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Molecular characterization and susceptibility testing of *Helicobacter pylori* strains isolated in western Argentina

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SUMMARY

Objective: To characterize *Helicobacter pylori* isolates from western Argentina using virulence markers and antimicrobial susceptibility patterns in order to assess the association between virulent genotypes, antimicrobial resistance, and disease. DNA fingerprinting was also evaluated for the segregation of virulent or resistant strain clusters.

Methods: Genotyping of 299 *H. pylori* isolates was performed by PCR using specific primers for the *cagA*, *vacA* and *iceA* genes. Random amplification of polymorphic DNA (RAPD)-PCR and rep-PCR genetic clustering were assessed using five random primers and BOXA1R and ERIC primers, respectively. Resistance to clarithromycin (Cla) and metronidazole (Mtz) was assessed by the agar dilution method.

Results: It was observed that 40.8% of the genotypes were *cagA*-positive; 66.9% were *vacA s1m1* genotype and the *iceA1* allele was found in 40.8%. A significant correlation ($p = 0.0000$) was observed between *cagA* positivity and *vacA s1m1*/*iceA1* genotypes. Triple virulent genotypes were statistically associated with peptic ulcer (PU) ($p = 0.0001$) and Cla resistance ($p = 0.0000$). RAPD fingerprints obtained with AO2 primers identified clusters that were strongly associated with PU, virulence markers, and resistance to Cla and Mtz.

Conclusions: The *H. pylori* isolates that harbored two or three virulence markers were more resistant to Cla and Mtz. Combined analysis of virulent genotypes and resistance patterns may permit identification of high-risk patients to prevent PU later in life or to avoid antimicrobial treatment failure.

Introduction

Helicobacter pylori is a curved Gram-negative bacterium frequently present in the human stomach. Once the host has been colonized, this microorganism persists for years or decades. This microorganism has a worldwide distribution and is a major etiological agent in the development of peptic ulcer (PU) and gastric cancer.[1]

There are established genetic markers that contribute to the pathogenicity of *H. pylori*, i.e., the *cagA* locus, *vacA* alleles and the two allelic variants of the *iceA* gene. Strains expressing the CagA protein, which is a part of a large pathogenecity island, have been found to induce more severe inflammation, a higher degree of gastric atrophy, and higher incidence of duodenal ulcer. These effects could be explained by the increased density of *H. pylori* in the antrum, which causes more profound inhibition of mucin synthesis. Although the *vacA* gene is present in all *H. pylori* strains, it is expressed in only 50–65% of them, thus inducing vacuolization in gastric epithelial cells.[2] Among the different allelic types of *vacA*, *vacAs1* and *vacAs1m1* are toxic and strongly associated with duodenal ulceration. The signal sequence of *vacAs1*, but not *s2*, is closely associated with in vitro cytotoxin activity, PU, and with the presence of the *cagA* gene.[3,4] The *m1* allele is associated with higher levels of toxin activity and more severe gastric epithelial damage than the *m2* allele.[3,4]

The genetic organization of the *iceA* gene shows two distinct *iceA1* and *iceA2* allelic variants. The *iceA1* gene, whose expression is induced by gastric epithelial cell contact, is associated with virulence related to the production of interleukin-8.[5–7] The *iceA2* gene is completely unrelated to *iceA1* in sequence, although the *iceA1* and *iceA2* genes occupy the same locus in different strains.[1,8]

In a given community, apart from genetic diversity among strains, there are indications of significant geographical and ethnic differences associated with these virulence markers. However, the prediction of disease outcome based on combined *cagA*, *vacA* and *iceA* status has provided contrasting results in several geographically distinct populations.[2,6,9]

The eradication of *H. pylori* can contribute to the treatment and prevention of several pathologies associated with infection, such as PU or gastric cancer.[10] Treatment regimens combine two or more

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antibiotics, such as metronidazole (Mtz), clarithromycin (Cla), tetracycline (Tet), and amoxicillin (Amx), with a proton pump inhibitor (PPI). However antibiotic regimens against *H. pylori* are frequently ineffective in developing countries due to strains with increased resistance to these antimicrobial agents, which have previously been used to treat respiratory or anaerobic and parasitic infections. Resistance to Cla is associated with a greater risk for failure with Cla-based treatments.[11]

Although there is no theoretical basis for predicting an association between antibiotic resistance and virulence markers, several studies have been carried out with the purpose of finding a general association between them.[12,13]

The discriminatory ability of random amplification of polymorphic DNA (RAPD)-PCR and rep-PCR fingerprinting encourages the use of these methods for the analysis of *H. pylori* diversity, persistence, and virulence.[14] It has been hypothesized that clonal grouping by fingerprinting may be associated with disease or virulence factors, but studies using RAPD- and rep-PCR have reached different conclusions.[15]

The aim of this study was to characterize *H. pylori* strains from western Argentina using virulence markers and antimicrobial susceptibility patterns, in order to determine possible associations between virulent gene *cagA*, *vacA* and *iceA* alleles and clinical outcome and antimicrobial resistance. Additionally RAPD-PCR and rep-PCR genetic clustering of 34 isolates was evaluated to assess their usefulness for the segregation of virulent or resistant strain clusters.

