Novel *VacA* S1d Signal Sequence Found in *Helicobacter Pylori* from Mexican Children with Recurrent Abdominal Pain

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Abstract

Helicobacter pylori is the causal agent of various gastric diseases and its virulence is associated with high genetic diversity at both a chromosomal and gene level. Genotype I strains are described as being highly virulent. The *vacA* gene is highly polymorphic and presents different subtypes with each group of alleles apparently having a specific geographic localization. In Mexico, as in other parts of Latin-America, s1b/m1 allele strains are predominant, although other subtypes have also been found. However, there are strains that could not be characterized using their *vacA* alleles suggesting even more variability. The aim of this study was to determine the Signal Sequence (ss) type of 23 *H. pylori* strains isolated from children with recurrent abdominal pain, which could not be characterized according to the *vacA* ss using Atherton's criteria. Five strains were sequenced from their ss region. The ss analysis showed the substitution of two base pairs at the 3' end of the complementary sequence for the SS3-F primer alignment site. Based on sequence analysis, we designed a primer set, which was used in a PCR protocol to determine the ss type presented in strains obtained from children. All of the strains yielded a PCR product. Genealogic and population structure analyses of the ss region of our strains with respect to the s1a, s1b and s1c subtype revealed a novel ss subtype, which we named s1d. Although the s1d and s1b subtypes are closely related, both s1b and s1d subtypes have perfectly separated lineages. The s1d subtype is exclusive in Latin-American populations. The cytotoxic activity of the s1d/m1 allele was variable, as was seen with the s1b/m1 allele. In conclusion, this present study identified a new *vacA* signal sequence subtype in *H. pylori* strains isolated from Mexican children with recurrent abdominal pain. Genealogic analysis showed that s1d subtype of *vacA* is a homogenous group and is present only in Latin American populations.

Keywords: *vacA* s1d signal sequence; *Helicobacter pylori*; Children; Recurrent abdominal pain

of the most common chronic human infections. *H. pylori* play an important role in the pathogenesis of peptic ulcer disease, distal gastric adenocarcinoma and gastric lymphoma [1]. Disease development results from a number of contributing factors, such as host susceptibility, environmental factors and bacterial genotype [2].

Several virulence factors have been associated with different gastric pathologies, some of these are: urease and catalase production; the presence of adhesins, such as BabA [2,3]; *cag* pathogenicity island (*cag*-PAI) [4,5]; *CagA* expression [6,7]; and the production of a vacuolating cytotoxin encoded by the *vacA* gene [8]. The variability of its genome is due to its ability to transform naturally and the incorporation of foreign DNA into its genome. The *vacA* gene has a mosaic structure consisting of several regions that are conserved and other regions that are highly divergent. Among the divergent regions of the *vacA* gene, there are basically two regions: the signal sequence (ss) region; and the middle region. For the signal sequence region, four subtypes of *vacA* have been described (s1a, s1b, s1c, and s2), and for the middle region, two subtypes (m1 and m2), all of which can be differentiated using PCR sequencing [8]. All combinations of the *vacA* signal sequence and middle allele types have been described worldwide. Extensive studies have shown that the *vacA* sequence signal types have a specific geographic distribution and some genotypes are more associated with disease than others [5-7]. In particular, the *vacA* s1c allele is more frequent in people from East Asia [9,10]. In Mexico, as in the rest of Latin America, the *vacA* s1b type is more frequent [11-15], while in most of the Western countries, *vacA* s1a type is more prevalent [5-7].

In a previous studies performed by our group, the *vacA* gene of *H. pylori* strains isolated from adults and children were characterized and *vacA* s1b/m1 allele was found to be the most prevalent in the studied population [11,13]. However, in this same study, we found strains isolated from children that could

Introduction

Helicobacter pylori is a gram-negative, microaerophilic bacterium that colonizes the human gastric mucosa; it is one

not be characterized according to the subtypes of the *vacA* gene signal sequence. With this in mind, we considered that there could be a greater variability in this region than had been described previously. In the current study, we randomly chose five strains isolated from children with recurrent abdominal pain that could not be formally typed according to the *vacA* signal sequence. Based on sequence analysis, we designed a new primer and completed the ss characterization using PCR.

