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Frequency and characterization of *vapD* gene in *Helicobacter pylori* strains of different *vacA* and *cag-PAI* genotype

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RESUMEN

Diversos estudios, realizados con diferentes técnicas de tipificación molecular han demostrado que el contenido genético y arreglo cromosómico entre cepas de *Helicobacter pylori* son altamente variables. Además, se ha observado esta variabilidad en diferentes genes, como *vacA* que presenta una estructura de mosaico y *cag*-PAI, que presenta una organización diferente en el cromosoma. Un estudio preliminar sugiere que el gen *vapD*, es un gen variable de cepa, presente en aproximadamente 60% de las cepas de *H. pylori*. El gen *vapD* está relacionado con el gen de la proteína D asociada a virulencia de *Dichelobacter nodosus*. El objetivo de este estudio fue caracterizar y determinar la frecuencia del gen *vapD* en 301 cepas de *H. pylori* con diferentes genotipos de *vacA* y cag-PAI, provenientes de 27 pacientes con diferentes patologías gástricas. Los resultados mostraron que el 70% de los pacientes presentan infección múltiple con cepas de *H. pylori* de diferentes genotipos de *vac*A y *cag*-PAI. El 52% de ellos estuvieron colonizados con cepas *vapD* positivas y *vapD* negativas. La caracterización de *vapD* en cepas individuales, mostró una frecuencia del 38%. Este es el primer estudio que muestra la frecuencia de *vapD* en cepas de *H. pylori* aisladas de pacientes mexicanos. Con respecto a los genes *vacA* y *cag*-PAI, el genotipo más frecuente entre las cepas fue s1-m1/*cag*-PAI+, aisladas de pacientes con dolor abdominal crónico, úlcera gástrica y úlcera duodenal. El gen *vapD* no tiene asociación significativa con un alelo específico de *vacA* o el genotipo de *cag*-PAI y la patología gástrica.

Palabras clave: *Helicobacter pylori*, infección múltiple, genotipos de *vacA*, *vapD*, *cag*-PAI.

ABSTRACT

Several studies using different molecular-typing techniques have demonstrated that the gene content and the chromosomal arrangement among Helicobacter pylori *strains are highly variable. Furthermore, this variability has been observed in different genes, such as* vacA*, which has a mosaic structure, and in* cag*-PAI, which has a different chromosomal organization. Preliminary study suggests that* vapD *is a strain-variable gene and it is present in about 60% of* H. pylori *strains.* vapD *gene is closely related to the virulence-associated protein D gene of* Dichelobacter nodosus*. The aim of this study was to characterize and to determine the frequency of* vapD *gene, in 301* Helicobacter pylori *strains with different* vacA *and* cag*-PAI genotypes isolated from 27 Mexican patients with different gastric pathologies. The results showed that 70% of the patients presented multiple infections with* H. pylori *strains of different,* vacA*, and* cag*-PAI genotypes. Fifty two percent of the patients were colonized with* vapD*-positive and -negative strains. Regarding the* vapD *gene characterization by individual strains, it presented a frequency of 38%. This is the first characterization report that shows the frequency of* vapD *gene in* H. pylori *strains from Mexican patients. With respect to* vacA *and* cag*-PAI, the s1-m1/*cag*-PAI positive genotypes were more frequent in strains isolated from patients with recurrent abdominal pain (RAP), gastric ulcer (GU), and duodenal ulcer (DU). Statistical analysis showed that the* vapD *gene did not have association with any specific family of* vacA *or* cag*-PAI genotypes and specific gastric pathology.*

Key words: Helicobacter pylori, vacA, cag*-PAI*, vapD *typing, multiple infections.*

