutive genes, is useful to determine population genetic structure and its evolutionary relationship among the isolates. The multilocus sequence typing (MLST) technique employs seven constitutive genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, *yphC*) to identify the evolutionary relationships among *H. pylori* isolates [14]. The accuracy of this technique has been widely demonstrated to situate the different strains within a population structure and even analyze intrapersonal transmission, as well as the relationship within the same individual [15–17]. Therefore, the objective of the present study was to isolate and identify clinical cultures of *H. pylori* from human stomach biopsies and characterize the isolates according to their colonial and microscopic morphology, phenotypic characteristics, and molecular genotyping to identify virulence factors associated with cancer and peptic ulcer.

2. Materials and Methods

2.1. Bacterial Isolates from Gastric Biopsies

The isolation of *H. pylori* was performed from 22 biopsies of the gastric mucosa obtained from three anatomical regions (gastric corpus, gastric antrum, and duodenum) of patients of Fray Antonio Alcalde (Antiguo Hospital Civil), Guadalajara, Jalisco, México. The patient selection criteria were randomized from patients' biopsies with the gastroen-

terologist's indication for different reasons, and no additional intervention was performed than the one indicated by their specialist. The experiment was carried out in accordance with the principles of the Declaration of Helsinki [18]. The biopsies were taken from May to July 2022 and placed in phosphate-buffered saline (PBS) solution on ice to preserve the samples. Then, the samples were transported to the Centro Público de Investigación (CIATEJ, CONAHCYT) laboratory, Guadalajara, over a period no longer than 3 h. The samples were placed in a sterile glass macerator and disaggregated with 100–200 µL of PBS. Then, the macerated samples were sown by dripping and striation in Petri plates with Casman agar base (Dibico, Cuautitlán Izcalli, Estado de Mexico, Mexico) medium supplemented with 5% aged goat blood to favor hemolysis (15 days after expiration date) and polymyxin B sulfate (3500 U L^{-1}), trimethoprim (5 mg L^{-1}), vancomycin hydrochloride (3 mg L^{-1}), and amphotericin B (2 mg L^{-1}) (all the antibiotics were purchased from Sigma-Aldrich®, St. Louis, MO, USA). The inoculated plates were incubated at 37 °C and 5% CO_2 (NuAire US Autoflow Automatic CO2 Water-Jacket Incubator, NU-4750/D/E) for three to five days [14]. The colonies with *H. pylori* morphological characteristics were recultured in Remel Casman agar base medium supplemented with aged goat blood and incubated at 37 °C and 5% CO_2 for three to five days for isolation of *H. pylori*.

2.2. Characterization of Bacterial Isolates

2.2.1. Morphological Characterization

For the morphological identification of the bacterial isolates named HCGDL-MR01, HCGDL-MR13, and HCGDL-MR17, Gram staining was performed. Approximately 20 µL of bacterial sample was placed on a slide and fixed with heat. Violet crystal dye was added and left to act for a minute, then the excess dye was removed. It was covered with an iodine solution and left for a minute, then washed with alcohol:acetone (1:1), and the excess was rinsed off with tap water. Safranin was added and left to act for a minute. Finally, it was washed with tap water until the rest of the dye was removed, left to dry, and observed in an Olympus BH2-RFCA optical microscope (Olympus Corporation, Tokyo, Japan). The reference strain ATCC 43504 of *H. pylori* was employed as a control.

2.2.2. Biochemical Characterization

For the urease test, urea agar base medium (BD BBL™, Becton Dickinson, Le Pont de Claix, France) was used. The bacterial biomass was taken with a bacteriological loop and placed in a microtube with urea agar base medium, and a few minutes (5–30 min, depending on the amount of biomass used) passed to observe a change in the color of the medium from yellow to pink, which indicates the presence of the enzyme urease. The catalase test was performed by placing the bacterial biomass on 3% H_2O_2 (Merck KGaA, Darmstadt, Germany). Bubble formation was observed in positive catalase strains due to oxygen and water production starting from H_2O_2. Finally, the oxidase test was performed with 1% tetra-methyl-p-phenylamine dihydrochloride (BBL™ In-vitro Oxidase Reagent Dropper, Becton Dickinson, Franklin Lakes, NJ, USA) to detect the oxidase cytochrome enzyme presence. A transition of color to intense blue was observed in positive oxidase samples.

2.2.3. Molecular Characterization of the Bacterial Genome Isolates

The DNA extraction was performed with a Wizard® Genomic DNA Purification A1125 kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Identification of the *ureC*, *cagA*, *vacA* (*vacAm1*, *vacAm2*, *vacAs1a*, *vacAs1b*, *vacAs2*), *ureA*, and *tonB* virulence genes of the HCGDL-MR01, HCGDL-MR13, and HCGDL-MR17 bacterial isolates was performed through end-point PCR. The *H. pylori* ATCC 43504 strain was employed as a control. The primers used (IDT™, Coralville, IA, USA) for the study and PCR thermal profiles are listed in Supplementary Tables S1 and S2, respectively.