Materials and methods

Biopsy samples, culture conditions, and identification

Fresh antrum biopsy specimens were taken during endoscopy from a total of 533 symptomatic patients of both sexes, inhabitants of the city of San Luis (Argentina), aged between 21 and 80 years, and with no previous record of antibiotic eradication therapy. *H. pylori* strains were successfully isolated from 299 (56.1%). Endoscopic diagnoses were as follows: 76 (25.4%) patients with PU (34 with gastric ulcer and 42 with duodenal ulcer), 17 (5.7%) patients with duodenitis, 10 (3.3%) patients with gastric cancer, and 196 (65.6%) patients with chronic gastritis (CG). All sample biopsies, one from each patient, were fixed in formalin 10% v/v and sent for histopathological evaluation. All endoscopic diagnoses were confirmed by histopathological analysis.

Cultures were prepared by smearing single biopsy specimens on Petri plates containing Mueller–Hinton agar (MHA; Oxoid, Basingstoke, Hampshire, UK) supplemented with 5–7% horse blood and Dent supplement (*H. pylori*-selective supplement; Oxoid). All cultures were incubated for 7–10 days at 37 °C in an atmosphere of 5% O_2, 10% CO_2, 85% N_2, and 98% humidity. *H. pylori* colonies were presumptively identified by their typical morphology. The isolates were confirmed as *H. pylori* by Gram-stain (negative and curved morphology) and catalase, oxidase, and urease activities.

Antimicrobial susceptibility

Resistance to antimicrobial agents was assayed by the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI, 2000)[16] using MHA supplemented with 7% horse blood. Serial dilutions of Cla (Abbott Laboratories, Argentina), Amx, Tet, and Mtz (Sigma Chemical Co., St. Louis, MO, USA) ranging from 128 to 0.008 mg/l were used. The bacteria were subcultured on MHA supplemented with 7% horse blood under the microaerophilic conditions mentioned above, at 37 °C for 48 h. The bacterial suspension in Mueller–Hinton broth was adjusted to a final concentration of a McFarland 0.5 standard. One microliter of the adjusted inoculum was delivered to the agar plates. The minimum inhibitory concentration (MIC) of Cla, Amx, Tet, and Mtz was determined after 72 h of incubation under a microaerobic atmosphere at 37 °C, and recorded as the lowest concentration of drug inhibiting visible growth. Resistance was defined as a Cla MIC of $\geq$2 mg/l, Mtz MIC of $\geq$8 mg/l, Tet MIC of $>$2 mg/l, and Amx MIC of $>$1 mg/l. The reference strain *H. pylori* NCTC 11638 was used as control. All tests were performed in duplicate.

Virulence markers

Genomic DNA was isolated from lysed *H. pylori* cells following phenol–chloroform extraction and ethanol precipitation as described previously.[17] Virulence markers were determined by PCR reactions carried out in a Thermal Cycler 2400 (Perkin Elmer Applied Biosystems, Foster City, CA). The primers CAGA-F and CAGA-R were used for detection of the *cagA* gene, which yields a fragment of 349 bp. The analysis of the *vacA s1/s2 vacA m1/m2* region was performed using primers VACA-F and VACA-R. For the *iceA1* allele analysis, primers iceA1F and iceA1R were employed.[5]

Clustering of antimicrobial resistance and virulence markers by genetic techniques

Thirty-four *H. pylori* strains were genotyped by RAPD and rep-PCR. A similar number of resistant and susceptible isolates were employed. They were selected according to their antibiotic resistance pattern: Cla-resistant (ClaR; 14 strains), Mtz-resistant (MtzR; 10 strains, of which three were both ClaR and MtzR), Cla-susceptible (ClaS; 20 strains), and Mtz-susceptible (MtzS; 24 strains). All strains were susceptible to Amx and Tet. Duplicates from the antrum and corpus of two patients were included. Endoscopic diagnoses were as follows: eight (23.5%) patients with PU (two gastric ulcer and six duodenal ulcer), three (8.8%) patients with duodenitis, and 23 (67.6%) patients with CG.

RAPD-PCR

The genomic DNA from bacterial samples was amplified with five random primers: AO1, AO2, AO3, AO4, and AO5 (Promega, Madison, WI, USA). The PCR reactions were carried out as described elsewhere.[14] Each isolate was tested with the selected primers under the same conditions at least three times.

Rep-PCR

The PCR reaction was performed using BOXA1R and ERIC primers (Promega, Madison, WI, USA).[14] Each isolate was tested with the selected primers under the same conditions at least three times.

Statistical analysis

Clustering analysis and the association between *H. pylori* genotypes and clinical disease, as well as among virulence markers and antibiotic resistance was evaluated using the Chi-square test with Statix version 3.5 software. The significance level was set at a p-value of <0.05.

Results

A total of 299 (56.1%) *H. pylori* strains were isolated from 533 symptomatic patients. The isolates were characterized by virulence markers and antimicrobial susceptibility.