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INTRODUCTION

Helicobacter pylori plays an important role in the pathogenesis of peptic ulcer disease, distal gastric adenocarcinoma, and gastric lymphoma.[1,2] Several putative virulence factors have been proposed, some of them are: urease and catalase production;[3-5] presence of adhesins, such as BabA;[6] the *cag* pathogenicity island (*cag*-PAI);[7,8] CagA expression,[9,10] a high molecular weight protein encoded by the *cagA* gene located at 3' end of the *cag*-PAI; and VacA, a vacuolating cytotoxin encoded by the *vacA* gene.[10,11] Genetic diversity among bacterial strains has also been related to pathogenicity.[12, 13] Previous studies using molecular-typing techniques, such as random amplification of polymorphic DNA (RAPD),[14] DNA fingerprinting, multilocus enzyme electrophoresis (MLEE), and pulsed-field gel electrophoresis (PFGE) have demonstrated that the gene content and the chromosomal arrangement are highly variable among different *H. pylori* strains.[15-17] Genetic diversity among *H. pylori* isolates is particularly striking in the vacuolating cytotoxin (*vacA*) gene.[18] *vacA* has a mosaic structure; it consists of several regions that are conserved and other regions that are highly divergent.[18] Two families of *vacA* alleles (types m1 and m2) can be differentiated by the analysis of the middle region of *vac*A and three types of *vacA* signal sequences (s1a, s1b, and s2,) can be differentiated.[18] Cao and Cover characterized a 4.2-kb chromosomal region downstream from the *vacA* locus, identifying 5 open reading frames (ORFs), 4 of which (*fepC*, *hemU*, *vdlC*, *vdlD*) were present in all characterized strains.[19] The last of these, a 285 bp ORF, was closely related to the virulence-associated protein (*vapD*) D gene of *Dichelobacter nodosus*[20,21] which encodes a putative 94-amino-acid product (VapD) that exhibits a high-level homology with the products encoded by other bacteria, such as *Haemophilus influenzae*[22] and *Neisseria gonorrhoeae.*[23] The *vapD* gene was present in about 60% of *H. pylori* strains and showed that this *vapD* region had an additional high-level genetic diversity regarding the *vapD*-negative strains.[19] Currently, the *D. nodosus* VapD protein function is unknown but the *vapD* gene is present in all pathogenic *D. nodosus* strains.[20,21,24]

Previous studies showed that our Mexican patients had multiple infections with *Helicobacter pylori* strains with different vacA and cag-PAI genotypes.[25,26] However, there are few worldwide *vapD* gene studies, including our country, showing the frequency of the gene in *H. pylori* strains.

The aims of this study were to characterize and determine the frequency of *vapD* and the *vacA* and *cag*-PAI genes by the PCR method, in 301 *H. pylori* strains isolated from 27 Mexican patients: seven children with recurrent abdominal pain (RAP), twenty adults, six with gastric ulcer (GU), nine with duodenal ulcer (DU), and five with non-ulcer dyspepsia (NUD).

METHODS

Gastric biopsies, strains and culture. Antrum and corpus gastric biopsies were obtained from 27 patients (7 children and 20 adults) who had been admitted to the Hospital de Pediatría and the Hospital General respectively, in Mexico City, Mexico.

All 7 children presented recurrent abdominal pain (RAP), whilst the adults were diagnosed as follows: 6 with gastric ulcer (GU), 9 with duodenal ulcer (DU), and 5 with non-ulcer dyspepsia (NUD).

The biopsies were transported to the laboratory in Brucella broth with 10% glycerol and frozen at -70°C until tested. The biopsy specimen was homogenized with a glass tissue grinder and a 50 μL sample was inoculated onto Casman agar plates with 10% sheep blood supplemented with an antibiotic mix (vancomycin 10 μg/mL, polymixin B 3.3 μg/mL, bacitracin 20 μg/mL, nalidixic acid 10.7 μg/mL, amphotericin B 5 μg/mL and trimethoprim 5 μg/mL). The plates were incubated at 37°C under microaerophilic conditions (BBL CampyPak plus, Beckton Dikinson) for a maximum of 10 days.[27,28] Cultures exhibiting typical *H. pylori* morphology were confirmed by gram stain and by positive urease, catalase, and oxidase tests. An average of 10 single colonies was isolated from the antrum and corpus cultures taken from each of the 27 patients. Each colony was individually grown in Casman agar plates as described above. Two *H. pylori* strains were used as controls: the 60190 strain (*vapD*-positive, s1-m1 *vacA* alleles and *cag*-PAI+) as positive control and the Tx30a strain (*vapD*-negative, s2-m2 *vacA* alleles and *cag*-PAI-) as negative control.[18,19]