The PCR reaction was prepared as follows: 1.2 µL PCR DreamTaq 10X buffer, 1.2 µL dNTPs 2 mM, 0.5 µL each oligonucleotide (6 pmol μL^{-1}), 5.4 µL milliQ water (UltraPure™ DNase/RNase-Free Distilled Water, ThermoFisher Scientific, Waltham, MA, USA), 0.2 µL

Taq DNA polymerase (5 U μL^{-1}, Dream Taq, Thermo Scientific, Waltham, MA, USA), and 3 µL genomic DNA (100 ng), with a final volume = 12 µL. The PCR reactions were performed in a BioRad T100™ (Hercules, CA, USA) thermocycler. The amplification products were observed in 1% agarose gel electrophoresis in 5 mM sodium borate (SB) buffer (1.905 g of $Na_2B_4O_7$ $10H_2O$ in 1 L of H_2O, pH 8.5) at 80 V. Amplicons were visualized with ultraviolet fluorescence after staining with GelRed® (Biotium, Inc., Fremont, CA, USA) in a Gel Doc™ XR System (ThermoFisher Scientific, USA) transilluminator.

2.3. Statistical Clustering Analysis

A clustering analysis in STATGRAPHICS Centurion XV.II (India) was performed with the genotype of each bacterial strain to cluster them according to their genetic characteristics. The data used for the clustering analysis were according to the presence (codification 1) or absence (codification 0) of virulence genes *cagA*, *vacAs1m1*, *ureA*, *ureC*, and *tonB*. The grouping method consisted of the construction of a distance tree, which was clustered with the unweighted pair group method with arithmetic mean (UPGMA) method.

2.4. 16S rRNA Gene Phylogenetic Analysis of Helicobacter pylori Strains

The PCR reactions for amplification of the 16S rRNA gene were prepared in a final volume = 50 µL as follows: 25 µL Go Taq® Master Mix 2X (Go Taq® DNA polymerase, Promega, USA, dNTPs, $MgCl_2$, reaction buffer), 2.5 µL each oligonucleotide fD1/rD1 [19] (Supplementary Tables S1 and S2) (6 pmol μL^{-1}), 10 µL genomic DNA (100 ng), and 10 µL milliQ water (UltraPure™ DNase/RNase-Free Distilled Water, ThermoFisher Scientific, USA). The PCR reaction was performed in a BioRad T100™ (Hercules, CA, USA) thermocycler. The amplification products were confirmed in 1% agarose gel electrophoresis in 5 mM SB buffer at 80 V and visualized with ultraviolet fluorescence after staining with GelRed® (Biotium, Inc., Fremont, CA, USA) in a Gel Doc™ XR System (ThermoFisher Scientific, USA) transilluminator.

The amplicons were directly quantified from the agarose gel and purified by a Wizard® PCR Preps DNA Purification System A7211 kit (Promega, USA) according to the manufacturer's instructions. Each amplicon was sequenced with five reactions following the Sanger method (Macrogen Inc., Seoul, Republic of Korea, https://dna.macrogen.com/), using oligonucleotides 800R (5′-TACCAGGGTATCTAATCC-3′) [20], 1100R (5′-GGGTTGCGCTCGTTG-3′) [21], and rD1. The assemblage of five 16S rRNA gene sequences were performed using SnapGene® 5.0.8 program (from Domatics; at snapgene.com). A phylogenetic analysis was performed. For this purpose, a sequencing alignment was performed with the 16S rRNA gene sequence of the isolated strains in MUSCLE [22], followed by the phylogenetic tree construction with MEGA v.10.0.5 [23] according to the following parameters: maximum likelihood method with 1000 bootstrap replicates, 5 gamma distribution, and Kimura 2-parameter model.

On the other hand, the sequences of each 16s rRNA gene were analyzed in the EzBioCloud database [24] to identify them at the species level.

2.5. Multilocus Sequence Typing Phylogenetic Analysis of H. pylori Isolates

For the multilocus sequence typing (MLST) analysis, PCR was performed for the *atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, and *yphC* constitutive genes according to Achtman et al. [14]. The PCR products were purified with ExoSAP-IT® (Affymetrix, Cleveland, OH, USA) and sequenced by the Sanger method (Macrogen Inc., Seoul, Republic of Korea, https://dna.macrogen.com/). The sequences were then compared with *H. pylori* PubMLST database alleles (https://pubmlst.org/) to determine their relationship with the sequence types (STs). An alignment was performed with ClustalW [25] and edited in Bioedit [26]. The seven constitutive genes were concatenated in MEGA v.10.0.5 [23]. A phylogenetic tree construction was also performed according to the following parameters: the maximum likelihood method, 1000 bootstrap replicates, and 5 gamma distribution Kimura 2-parameter model. All the analyses included the three isolates of this study, 198 STs

previously obtained from the PubMLST database (https://pubmlst.org/), and the *H. pylori* ATCC 43504 strain control.

3. Results

3.1. Bacterial Isolates from Gastric Biopsies

Table 1 shows the clinical features of the patients included in this study for obtention of gastric biopsies and latter bacterial isolation. From 22 gastric biopsies, 3 bacterial strains were isolated (HCGDL-MR01, HCGDL-MR13, HCGDL-MR17), which corresponded to patients 1, 13, and 17, respectively. The isolates were then cultured in synthetic in vitro medium and identified as *H. pylori* first by colonial morphology, which we described as small "dew drops", round and translucid colonies (Figure 1). Then, characterization was followed by the urease test and identification of the *ureC* gene. Patient 7 presented a positive urease test result; however, no bacterial growth was observed after the first culture in vitro, resulting in the loss of the strain.