Table 1
Association between virulent genotypes and gastric pathologies

Genotype	Pathology				
	Chronic gastritis (n = 196)	Duodenitis (n = 17)	Peptic ulcer (n = 76)	Gastric cancer (n = 10)	Total (n = 299)
cagA-pos	85 (43.4)	6 (35.3)	24 (31.6)	7 (70)	122 (40.8)
s1m1	143 (72.9)	0	50 (65.8)	7 (70)	200 (66.9)
iceA1	82 (41.8)	1 (5.9)	35 (46.1)	4 (40)	122 (40.8)
iceA1/iceA2	11 (5.6)	0	12 (15.8)[c]	3 (30)	26 (8.7)
Triple-virulent[a]	21 (10.5)	0	22 (30.6)[d]	1 (33.3)	44 (14.7)
Double-virulent[b]	52 (26.5)	0	41 (53.9)[d]	2 (20)	95 (31.8)
cagA-neg	111 (56.6)	11 (64.7)	52 (68.4)	3 (30)	177 (59.2)
s2m2	53 (27.0)	17 (100)[e]	26 (34.2)	3 (30)	99 (33.1)
iceA2	103 (52.5)	16 (94.1)[c]	29 (38.2)	3 (30)	151 (50.5)

Numbers in parentheses are percentages.
[a] cagA-pos/s1m1/iceA1 (n = 44).
[b] cagA-pos/s1m1/iceA2 (n = 62); cagA-neg/s1m1/iceA1 (n = 26); cagA-pos/s2m2/iceA1 (n = 7).
[c] Statistically significant differences: [c]p = 0.012: [d]p = 0.001; [e]p = 0.0009.

Genotyping of virulence genes

The results of *H. pylori* strain genotyping are summarized in Table 1. It was observed that 40.8% (122/299) of the genotypes were *cagA*-positive and 59.2% (177/299) were *cagA*-negative. The *vacA s1m1* alleles were predominant, occurring in 66.9% (200/299), while *s2m2* was observed in 33.1% (99/299) of the isolates (p = 0.001). No strains harboring *s1m2* or *s2m1* genotypes were obtained in this study. All *H. pylori* isolates possessed the *iceA* gene; 40.8% (122/299) were positive for the *iceA1* allele, 50.5% (151/299) were *iceA2*, and a PCR product of *iceA1*/*iceA2* was obtained in 8.7% (26/299) of the strains. The study of the association of the main virulence genes showed 44 triple-positive (*cagA*-positive/*s1m1*/*iceA1*) and 95 double-positive (*cagA*-positive/*s1m1*; *cagA*-positive/*iceA1* or *s1m1*/*iceA1*) strains.

Table 2 shows the distribution of *cag* status and the *vacA* and *iceA* allelic combinations. A statistically significant correlation was observed between *cagA*-positive status and *s1m1* (p = 0.0000), while most *s2m2* strains carried a *cagA*-negative (p = 0.0000) genotype. A significant association was also observed between *cagA*-negative status and *iceA1*/*iceA2* (p = 0.0006). Statistical analysis showed a significant association between virulent gene *cagA*-positive and *s1m1*/*iceA1* genotypes (p = 0.0000), while *cagA*-negative was associated with *s2m2*/*iceA1* and *s2m2*/*iceA1*/*iceA2* (p = 0.0000).

Association between genotypes and gastric pathologies

The association between virulence markers and pathologies is shown in Table 1. A significant association between PU and *iceA1*/*iceA2* (p = 0.0122) was observed. On the other hand, no statistical correlation was found between this pathology and *iceA1* or *cagA* status (p = 0.0579 and p = 0.259 respectively), but an association between PU and *iceA1* may be assumed as a clear tendency. Of the patients with duodenitis, 64.7% were *cagA*-negative, and the *s2m2* (p = 0.0009) and *iceA2* (p = 0.012) genotypes were strongly associated with this pathology. Most of the isolates characterized came from patients suffering from CG. In this group, the most abundant genotype was *s1m1* (72.9%), followed by the *cagA*-negative (56.6%) and *iceA2* (52.5%) genotypes. For this pathology, no significant association with virulence markers was observed. Double- and triple-positive virulent strains were significantly associated with PU (p = 0.001).

Table 2
Association between *cagA* status and *vacA* and *iceA* subtypes

Genotype	*cagA* status	
	cagA-pos (n = 122)	*cagA*-neg (n = 177)
vacA		
s1m1	106 (86.9)[a]	94 (53.1)
s2m2	16 (13.1)	83 (46.9)[a]
iceA		
iceA1	51 (41.8)	71 (40.1)
iceA2	71 (58.2)	80 (45.2)
Mixed infection *iceA1/iceA2*	0	26 (14.7)[b]
Genotype combinations		
s1m1/iceA1	44 (36.1)[c]	26 (14.7)
s2m2/iceA2	9 (7.4)	19 (10.7)
s1m1/iceA2	62 (50.8)	61 (34.5)
s2m2/iceA1	7 (5.7)	45 (25.4)[c]
s1m1/iceA1/iceA2	0	7 (4.0)
s2m2/iceA1/iceA2	0	19 (10.7)[c]

Numbers in parentheses are percentages.
Statistically significant differences: [a]p = 0.0000; [b]p = 0.0006; [c]p = 0.0000.