DNA isolation. Each single colony of *H. pylori* was cultured as mentioned above. DNA was isolated from the cultured bacteria by harvesting cells from a plate in phosphate-buffered saline, followed by centrifugation. The pelleted cells were resuspended in 200 μL of proteinase K solution (10 mM Tris-HCl [pH 7.8], 5 mM EDTA, 0.5% sodium dodecyl sulfate [SDS], and 50 mg of proteinase K per mL), and the mixture was incubated at 55°C for 30 min. The DNA

was extracted using phenol-chloroform-isoamyl alcohol following standard procedures.[29] DNA was precipitated by adding 1/10 volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol. After centrifugation, the DNA pellet was washed with 70% ethanol and dried at room temperature. The DNA was dissolved in 50 μL of TE buffer (10mM Tris-HCl [pH8.3], 0.1mM EDTA) and its concentration was determined in a spectrophotometer (GENios, TECAN Austria GMBH).

***vacA* and *cag*-PAI genotyping by specific PCR amplification.** The *vacA* signal sequence and mid-region were typed by allelic type-specific PCR, as previously described. Briefly, each strain was typed as *vacA* signal region, s1 or s2, by performing a PCR assay using the conserved forward and reverse primers VA1-F and VA1-R, previously described by Atherton *et al*.[18] The *vacA* mid-regions were typed as m1 or m2 by performing two separate PCR assays, each one with two allelic type-specific primers. Conditions for thermal cycling were 35 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min. PCR detection of *cag*-PAI followed previously determined procedures.[30] Briefly, each strain was typed with or without *cag*-PAI by a specific PCR assay using the primers previously described. PCR was performed with an initial denaturizing cycle at 94°C for 5 min, and then 35 cycles at 94°C for 1 min, 57°C for 1 min and 72°C for 1 min each. All product sizes were differentiated using 1% agarose gel electrophoresis, stained with ethidium bromide, and examined under UV illumination.

***vapD* probe obtaining and characterization by specific PCR amplification.** A set of oligonucleotide primers for the *vapD* gene was designed: D1 (5' AGAGATGCGGTGAATGG 3') (nucleotides 305-321) and D2 (5' AAGCGTTATGAGTGGTGTG 3') (nucleotides 786-803) using a LASERGENE software program (DNASTAR, Inc. USA) and derived from the chromosomal region containing the *vapD* ORF (nucleotides 399 to 684) from the previously characterized 60190 *H. pylori* strain [GeneBank / EMBL:U94318].[19] The primers bond to the sequence flanking outside the *vapD* ORF, and we expected a 498bp PCR product. The PCR mix master consisted of 10ng of *H. pylori* DNA and 5 μL PCR buffer 10 X solution (Gibco BRL, Gaithersburg, MD), 1.5mM $MgCl_2$, 25 pmol of each primer, 10nM dNTPs and 5U of Taq polymerase (Gibco BRL). PCR was performed under the following conditions: 35 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 1 min each, in a DNA Engine PTC-200 (MJR Research, Inc. Waltham, Massachusetts). The product was electrophoresed on a 1% agarose gel, stained with ethidium bromide, and examined under UV light. The 498–bp product PCR was excised from gel and purified using the QIAquick PCR purification kit from QIAGEN and was used as a probe.