Table 1. Clinical features of the study patients recruited from Antiguo Hospital Civil de Guadalajara for obtention of gastric biopsies.

Patient	Previous Diagnosis from Endoscopy	Age (Years)	Gender	Urease Test
1	GERD	68	F	+
2	GERD, FLD	54	F	-
3	Dyspepsia, UGIB	40	F	-
4	Dyspepsia	70	F	-
5	Dysphagia, dyspepsia	67	M	-
6	Esophageal CA	57	F	-
7	GERD	53	M	+ (no growth)
8	UGIB	60	M	-
9	Dyspepsia, UGIB	61	F	-
10	GERD	48	F	-
11	Colon CA	74	M	-
12	Dyspepsia	28	F	-
13	Anemia	83	F	+
14	UGIB, ulcer	72	F	-
15	HC	58	F	-
16	Hepatic METS	54	F	-
17	GERD	60	F	+
18	Esophageal stenosis	49	F	-
19	Dyspepsia	62	F	-
20	Dyspepsia	69	F	-
21	UGIB	77	F	-
22	UGIB	42	M	-

GERD: gastroesophageal reflux disease, UGIB: upper gastrointestinal bleeding, METS: metastasis, CA: cancer, FLD: fatty liver disease, HC: hepatic cirrhosis. F = feminine; M = masculine. + = positive *H. pylori* test, - = negative *H. pylori* test.

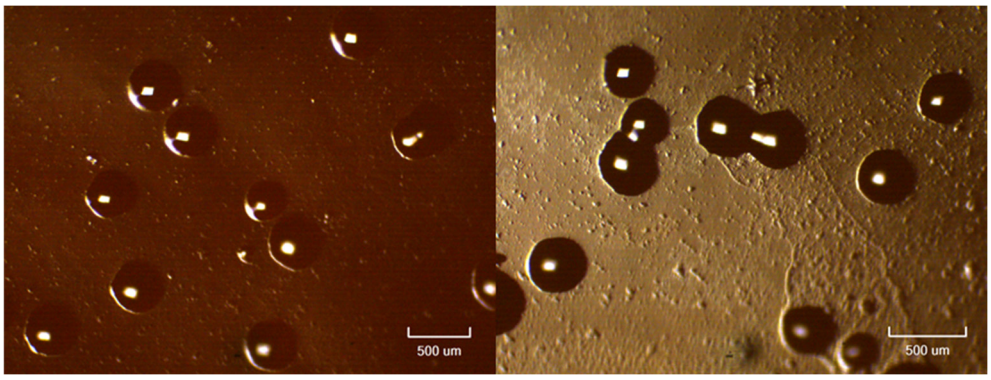

Figure 1. Typical *Helicobacter pylori* colonial morphology of the HCGDL-MR17 isolate at 72 h growth. Cultured in Remel Casman agar base supplemented with 5% goat blood and incubated at 37 °C and 5% CO_2. Left image = 40×, right image = 50×. Observations were performed with Nikon SMZ745T stereo microscope, Lumenera Infinity 1 CMOS digital camera, and Infinity Capture Application (version 6.5.4, Lumenera Corporation, Ottawa, ON, Canada).

3.2. Microscopic Morphological Characterization of Helicobacter pylori Isolates

Gram staining was applied to the bacterial isolates observed in the optic microscope. The *H. pylori* isolates were confirmed jointly with the urease test. Figure 2 shows the Gram-negative morphological characteristics, which we described as the typical "seagull wing" curved–shaped in "C" or "S" of approximately 2–3 µm length.