Antimicrobial susceptibility

The antimicrobial susceptibility of the 299 isolates is shown in Table 3; MIC_{50} and MIC_{90} values are shown. All strains were sensitive to Amx and Tet, while 83 (27.8%) strains were ClaR and 113 (37.8%) were MtzR. Only three strains were both ClaR and MtzR.Correlation of virulence markers with antimicrobial susceptibility

The distribution of virulence markers by antimicrobial susceptibility pattern is shown in Table 4. The combined genotype *cagA*-positive, *vacA s1m1*, *iceA1* was predominant in ClaR (p = 0.0000) and MtzR (p = 0.0000) strains. Also, a significant association was observed between the combined genotype *cagA*-negative, *vacA s1m1*, *iceA1* (p = 0.0000) and ClaR.

Analysis of RAPD fingerprinting

Only AO2 and AO3 RAPD primers generated reproducible RAPD fingerprints. They were able to discriminate 34 different profiles with 100% of typeability. Duplicate isolates coming from the same patients were not discriminated by any of the genotyping techniques assayed. The number and size of bands obtained with RAPD for the AO2 primer varied from four to 12 and 140 to 3000 bp, respectively. The segregation of PU was significant (p = 0.03) in cluster III. The dendrograms based on the RAPD profiles obtained with the AO2 primer, including virulence markers, antimicrobial susceptibilities, and pathology, are summarized in Figure 1. It was determined whether the specific virulence markers and antimicrobial susceptibilities were associated with the RAPD cluster. Isolates containing two or three combined virulence markers (9/16; 56.3%) were included in cluster III, whereas isolates that harbored no or only one virulence marker (15/18; 83.3%) were placed in cluster II (p = 0.0007). There were also significant differences (p = 0.0000) in the segregation of susceptible and resistant strains. Cluster III constituted 13/24 (54.2%) ClaR, MtzR or

Table 3
MICs of drugs and percentages of resistance among strains

Drugs	MIC (mg/l)			% Resistance
	MIC_{50}	MIC_{90}	Range	
Clarithromycin	8	32	0.008–128	27.8 (n=83)
Metronidazole	16	64	0.008–128	37.8 (n=113)
Amoxicillin	0.032	0.5	0.008–128	0
Tetracycline	0.016	0.25	0.008–128	0

MIC, minimum inhibitory concentration.
Numbers in parentheses are *Helicobacter pylori* isolates.

ClaR/MtzR strains, and cluster II grouped 29/44 (65.9%) of susceptible strains.

With the AO3 primer, well-resolved patterns of seven to 13 fragments of 150 to 2000 bp were obtained. Neither the segregation of PU isolates nor the combined virulence markers were significant with this primer. In contrast, significant differences were observed in the segregation of susceptible and resistant strains (p = 0.032). Cluster II constituted 16/24 (66.7%) ClaR, MtzR or ClaR/MtzR strains, and cluster I grouped 24/44 (54.5%) of susceptible strains with the exception of three ClaR strains (MIC = 2 mg/l).

Analysis of rep-PCR fingerprints

Thirty-one different BOX A1R profiles and 33 ERIC profiles were obtained with 100% of typeability. The BOX A1R patterns consisted of 7–14 bands with sizes ranging from 150 to 4000 bp. The dendrogram based on rep fingerprints obtained with the BOX A1R primer, including virulence markers, antimicrobial susceptibilities, and pathology, is summarized in Figure 2. A distinct pathological cluster that was segregated with combined virulence markers and ClaR and MtzR strains was observed with this primer. Isolates with two or three combined virulence markers (9/17; 52.9%) were included in cluster III, while isolates that harbored no or only one virulence marker (11/17; 64.7%) were placed in cluster II (p = 0.0008). There were also significant differences (p = 0.0000) in the segregation of susceptible and resistant strains. Cluster III constituted 12/24 (50%) ClaR, MtzR or ClaR/MtzR strains, and cluster II grouped 19/44 (43.2%) of susceptible strains.

The ERIC patterns consisted of approximately 7–13 bands per isolate. The molecular sizes of the fragments ranged from 50 to 5000 bp. With this primer, isolates with two or three combined virulence markers (7/17; 41.2%) were included in cluster III, while isolates that harbored no or only one virulence marker (11/17; 64.7%) were placed in cluster II (p = 0.05).

Discussion

Many studies have mentioned the occurrence of bio-geographical variations in virulence factors and resistance rates of *H. pylori* strains that may vary from one region to another, even in the same country.[18,19]

The combination of the distinct *cagA*, *vacA*, and *iceA* genotypes illustrates the mosaic composition of the *H. pylori* genome; strains that are typed as *cagA*-positive/*vacA s1m1*/*iceA1* can be considered the most pathogenic and are found predominantly in patients with ulcer disease. In contrast, strains typed as *cagA*-negative/*vacA s2m2*/*iceA2* appear to be the least pathogenic and do not occur in PU patients.[12,20]

In this study it was found that 40.8% of the *H. pylori* strains were *cagA*-positive, which is slightly higher than the result obtained in a previous study in the same population[21] and similar to the value reported in a study from Chile (38%),[22] but lower than reports from eastern Argentina (77%)[9] and Brazil (75.3%).[23] A significant correlation was observed between *cagA* positivity and *vacA s1m1*. This double virulence genotype is commonly linked to increased *H. pylori* virulence.[24] On the other hand, *cagA*-negative status was significantly associated with the less virulent *vacA s2m2* genotype (Table 2). It has been reported that individuals colonized with *cagA*-positive *H. pylori* strains are at increased risk of developing peptic ulceration.[5,12] In this study, however, no correlation was observed between *cagA* status and PU, probably due to the low incidence of *cagA*-positive strains circulating in this geographical region.