Southern hybridization. The PCR amplification products were electrophoresed on a 1% agarose gel and the capillaries were transferred onto an Hybond-N+nylon membrane (Amersham-Pharmacia Biotech) using a 20 X SSC (NaCl 3M, Na citrate 0.3M; pH 7) as a blotting buffer.[29] DNA was fixed to the membrane by exposure to UV transillumination for 5 min. The Southern blot membrane was hybridized overnight in Dig buffer (formamide 50%, blocking agent 1%, N-Sarkosyl 0.1%, SDS 0.02%, 5 X SSC) at 52°C using a digoxigenin-labeled (Boehringer Manheim) hybridization *vapD* probe (20 ng mL^{-1} of hybridization buffer) and revealed using a DIG Oligonucleotide 3'-End Labeling kit (Boehringer Manheim).

Sequencing. Randomly chosen PCR products were purified using the QIAquick PCR purification kit from QIAGEN and sequenced on both strands using the fluorescent dideoxy terminator method (ABI PPRISM Dye Terminator Cycle Sequencing Ready Reaction Kit [Perkin Elmer]) running samples on an automatic ABI prism 3100 sequencer (Perkin Elmer). The DNA sequence was edited using the LASERGENE (DNASTAR, Inc.) software package. The analysis and detailed alignments were carried out with the CLUSTAL program. A database search for homologous genes and proteins was performed by the BLAST network service of the National Centre for Biotechnology Information (NCBI).

Statistical methods. Descriptive statistics were frequencies and percentage. The χ^2 test with Yates's continuity correction and Fisher's exact test were used for the analysis of two-by-two tables of categorical data. All tests were two-tailed and the significance level was fixed at 0.05.

RESULTS

Genotyping of *vacA and cag-PAI*. A total of 301 strains, isolated from 27 patients, were characterized for *vacA* and *cag*-PAI: 75 strains were studied from 7 children with RAP, 71 from 6 adults with GU, 99 from 9 adults with DU, and 56 from 5 adults with NUD.

Regarding the strains isolated from children with RAP, 49%, 18%, and 4% were s1/m1, s1/m2, and s2/m2 *vacA* genotypes respectively; 29% was not able to be typed for *vacA* signal sequence with conserved

primers or with mid-region with allelic type-specific primers;[18] 65% of the strains were *cag*-PAI$^+$ and 35% were *cag*-PAI$^-$. Regarding the strains that were isolated from adults with GU, 62% and 39% were s1/m1 and s2/m2 *vacA* genotypes respectively; 58% were *cag*-PAI$^+$, and 42% were *cag*-PAI$^-$. From the strains that were isolated from adults with DU, the 61%, 17%, and 21% were s1/m1, s1/m2 and s2/m2 *vacA* genotypes respectively, and 75% of them were *cag*-PAI$^+$ and 25% were *cag*-PAI$^-$. Finally, from the strains isolated from patients with NUD, 5% were s1/m1 and s1/m2 *vacA* genotypes respectively and 90% were s2/m2; and 5% were *cag*-PAI$^+$ and 95% were *cag*-PAI$^-$.

In general, strains with s1/m1 and *cag*-PAI$^+$ genotypes were the most frequently found in both the antrum and corpus from patients with RAP, GU and DU. Meanwhile, the s2m2 and *cag*-PAI$^-$ genotypes were more common in patients with NUD *(Table I)*.

***vapD* typing.** As described before, a set of primers (D1 and D2 position nucleotides 305-321 and 786-803, respectively) was designed with respect to the 4.2-kb chromosomal *vapD* region from *H. pylori* 60190 (GeneBank /EMBL accession No.U94318) and was used as positive control for *vapD* characterization. A 498bp DNA fragment from *H. pylori* 60190 (positive control) was amplified by PCR. From all the 301 single tested colonies, 38% (114) of the strains were *vapD* positive and 59% were *vapD* negative; 3% of the strains could not be characterized. Regarding the patients, 52% were colonized by *H. pylori vapD*-positive strains in the antrum and/or corpus *(Table I)*.

Multiple colonization. Seventy percent of the patients were infected in both the antrum and corpus by *H. pylori* strains of multiple *vacA*, or *cag*-PAI or *vapD* genotypes. In the RAP children's group, five were colonized by *H. pylori* strains with different *vacA* types, three of them simultaneously presented *cag*-PAI-positive and negative colonies. In addition, one of them had both *vapD*-positive and -negative strains *(Table I)*. Regarding the DU group, three pa-

Table I. Relation of *vacA*, *cag*-PAI and *vapD* genotypes of *Helicobacter pylori* isolates from different patients with multiple infection.