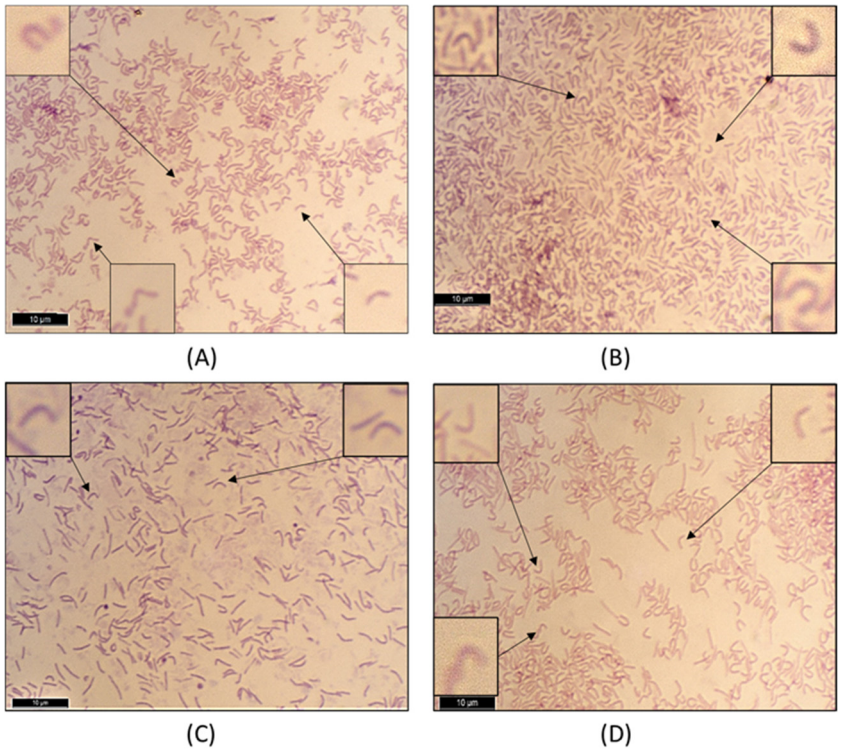

Figure 2. Microscopic morphological characterization of Gram staining bacterial isolates of patients from Antiguo Hospital Civil de Guadalajara. (**A**) HCGDL-MR01; (**B**) HCGDL-MR13; (**C**) HCGDL-MR17. (**D**) ATCC 43504 *H. pylori* reference strain. Micrographs were taken at 100×. Observations were performed with Olympus BH2-RFCA microscope, Leica digital camera DFC450C, and software (Application Suite LAS v.4.1.0, Wetzlar, Germany). Arrows indicate typical *H. pylori* morphology in culture with "seagull wing" in "C" or "S" curved–shaped. The boxes show an amplification of this typical morphology.

3.3. Biochemical Characterization of the Bacterial Isolates

Urease, catalase, and oxidase were positive for the three study isolates (Table 2). The urease test showed a positive result with a change in coloration from yellow to pink in a lapse from 5 to 30 min, depending on the biomass employed from each bacterial isolate. The presence of catalase was observed by oxygen formation starting from hydrogen peroxide. Lastly, a coloration change to intense blue was observed for all the strains, which indicated the presence of the oxidase cytochrome enzyme.

Table 2. Biochemical characterization of bacterial isolates obtained from patients of Antiguo Hospital Civil de Guadalajara.

Bacterial Strain	Biochemical Test		
	Urease	Catalase	Oxidase
HCGDL-MR01	+	+	+
HCGDL-MR13	+	+	+
HCGDL-MR17	+	+	+
ATCC 43504 *	+	+	+

* Reference strain. + = positive.

3.4. Genomic Molecular Characterization of Bacterial Isolates

Amplification of the *ureC* gene through PCR was observed in the three cultures of study, which confirmed the identity of the isolates at the species level (Figure 3A). Identification of the *ureA* and *tonB* genes was observed, as well (Figure 3B,C). Regarding the identification of the *cagA* gene, only the HCGDL-MR01 strain presented the gene, indicating a greater virulence compared to the other two isolates (Figure 3D). The *vacAm2* allele was observed in strains HCGDL-MR01, HCGDL-MR13, and HCGDL-MR17, while the *vacAm1* allele was amplified in strain ATCC 43504 (Figure 3E,F); on the other hand, the *vacAs1b* allele was not identified in any of the isolates. In the three isolates, the *vacAs2* allele was determined, and the *vacAs1a* allele was amplified in the strain ATCC 43504 (Figure 3G–I). Table 3 lists the genotypes of the isolated strains.

Table 3. Genotypes of the study isolates from Antiguo Hospital Civil de Guadalajara.

Bacterial Strain	Genotypes
HCGDL-MR01	*ureC* (+), *cagA* (+), 16S (+), *tonB* (+), *ureA* (+), *vacAs2m2*
HCGDL-MR13	*ureC* (+), *cagA* (-), 16S (+), *tonB* (+), *ureA* (+), *vacAs2m2*
HCGDL-MR17	*ureC* (+), *cagA* (-), 16S (+), *tonB* (+), *ureA* (+), *vacAs2m2*
ATCC 43504 *	*ureC* (+), *cagA* (+), 16S (+), *tonB* (+), *ureA* (+), *vacAs1m1*

* Reference strain. (+) = presence, (-) = absence.