The prevalence of *vacA s1m1* alleles was significantly higher than that of *s2m2* (p = 0.001). Similar prevalence of this genotype has been observed in eastern Argentina[9,21] and other Latin-American countries, namely, Peru, Brazil, Costa Rica, and Colombia[5] and Mexico,[25] where *s1m1* was predominant in infected patients. However, the types of signal sequences and media region genotypes seem to vary with the geographical region. High rates of *vacA s1* genotype have been obtained in Argentina (74% and 75%), Cuba (73.8%), and Brazil (62%).[9,12,21,24] In the *H. pylori* isolates analyzed here, no mixed *vacA* genotypes were observed as was found in a previous study in western Argentina.[21] In Latin-American countries mixed *vacA* alleles have been detected at low rates, ranging from 0.8%[24] to 32%,[12] in contrast with other geographical regions, where mixed *vacA* are predominant, for instance Pakistan with 63.6% and Turkey with 48.4%.[26,27] Probably, a much larger number of *H. pylori* isolates should be analyzed in order to determine the presence of mixed *vacA* genotypes in the western Argentinean population.

No correlation was found between *vacA s1m1* strains and any of the gastric pathologies, but a significant association between *vacA*

Table 4
Distribution of virulence markers of *Helicobacter pylori* isolates and antimicrobial susceptibility pattern

Genotypes	Antimicrobial susceptibility pattern			
	ClaS	ClaR	MtzS	MtzR
cagA-pos *s1m1 iceA1*	10 (3.8)	34 (53.4)[a]	21 (11.8)	45 (47.2)[a]
cagA-pos *s1m1 iceA2*	58 (29.9)	4 (1.7)	9 (3.9)	22 (21.3)
cagA-pos *s2m2 iceA1*	4 (0.54)	3	10 (4.6)	3
cagA-pos *s2m2 iceA2*	6 (1.08)	3	8 (3.3)	3
cagA-neg *s2m2 iceA2*	15 (6.52)	4 (1.7)	29 (15.0)	21 (20.2)
cagA-neg *s1m1 iceA1*	7 (2.17)	19 (27.6)	23 (13.1)	11 (9.0)
cagA-neg *s2m2 iceA1*	34 (16.8)	11 (13.8)	6 (2.0)	2
cagA-neg *s1m1 iceA2*	57 (28.8)	4 (1.7)	55 (33.9)	5 (2.2)
cagA-neg *s1m1 iceA1/iceA2*	6 (1.6)	1	7 (2.6)	1
cagA-neg *s2m2 iceA1/iceA2*	19 (8.7)	0	18 (9.8)	0
Total	216	83	186	113

ClaS, clarithromycin-sensitive; ClaR, clarithromycin-resistant; MtzS, metronidazole-sensitive; MtzR, metronidazole-resistant.
[a] Three strains ClaR/MtzR.
Numbers in parentheses are percentages.