Patient	Disease	*vacA* allelic type	*cag*-PAI	*vapD* PCR-product		
				Positive	Negative	ND*
250	RAP	s1/m1	-		10	
64	RAP	s1/m1; s1/m2	+/-		13	
72	RAP	s1/m1; s1/m2	+/-	8		2
262	RAP	s1/m1	+	10		
92	RAP	s1/m1; ND¶	+		11	
236	RAP	s1/m2; ND¶	+	10		
128	RAP	s2/m2; ND¶	+/-	9	2	
33	DU	s1/m1	-		10	
46	DU	s1/m1	+	4	6	
52	DU	s1/m1	+	9	3	
60	DU	s1/m1; ND¶	+	7	3	
249	DU	s1/m1; s1/m2	+/-	9	4	
259	DU	s1/m1; s2/m2	+/-		10	
256	DU	s1/m1	+	1	10	
251	DU	s2/m2	-		13	
59	DU	s1/m2	+		10	
1C	GU	s2/m2; ND¶	+/-		10	
53	GU	s1/m1	+/-		10	
62	GU	s1/m1	+/-		10	
252	GU	s2/m2	-	14	2	1
248	GU	s1/m1	+	11	3	
261	GU	s1/m1	+		10	
48	NUD	s1/m1; s2/m2	-		5	5
254	NUD	s2/m2	-	11		
179	NUD	s2/m2	-	1	11	
21	NUD	s1/m1; s2/m2	+	10		
75	NUD	s2/m2	-		13	

Abbreviations: RAP, recurrent abdominal pain; DU, duodenal ulcer; GU, gastric ulcer; NUD, non-ulcer dyspepsia
* No product generated by PCR
¶The signal sequence or mid region type of the *vacA* gene could not be determined.

tients were colonized with strains of different *vacA* genotypes; two of them simultaneously presented *cag*-PAI-positive and negative colonies and five patients were colonized with *vapD*-positive and -negative strains. From the GU group, one patient was infected by *H. pylori* strains of different *vacA* types, three were simultaneously infected by *cag*-PAI-positive and negative strains and two patients were infected by *H. pylori* strains of different *vapD* genotypes. Finally, in the NUD group, two patients were infected by *H. pylori* strains of different *vacA* types, one was infected by strains of different *vapD* types, and one more had strains that could not be *vapD* characterized *(Table I)*.

Statistical analysis. Statistical analysis among *H. pylori* genotypes showed association between the s1-m1/*cag*-PAI+ genotype and DU ($p = 0.005$), while that the s2-m2/*cag*-PAI- genotype was associated with DNU ($p = 0.008$). The presence of the *vapD* gene did not show association with the *vacA* or *cag*-PAI genotype neither with any disease-specific condition ($p = 0.48$).

Southern blot hybridization. To investigate if the PCR products amplified with a set of primers used in this study, corresponding to the *vapD* gene, seven PCR products from different strains were randomly selected plus 60190 and Tx30a strains (positive and negative control, respectively) and transferred to a Hybond-N+nylon membrane. The transferred PCR products were derived from the following strains: MxHp72a6, MxHp252a2, MxHp254a8, MxHp254c9, MxHp262c11, Mx262c13, and MxHp72a10 *(Figure 1)*. The *vapD*-specific probe hybridized strongly with all PCR-products tested, under highly stringent conditions *(Figure 1)*. In contrast, there was no hybridization with the *vapD*-negative *H. pylori* (Tx30a) strain.