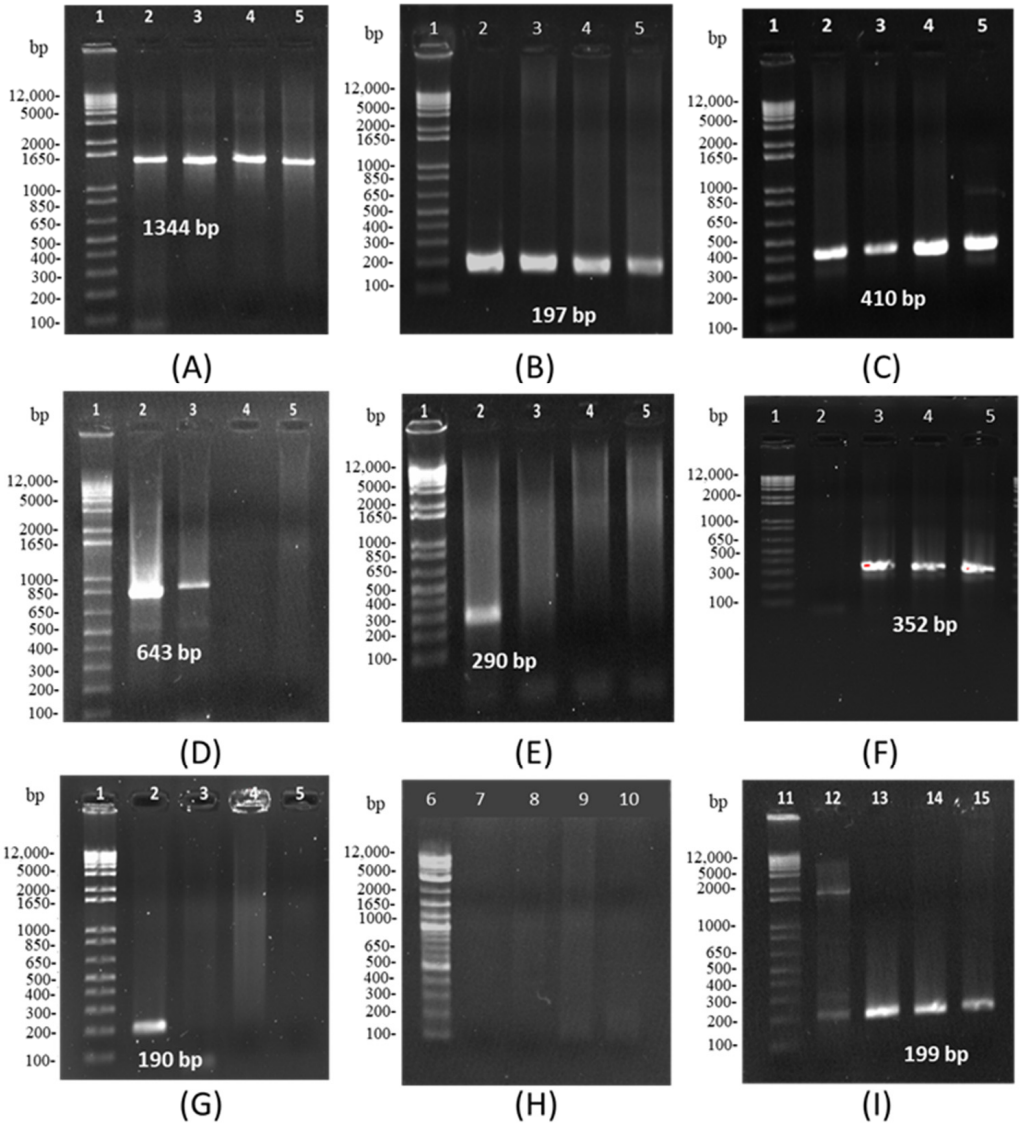

Figure 3. Genotyping of the *H. pylori* study isolates obtained from patients of Antiguo Hospital Civil de Guadalajara. (**A**) *ureC* gene, 1: MW (1 kb-Plus, Invitrogen^{TM}); 2: ATCC 43504; 3: HCGDL-MR01; 4: HCGDL-MR13; 5: HCGDL-MR17; (**B**) *tonB* gene, 1: MW; 2: HCGDL-MR17; 3: HCGDL-MR13; 4: HCGDL-MR01; 5: ATCC 43504; (**C**) *ureA* gene, 1: MW; 2: ATCC 43504; 3: HCGDL-MR01; 4: HCGDL-MR13; 5: HCGDL-MR17; (**D**) *cagA* gene, 1: MW; 2: ATCC 43504; 3: HCGDL-MR01; 4: HCGDL-MR13; 5: HCGDL-MR17; (**E**) *vacA* gene *m1* allele, 1: MW; 2: ATCC 43504; 3: HCGDL-MR01; 4: HCGDL-MR13.; 5: HCGDL-MR17; (**F**) *vacA* gene *m2* allele, 6: ATCC 43504; 7: HCGDL-MR01; 8: HCGDL-MR13; 9: HCGDL-MR17; 10: MW; (**G**) *vacA* gene *s1a* allele, 1: MW; 2: ATCC 43504; 3: HCGDL-MR01; 4: HCGDL-MR13; 5: HCGDL-MR17; (**H**) *vacA* gene *s1b* allele; 6: MW; 7: HCGDL-MR17; 8: HCGDL-MR13; 9: HCGDL-MR01; 10: ATCC 43504; (**I**) *vacA* gene *s2* allele; 11: MW; 12: ATCC 43504; 13: HCGDL-MR01; 14: HCGDL-MR13; 15: HCGDL-MR17. Electrophoresis conditions: 1% agarose, dehydrogenated disodium borate buffer (pH 8.5), 80 V.

3.5. Clustering Analysis

The clustering analysis allowed us to observe differences among the strains regarding their virulence. As observed in Figure 4, HCGDL-MR13 and HCGDL-MR17 were similar and considered less virulent, whereas the *H. pylori* ATCC 43504 reference strain was the most virulent, followed by HCGDL-MR01. This fact matches with previously observed results.

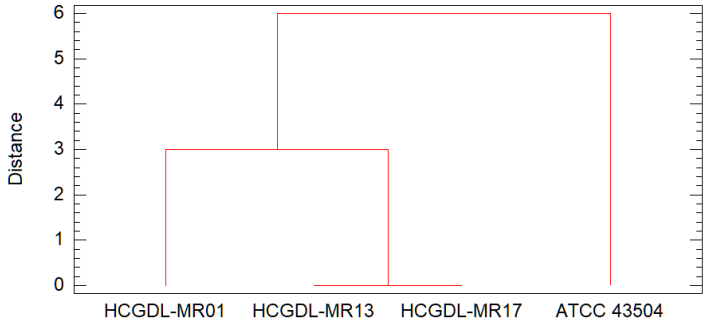

Figure 4. Relationship of bacterial isolates from Antiguo Hospital Civil de Guadalajara according to *H. pylori* genotypes. Construction of a distance tree using clustering with the unweighted pair group method with the arithmetic mean (UPGMA) method.