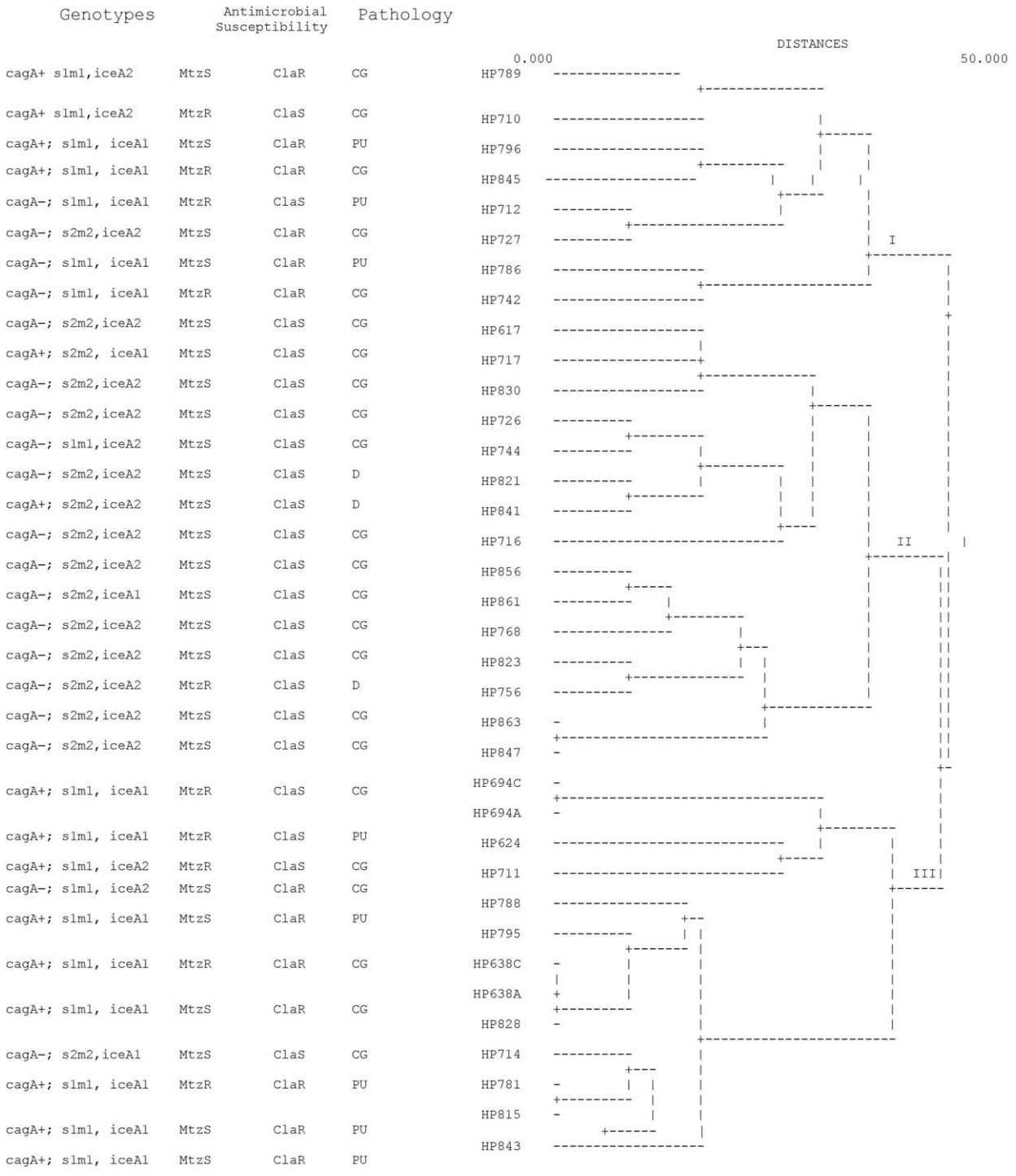

Figure 1. Dendrogram of *Helicobacter pylori* strain AO2-PCR DNA fingerprints. Three separate clusters (I, II, and III) at a genetic distance of approximately 57% are indicated. Cluster III consists of 13 isolates from eight patients suffering from chronic gastritis (CG) and five patients with peptic ulcer (PU). The segregation of PU was significant ($p = 0.03$) in cluster III. There were significant differences in the segregation of combined virulence markers ($p = 0.0007$) and in the segregation of susceptible and resistant stains ($p = 0.0000$). Fingerprint analyses were carried out using SYSTAT software version 5.0 (Systac Inc., Melbourne, FL), and the matrix of the S_{sm} correlation coefficients were clustered by the unweighted paired group mean average method (UPGMA). The similarity between all fingerprints obtained for each method is expressed as a Dice coefficient.

s2m2 strains and duodenitis was observed. These results are consistent with the fact that less virulent *vacA s2m2* genotypes are associated with lower gastric epithelial damage, because very little or no toxin is produced. However, in order to confirm this association, further studies are required with more patients suffering this less severe pathology.

The prevalence of *iceA2* was higher than that of the *iceA1* allele; however, no statistically significant difference was obtained. Similar frequencies of occurrence of the *iceA1* and *iceA2* alleles were reported by Leanza et al. in 2004 in Argentina.[9] Similarly, no statistically significant difference between *iceA2* (44.9%) and *iceA1* (30.8%) has been reported in Colombia. Our results contrast with the reports of high prevalence of *iceA1* alleles in the USA (78.6%) and Brazil (74.2%).[6,12] The rate of detection of isolates with both *iceA1*/*iceA2* alleles, which may be considered as mixed *H. pylori* infections, was 8.7% (26/299) (Table 1). In this work, the *iceA1*/