Nucleotide sequence accession numbers and sequence analysis of the *vapD* gene. The seven PCR products derived from the strains positive for Southern blot were selected for sequencing. The *vapD* gene sequence of the seven Mexican *H. pylori* strains were submitted to the GenBank/EMBL Data Bank and were assigned: MxHp72a6 strain [GenBank/EMBL:AY781665]; MxHp72a10 strain [GenBank/EMBL:AY781666]; MxHp262c13 strain [GenBank/EMBL: AY781667]; MxHp262c11 strain [GenBank/EMBL:AY781668]; MxHp254c9 strain [GenBank/EMBL:AY781669]; MxHp254a8 strain [GenBank/EMBL:AY781670]; and MxHp252a2 strain [GenBank/EMBL:AY781671].

The sequence analysis of all the studied strains, including *H. pylori* 60190 strain [GenBank/EMBL: U94318] was edited using the LASERGENE (DNASTAR, Inc.) software package. The detailed alignments were carried out via the CLUSTAL program. A database search for homologous genes and proteins was performed by the BLAST network service of the National Centre for Biotechnology Information (NCBI).

As expected, the nucleotide sequence of the *vapD* gene from the Mexican strains was highly homologous to the *vapD* ORF from *H. pylori* 60190 strain (97.5%). The nucleotide sequences of the *vapD* gene of five Mexican *Helicobacter pylori* strains were all closely related to each other (identity between 97.3 and 99.3%) and their deduced protein was 100% homologous to each other *(Figure 2)*. The sequence analysis of the *vapD* ORF from two Mexican *H. pylori* strains (MxHp262c13 [GenBank/EMBL:AY781667] and MxHp262c11 [GenBank/EMBL:AY781668]) presented internal stop codons in the conceptual translation of the coding re-

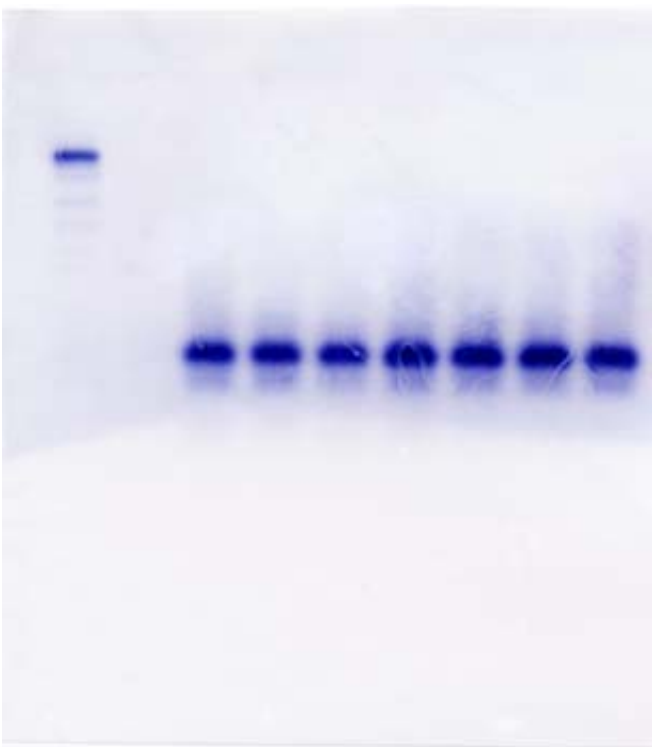

Figure 1. Southern blot hybridization of PCR products with *vapD*-specific probe. The PCR products electrophoresed on a 1% agarose gel were transferred to a nylon membrane and hybridized with a digoxigenin-labeled *vapD* probe under highly stringent conditions. The *vapD* probe hybridized strongly with the PCR products (lines 3-8), line 2 and 9 correspond to negative and positive control respectively. The line 1 corresponds to 100 bp weight marker.

gion, due to a frame shift caused by an insertion of a nucleotide (A) around position 55 *(Figure 2)*.

The comparison between *vapD* products from the Mexican *H. pylori* strains and the database showed that VapD exhibited a high-level homology with products encoded by other bacteria, such as the chromosomal *vapD* gene from *D. nodosus* (68%), the chromosomal *vapD* gene from *H. influenzae* (40.7%), and the ORF5 from the *Neisseria gonorrhoeae* cryptic plasmid (49.8%).