3.6. Phylogenetic Analysis of the 16S rRNA Gene Bacterial Isolates

The phylogenetic analysis performed from the amplification of the 16S rRNA gene confirmed the identity of the study isolates and allowed us to determine the relationship within the *H. pylori* ATCC 43504 reference strain (Figure 5). The phylogenetic tree shows that the three clinical strains are related with CHC155 and VN1291, both strains isolated from Vietnamese patients with gastric and duodenal cancer, respectively [27]. On the other hand, HCGDL-MR01 and HCGDL-MR13 are phylogenetically related when compared with HCGDL-MR17. However, the genotyping analysis showed that HCGDL-MR13 and HCGDL-MR17 are more similar in terms of virulence, and both are considered less virulent when compared with HCGDL-MR01.

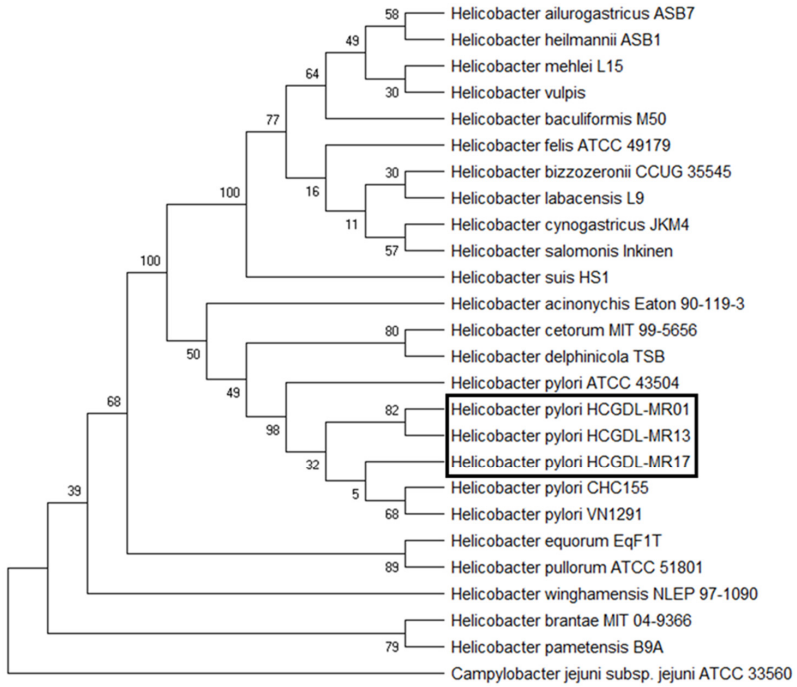

Figure 5. 16S rRNA phylogenetic analysis of the bacterial isolates of study from Antiguo Hospital Civil de Guadalajara against bacterial sequences of *Helicobacter* genus. Bacterial isolates highlighted in the bold square. Alignment performed in MUSCLE [22]. Parameters MEGA: maximum likelihood method with 1000 bootstrap replicates, 5 gamma distribution, Kimura 2-parameter model. *H. pylori* ATCC 43504 reference strain.

The 16S rRNA gene sequencing for the three isolates is found in GenBank (https://www.ncbi.nlm.nih.gov/genbank/ accessed on 6 June 2024) with accession numbers PP263608 (HCGDL-MR01, 1363 bp), PP263609 (HCGDL-MR13, 1534 bp), and PP263610

(HCGDL-MR17, 1464 bp). These sequences were analyzed in the EzBioCloud database, where it was determined that the isolated strains indeed belong to *Helicobacter pylori*, with a similarity percentage of 99.70% for the HCGDL-MR01 strain, 99.72% for the HCGDL-MR13 strain, and 99.72 for the HCGDL-MR17 strain.

3.7. Multilocus Sequence Typing Phylogenetic Analysis

The phylogenetic analysis performed with the seven constitutive genes of the isolated HCGDL-MR01, HCGDL-MR13, and HCGDL-MR17 strains were found to be related to the European strains situated within the hpEurope population structure. The ATCC 43504 strain was also found within this group (Figure 6). When the isolated strains were compared to those in the present study with the allele sequences of the PubMLST database (https://pubmlst.org/), no relationship was observed in any allele profile, thus demonstrating that the HCGDL-MR01, HCGDL-MR13, and HCGDL-MR17 strains have non-reported sequence type (ST) alleles. The sequences were submitted to the pubMLST database and the allele numbers 3658, 3679, 3348, 3766, 3785, 3408, 3729, 3740, 3709, 3782, 3824, and 3828 were assigned; the sequence types ST4387 (HCGDL-MR01 and HCGDL-MR13 strains) and ST4454 (HCGDL-MR17 strain) were also assigned.