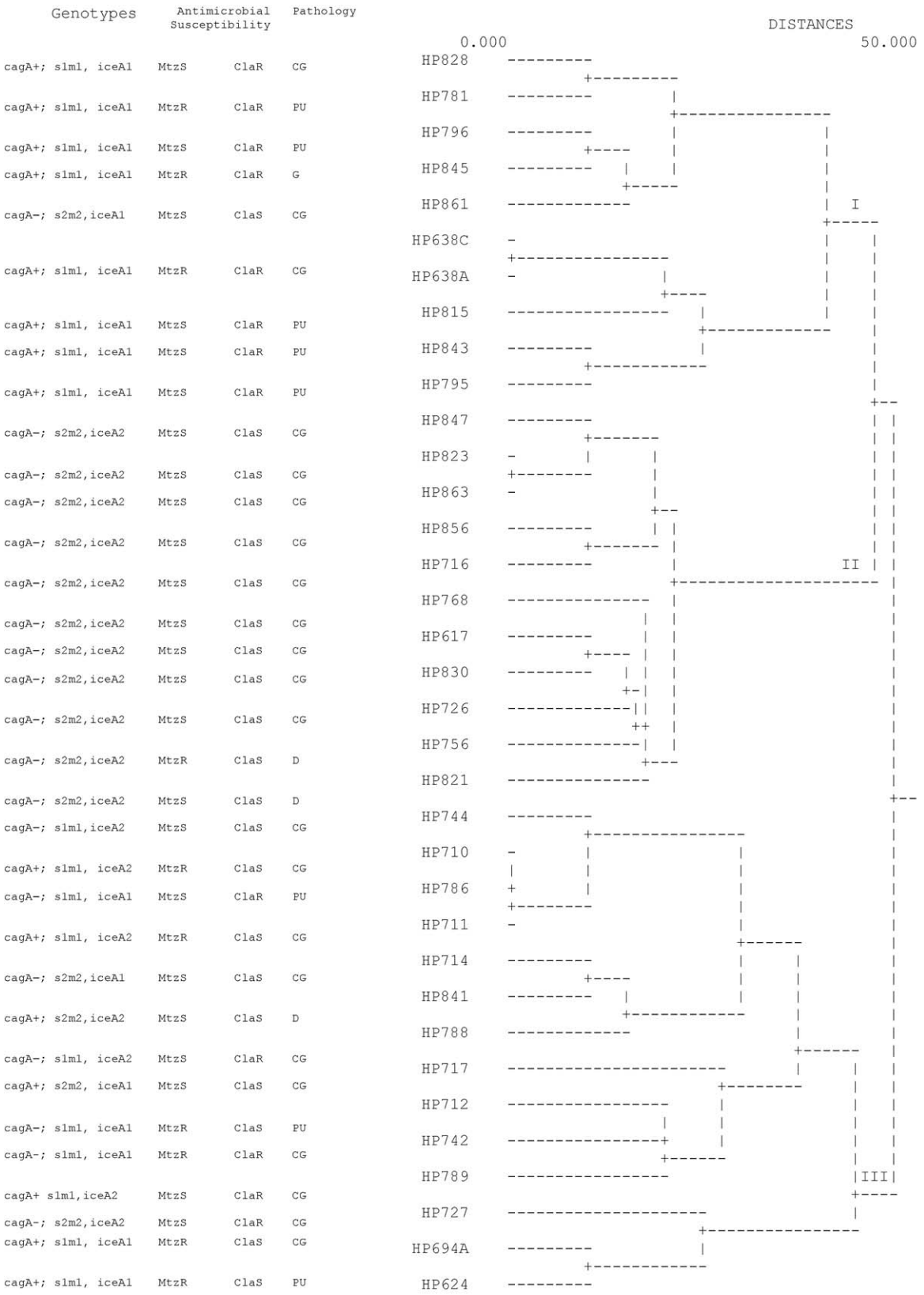

Figure 2. Dendrogram of *Helicobacter pylori* strain BOX A1R DNA fingerprints. Three separate clusters (I, II, and III) at a genetic distance of approximately 63% are indicated. Cluster III consists of 15 isolates from 11 patients suffering chronic gastritis (CG), three patients with peptic ulcer (PU), and one patient with duodenitis. The segregation of PU was significant ($p = 0.03$) in cluster III. There were significant differences in the segregation of combined virulence markers ($p = 0.0008$) and susceptible and resistant strains ($p = 0.0000$).

iceA2 genotype was statistically associated with *cagA* negativity ($p = 0.0006$).

The number of mixed infections based on the *iceA1*/*iceA2* genotype obtained in this study was similar to that obtained in Brazil (7.74%),[28] but higher than the number reported in a previous study in eastern Argentina (2.3%).[9] Mixed infection with two or more *H. pylori* strains colonizing the same patient possibly represents a stable association during longtime colonization.[13] Also, it could be explained by the genetic rearrangement of a founding cell and its capacity to lose and acquire exogenous DNA during persistent colonization, resulting in a diversity of *H. pylori* lines following bacterial adjustment to changing host conditions.[29]

The different rates of multiple infections reported from the USA (4.3%), Japan (17%), Korea (20%), and Colombia (22%)[6] could reflect the ability of the genetic techniques used to detect specific polymorphic loci and the number of biopsy samples analyzed from a single patient, rather than real differences between populations.

More recently, besides the genetic characterization of the strains, host susceptibility factors have been taken into account to understand the complex host–pathogen relationship that determines the natural history of *H. pylori* infection. Among them is the association between genetic polymorphisms in pro-inflammatory interleukin, which varies among individuals within the same populations.[30] Also the secretor status of the host has been studied in different populations.[31] In order to match host receptors and thereby stabilize microbial colonization, *H. pylori* developed the BabA and SabA adhesins, which bind to the host fucosylated blood group ABO antigens (denoted the ABH antigens) and sialylated Lewis antigens, respectively.[32,33] The ABH antigens are also expressed along the oro-gastrointestinal mucosal lining in individuals of positive secretor status.[34] A majority of Caucasians (80%) are secretors, whereas 20% are non-secretors. Although we did not determine the secretor status of the cohort analyzed in this study, Argentina is considered to be a predominantly white country. A recent study on the average genome of the Argentine population established that it contains 79.9% of Caucasian race contribution.[35] We hypothesized that the high prevalence of less severe pathologies, such as chronic gastritis and duodenitis, and low rates of gastric cancer and PU diseases in our population, may be associated with the prevalence of secretor phenotype, which reduces bacterial density and the associated inflammation during *H. pylori* infections.