DISCUSSION

Previous studies have reported that infections by multiple *H. pylori* strains of different genotypes are particularly common in Mexico.[25,26] Those studies have shown that almost 85% of Mexican patients are colonized by multiple strains of *H. pylori* of different *vacA* and *cagA* genotypes both gastric antrum and corpus independently of the gastric pathology. In the current study, it was not surprising found that the 70% of our patients were infected with *H. pylori* strains with different *vacA* and *cag*-PAI genotypes in both the antrum and corpus and that this condition would be present in the four groups of patient studied. In addition, we found that more than half of the patients presented colonization in their stomach with *vapD* positive and negative *H. pylori* strains, confirming the multiple infections. On the other hand, we observed that the RAP group had the mayor number of patients infected with strains of different *vacA* genotype, while the UD group was who presents the major number of patients with infection with *vapD*-positive and negative strains, although they did not show any association.

When we made the *vapD* characterization of single colony isolates from each patient, we found that *vapD* is present in 38% of our strains (114 out of 301 strains), contrary to that reported by other stuies[19,31] where it was found in approximately 70% frequency in *H. pylori* strains. The difference between our frequency and the other studies is probably due to the fact that those studies work with the characterization of the total growth obtained for patients and did not work with individual isolates and we characterized single colonies isolated from the total growth obtained from the biopsies, with this procedure we avoiding false positives.