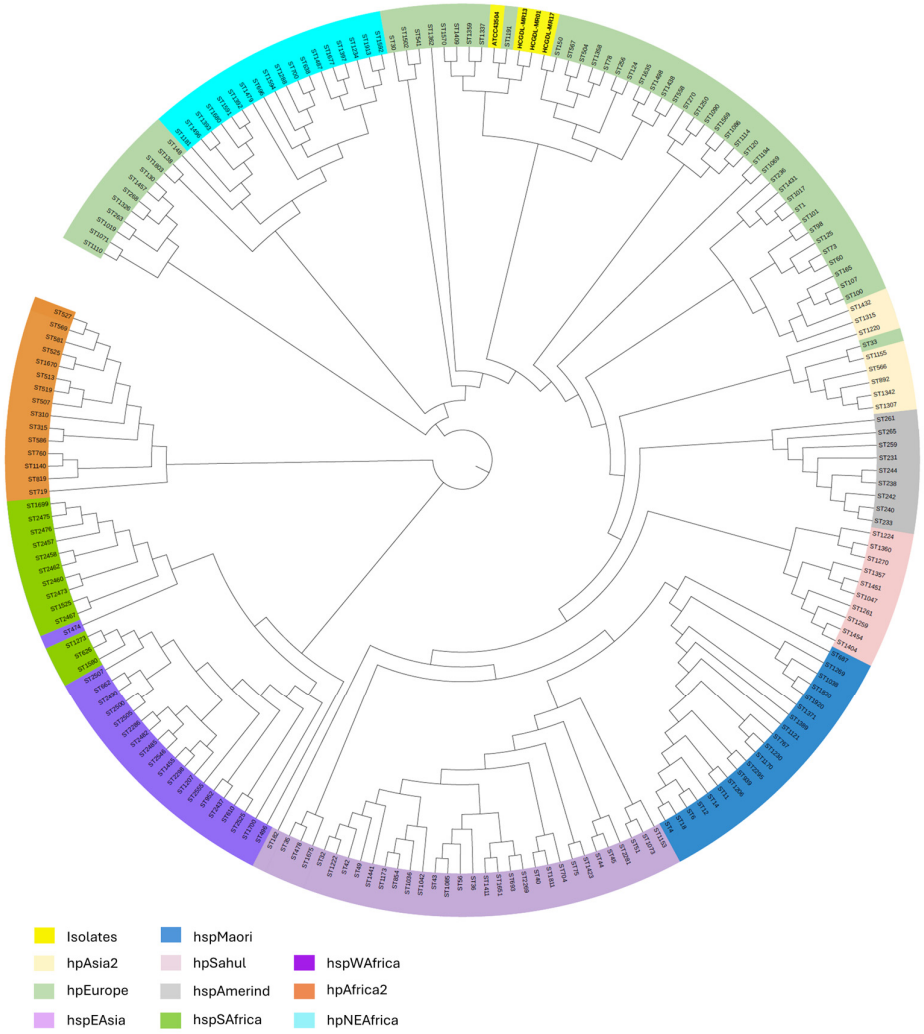

Figure 6. Phylogenetic analysis of the bacterial isolates against 198 STs of *H. pylori* bacterial strains obtained from the PubMLST database (https://pubmlst.org/), performed with DNA sequences of the seven housekeeping (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, *yphC*) genes. Alignment was performed with ClustalW. MEGA parameters: maximum likelihood method with 1000 bootstrap replicates, gamma distribution of 5, kimura 2-parameter method. *H. pylori* ATCC 43504 reference strain. Image edited in iTOL [28].

4. Discussion

Three bacterial cultures corresponding to *H. pylori* were isolated from human stomach biopsies and subsequently characterized. They showed differences in their genotype; therefore, they have differences in their virulence. However, all of them were shown to be related to each other and to the hpEurope population structure.

It should be noted that three of the four patients gave positive urease test results of gastroesophageal reflux disease (GERD), suggesting a relationship between the disease and bacterial infection. Nevertheless, the reports on such a relationship are controversial. Evidence has suggested that *H. pylori* plays a potential role in developing GERD since 82.5% of patients with the disease showed the bacterial infection [29]. However, other studies report a null relationship between *H. pylori* and GERD, where the bacterium was diagnosed in up to 77% in patients with GERD, whereas in *H. pylori*-negative patients were diagnosed with similar but no statistically significant different *H. pylori* rates (73.8%; $p = 0.106$). In the same study, no existing associations were reported between sex or age with the bacterial infection [30], while several risk factors of patients experiencing GERD have been described (obesity, smoking, lifestyle) [31]. Therefore, an existing relationship between *H. pylori* and such a disease cannot be concluded. On the other hand, although in the present study, all the cultures characterized were isolated from female patients over 60 years of age, this could be a coincidence due to the sample size, so further studies are needed to determine whether there is a relationship.

H. pylori presents in a spiral form, which is crucial to colonize the stomach. Jointly, with help from its flagella, the bacterial cells go through the stomach mucus to reach the gastric epithelial cells, where pH conditions are favorable [5,32]. However, *H. pylori* can also take a straight or curved rod or coccoid shape. These changes are adaptation mechanisms to environmental conditions [33]. In general, in vitro *H. pylori* cultures induce a spiral morphological change, giving place to curved bacilli, thus, this type of bacterial morphology was observed in the present study (Figure 2).