In this study, a statistically significant association between the virulent *cagA*-positive/*vacA s1m1*/*iceA1* genotypes was observed ($p = 0.0000$). The rates of triple- and double-combined positive were 14.7% and 31.8%, respectively. The triple-combined positive rate was lower than that obtained in a previous study in eastern Argentina (35.2%)[9] and other Latin-American countries, with 32.6% of *cagA*-positive/*vacA s1m1*/*bab*-positive genotype in Brazilian patients[23] and 56.2% in Cuban isolates.[24] Despite the low rates obtained in this work, there was a strong association between combined virulent genotypes and PU ($p = 0.0001$) (Table 1). Infection with these strains has been associated with a higher degree of inflammation and gastro-duodenal lesions. The same association has been observed between triple-virulent strains and PU in several reports.[6,12,24] The results obtained in this work showed that the majority of double- (69/95) and all triple-virulent strains have contact-dependent mechanisms to interact with modified epithelial cells, including a type IV secretion system that injects the virulence factors CagA into host cells.[36] Recent reports indicate that the translocation of CagA protein increases the risk of gastric diseases because it plays a key role in altering epithelial cell polarity to enable adhered *H. pylori* to turn the apical cell surface into a replicative niche.[36] It may be considered that Cag status alone is not enough to induce gastric pathology in our population, but when it is combined with other virulence genes it is clearly associated with PU.

The rate of ClaR obtained in this work (27.7%) is similar to that of previous studies carried out in the same region of Argentina (23.9%),[37] Chile (20%),[38] and Spain (21.4%),[39] but higher than Paraguay (2%).[40] There are no previous reports of *H. pylori* resistant to Mtz in Argentina. The rate of MtzR was found to be 37.8% in this work, in agreement with rates observed in developing countries, which range between 5% and 90%, and higher than in Western Europe and the USA.[41]

The correlation between virulence factors and antimicrobial resistance varies in different countries. Most studies have shown no significant correlation,[12] while in others, lower rates of resistance to Cla and Mtz have been reported in more virulent strains of *H. pylori*.[42] However, in studies carried out in North Wales and in Central Italy, a clear association has been found between more virulent genotypes and antibiotic resistance.[13,19] A similar association has been observed between ClaR *H. pylori* strains and *iceA1* in western Argentina.[37] In the present study, a significant association was found between combined virulence markers, PU outcome, and resistance to Cla and Mtz, suggesting that virulent strains are difficult to eradicate in this region. Moreover, it was found that a less virulent genotype *cagA*-negative/*vacA s2m2* was associated with susceptible isolates.

It has been reported that the efficacy of triple therapy treatment eradication of *H. pylori*, based on a per-protocol analysis, is comparable among peptic ulcer and non-ulcer dyspepsia patients, regardless of CagA status.[43] It is well accepted that antibiotic resistance is a leading cause of eradication treatment failure, so tailored treatment based on antibiotic susceptibility testing might be more effective in the achievement of a high eradication rate when high resistances rates to Mtz or Cla are present in PU strains. Also, another cause that would affect the efficacy of eradication treatment is the increased free and long-term use of PPIs in Argentina to manage acid-related diseases usually associated with the alimentary habits of the population (e.g., intake of 70 kg of red meat per capita/year), which require digestive juices high in hydrochloric acid. PPIs may induce aberrant forms and changes in the *H. pylori* orientation in the epithelial cells that negatively affect antimicrobial eradication treatment.[44]

Another factor to consider is that the hepatic metabolism of PPIs influences their inhibitory potency on hydrochloric acid secretion.

RAPD and rep-PCR techniques have shown a high discriminatory power. However, few studies have suggested that DNA fingerprints may be useful in defining disease-associated strains.[15] *H. pylori* strains from ulcer patients have been found to be more homogeneous as determined by PCR fingerprints.[45] Similarly, both AO2 and BOX A1R fingerprint patterns clearly defined a distinct pathological cluster for PU that segregated with combined virulence markers and ClaR and MtzR strains. Both AO2 and AO3 primers allowed the differentiation of ClaR and MtzR from ClaS and MtzS strains, while the ERIC primer allowed the segregation of virulence fingerprints, but no disease-associated strains. This is the first report where BOX A1R primer has successfully been used to discriminate *H. pylori* strains associated with PU in Argentina.

Rep and RAPD primers were capable of generating DNA fingerprints, which may include virulence-specific or antimicrobial resistance areas, and were useful in discriminating PU-related strains. Also, *H. pylori* isolates from western Argentina that harbored two or three virulence markers were more resistant to antibiotic treatment. This association appears to be uncommon and our finding is of special importance due to its implications for the treatment and evolution of the pathology.

Combined analysis of the *H. pylori* virulent genotypes and resistance patterns may permit identification of high-risk

patients, to prevent PU later in life or to avoid antimicrobial treatment failure.

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