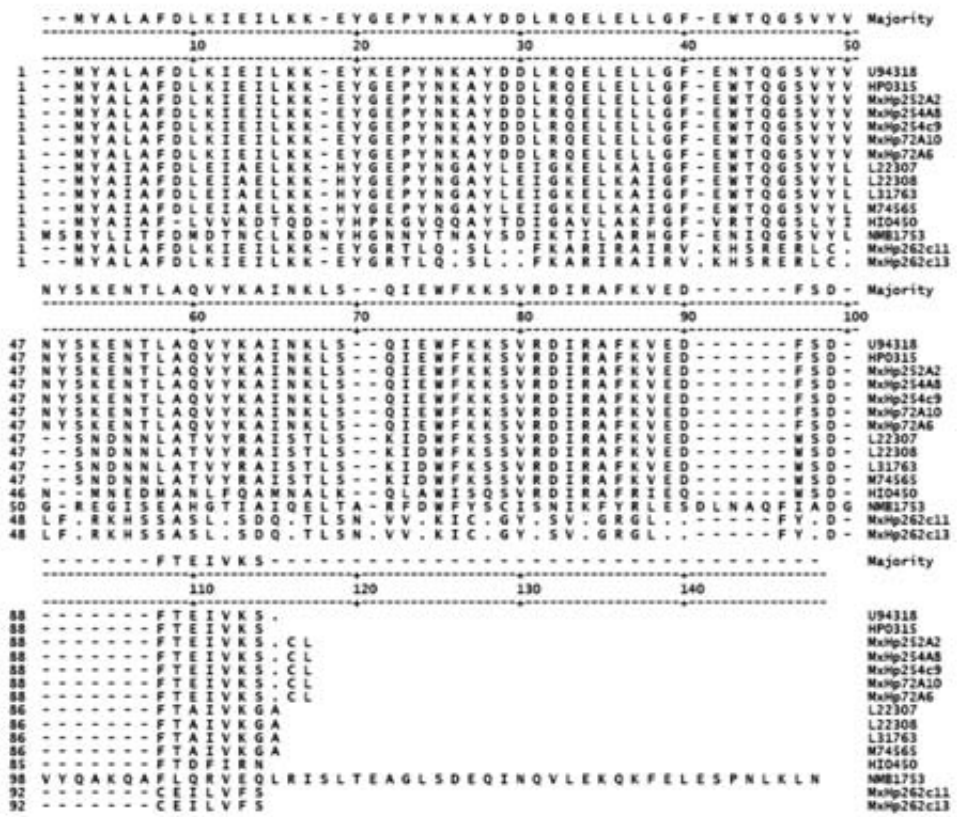

Figure 2. Alignment of the deduced *H. pylori vapD* products with homologous gene products from other bacterial species. Mexicanstrains: MxHp252 A2,MxHp254A8, MxHp254c9, MxHp72A10, MxHp72A6, Mx Hp262c11, and MxHp262c13; 60190 strain: U94318; 26695 strain: Hp0315;*Dichelobacter nodosus*:L22307,L22308, L31763, M74565; *Haemophilus influenzae*: HI0450; *Neisseria meningitidis* NMB1753. Periods indicate gapsthat were introduced to allow optimal alignment, and points indicate stop codons.

A number of putative virulence factors for *H. pylori* have been well documented, including CagA, VacA, IceA, *cag*-PAI, and BabA.[6-9,30] However, there are problems in separating putative virulence factors from true virulence factors due to a lack of criteria for establishing and determining a true virulence factor, especially when it is associated with a specific disease. The *cag*-PAI is involved in the induction of the cytokine expression in gastric epithelial cells, which is manifested in a marked increase in the interleukin-8 (IL-8) secretion.[7,9] Individuals infected with *H. pylori* that have a functional *cag*-PAI have an elevated mucosal level of IL-8, marked neutrophilic infiltration into the gastric mucosa, and an increased risk of developing a symptomatic outcome such as peptic ulcer or gastric cancer. However, the relationship between the presence of *cag*-PAI and the outcome is not consistent among different geographic regions, especially in East Asia, where more than 90% of isolates possess the *cag*-PAI, irrespective to the outcome.[6,32,33] In Western countries, *H. pylori* strains lacking the *cag*-PAI are found in a higher percentage compared with Asian countries, and the increased likelihood of a symptomatic outcome is seen. Different studies in the Mexican population, including this current study, have found that a single patient has both *cag*PAI-positive and –negative *H. pylori* strains in the gastric antrum and corpus independently of the gastric pathology, therefore, it has not been determined whether the presence of *cag*PAI-positive strains is linked to a specific gastric disease or not in our population.[25,26] Regarding to *vacA,* the gene for the vacuolating cytotoxin, it has been subtyped and it was thought that genotyping might show a disease-specific association.[18,34] Different studies showed that the s1 genotype was associated with duodenal ulcer disease and the s2 genotype was associated with asymptomatic patients, our finding confirmed these results. However, a number of prospective studies have been done to investigate the value of *vacA* genotyping.[6,13] A compilation of studies involving approximately 1,500 isolates from Europe, the US, and Asia has not substantiated the original hypotheses and, currently, there is an overwhelming amount of data showing that *vacA* genotyping is not useful for predicting symptoms, presentation, therapy responses, or inflammation degrees.[35] Actually, *vacA* genotyping is useful to predict *cagA* status. Current data do not support the notion that *vacA* is a virulence factor in its own right, nor that it is additive with *cagA*. Overall, the hypothesis that *vacA* genotyping might prove clinically useful, for example to predict presentation such as duodenal ulcer, has never been proved.[35] The results of this current study support those observations; strains with different *vacA* and *cag*-PAI genotype have been found in all patient groups without any association. In Mexico, the link between infection and strains of a specific *vacA* and *cag*-PAI genotype with gastroduodenal disease is not directly applicable since infection with multiple strains of different *vacA* and *cag*-PAI genotypes is common.[25,26]

Regarding the *vapD* gene, different studies have showed that the *vapD* gene has been associated to virulence in *H. influenzae,*[22,36] *Rhodococcus equi,*[37] and *D. nodosus,*[20] all of them facultative intracellular microorganisms. In *H. influenzae* and *Rhodococcus equi* studies, have proved to be highly induced by macrophage-related stress.[38] However, in *H. pylori*, it has not been determined if the *vapD* gene could play a role in the disease pathogenesis. Nevertheless, epidemiological and molecular studies are now required to assess the clinical significance of *vapD* –positive *H. pylori* strains and the action mechanism of *vapD* in the pathogenesis of gastric diseases.

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