Moreover, all the strains isolated in the present study gave a positive result for the *ureA* gene. This urease enzyme is highly conserved in all *H. pylori* strains, thus the reason why they are commonly used as diagnostics. In studies performed in animal models, *H. pylori* mutants that lack urease activity have shown a deficient colonization process, which demonstrates the role of this enzyme in pathogenesis [5].

The *vacA* s1/m1 genotype has been reported with great cytotoxic activity, followed by the *s1/m2* genotype with lower activity, and the s2/m2 genotype, which lacks cytotoxic activity [34]. On the other hand, Jeyamani et al. [35] demonstrated a relationship between the s1 allele and gastric diseases, especially ulcer, whereas the m allele did not show any relationship with the development of the disease, highlighting the importance of the s1 allele as a virulence marker. In the present study, the clinical isolates showed the s2/m2 genotype, suggesting an absence of the VacA cytotoxin and thus less virulence; the *H. pylori* ATCC 435604 reference strain was the only one with s1/m1 genotype and was the most virulent.

The HCGDL-MR01 strain is the only one isolated in the present study that has the *cagA*+ gene, which provides greater virulence independently of its *vacA* genotype. Different studies have demonstrated the relationship between the *cagA*+ genotype with the development of gastric cancer and peptic ulcer in different patients from distinct populations [36–38]. In contrast, *cagA* patients presented a lower risk for development of such pathologies. Furthermore, research has been performed with animals that confirm these findings. In a study with mice, Ohnishi et al. [39] reported that the expression of the oncoprotein CagA is sufficient to develop neoplasia—tumor formation in mice associated with CagA tyrosine-phosphorylation—demonstrating its essential role to interact with the SHP-2 oncoprotein and allow abnormal gastric epithelial cell proliferation and alter leukemogenesis. This fact supports the association between *H. pylori* infection and the development of lymphoma B cells. Another study performed with gerbils (*Meriones unguiculatus*) showed the importance of T4SS for colonization of the gastric corpus, resulting

in atrophic gastritis, representing a higher risk for the development of malignancies [40]. Based on this fact, we can determine that a person infected with the HCGDL-MR01 strain has more possibilities of developing cancer than those infected with the HCGDL-MR13 or HCGDL-MR17 strains, although other virulence factors should be contemplated, as well as the environmental conditions. This result is important, since knowing and genotyping the bacterial virulence factors, for example, the *cagA* and *vacA* genes of clinical strains, the potential damage and degree of severity that *H. pylori* can induce in each patient can be determined.

The hpEurope strains are found to be related with the hpAsia2; therefore, HCGDL-MR01, HCGDL-MR13, and HCGDL-MR17 also have some relationships with hpAsia2. This fact is considered important since Asian strains are considered highly pathogenic [17]; however, it should be noted that the strains in the present study presented new alleles (3658, 3679, 3348, 3766, 3785, 3408, 3729, 3740, 3709, 3782, 3824, and 3828) without showing any coincidence with the alleles in the database, except for the *efp* gene of the HCGDL-MR17 strain, which presents 100% coincidence with allele 264 of the pubMLST database. This allele is present in isolates from Venezuela, Portugal, Colombia, and Nicaragua, indicating that there is little variability in this gene. The relationship between Mexican strains within the hpEurope population structure was previously reported in 2019 [41]. The results obtained in present study corroborate this information. A relationship was observed among HCGDL-MR01 and HCGDL-MR13, which share the same sequence type (ST4387). In turn, these two strains are related to the reference strain ATCC 43504 used as a control, unlike strain HCGDL-MR17 (ST4454), which is phylogenetically more distant from the previous three. The phylogenetic analysis with 16S rRNA also shows a closer relationship between the HCGDL-MR01 and HCGDL-MR13 strains.

5. Conclusions

The bacterial isolates were identified at a species level as *H. pylori*. Different genotypes were obtained for each one of them, allowing a greater panorama of the possible effects of each of the strains based on their virulence factors. Relative to the *vacA* gene, all the clinical isolates showed the s2/m2 genotype, indicating an absence of the VacA cytotoxin, and, consequently, a lower virulence with respect to strain ATCC 43504. However, HCGDL-MR01 was the only strain carrying the *cagA* gene, which contributes to an increased risk of developing gastric cancer and peptic ulcer. The MLST analysis indicates that all the strains are grouped within hpEurope population structure and, in turn, are related to hpAsia2, which are considered highly pathogenic.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres15030123/s1, Table S1: Primers employed for molecular characterization of the *H. pylori* isolates from Antiguo Hospital Civil de Guadalajara; Table S2: Thermal profiles for amplification of different genes present in the *H. pylori* strain genome through end-point PCR. References [7,19,42–44] are cited in the Supplementary Materials.

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Informed Consent Statement: Patient consent was waived due to the fact that the biopsy samples provided by the Civil Hospital of Guadalajara "Fray Antonio Alcalde" were not obtained directly from patients but were provided by the hospital from biopsies that were performed on patients at the

direction of their physician to diagnose the presence of *H. pylori*, regardless of the purposes of the present study, and only a portion of each biopsy was taken to perform bacterial isolation.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author/s.

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