Materials and Methods

Strains and culture: Using a PCR-based typing system developed by Atherton, we reported the previous characterization of *H. pylori* strain populations isolated from children and adults according to the *vacA* allele, and other virulence genes, such as *cagA* and *vapD*. In the aforementioned study that was carried out previously by our group, we found that 10% of the strains (23) could not be typed according to their *vacA* ss [11,13]. In the current study, we used these 23 *H. pylori* isolates, all of which were isolated from children with Recurrent Abdominal Pain (RAP). Primer sets were used to determine the *vacA* s1 or s2 signal sequence type according to the Atherton's characterization [8]. Additionally, we used four *H. pylori* strains as controls for each *vacA* allele: the 60190 and 26695 strains, which are both s1a/m1; the 84183 strain, which is s1b/m1; and the Tx30a strain, which is s2/m2.

Stock cultures of the *H. pylori* strains stored at -70°C were regrown individually on Casman agar plates supplemented with 10% sheep blood. The plates were incubated at 37°C under microaerobic conditions [GasPak™ EZ Campy (Beckton Dickinson)] for a maximum of 5 days.

DNA Isolation: Each *H. pylori* strain was cultured as described above. DNA was isolated from the cultured bacteria by harvesting cells from a plate in phosphate-buffered saline solution 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), the mixture was then incubated at 55°C for 30 min. The DNA was extracted using phenol-chloroform-isoamyl alcohol following standard procedures [16]. DNA was precipitated by adding 1/10 volume of 3 M 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 (10 mM Tris-HCl [pH 8.3], 0.1 mM EDTA) and its concentration was determined by a spectrophotometer (GENios, TECAN Austria GMBH).

***vacA* typing by allelic type-specific PCR primers:** *vacA* signal sequence typing was performed by PCR with the VA1-F and VA1-R primers to categorize this region as type s1 or s2 [8]. Subsequently, we made individual PCRs to determine the family type of m1 or m2 mid-region according to Atherton's protocol using m1-specific primers (VA3-F and VA3-R) and m2-specific primers (VA4-F and VA4-R) [8].

***vacA* signal sequence sequencing:** We randomly selected five strains (72a10, 536A8, 268c3, 92a5, 64c1) from

children whose *vacA* signal sequence could not be typed previously using Atherton's criteria; a 623 bp *vacA* fragment (coordinates 519 to 1142 bp in the published *vacA* sequence of strain 60190) corresponding to the 5' end of the *vacA* gene was amplified by PCR for each strain using CYSS primers (5'-CGCTTTGATGGACACCCCAACAA-3') and new primer (5'-CCCATCCCAACCTCCATCAATCT-3') designed in this study [8]. PCR products were purified using the QIAquick PCR purification kit from QIAGEN and sequenced on both strands. The DNA sequence was analyzed using the LASERGENE (DNASTAR, Inc.) software package. The detailed alignments were performed using the CLUSTALW program. Representative sequences have been deposited in GenBank: Mx536A8 strain [GenBank/EMBL accession no. DQ061145], Mx92a5 strain [GenBank/EMBL accession no. DQ061150], Mx64c1 strain [GenBank/EMBL accession no. DQ061153], Mx72A10 strain [GenBank/EMBL accession no. DQ061154] and Mx268c3 strain [GenBank/EMBL accession no. DQ061156].

Based on sequence analysis, we designed a new SS5-F specific primer (5'-AGCGCCATACCGCAACAAA), which was used together with reverse primer VA1-R, as described previously, to complete the signal sequence genotyping of the strains isolated from children with RAP [8]. All PCR mixtures consisted of 10 ng of *H. pylori* DNA and 5 µl PCR buffer 10X solution (Gibco BRL, Gaithersburg, MD), 1.5 mM MgCl₂, 25 pmol of each primer, 10 nM dNTPs and 5U of Taq polymerase (Gibco BRL). Conditions for thermal cycling were: 35 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. The products were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

Genealogic and Population Structure Analyses: In order to assess the genealogic relationship between the *vacA* signal sequence types previously reported in the database GenBank and the ss sequences of our strains, a network of haplotypes was created. Genealogies based on individual allele (haplotypes) sequences were reconstructed using TCS 1.21 software (44). The haplotype network shows the connection between each of the alleles, inferring the number of mutational steps between them (when there is more than one mutational step between one haplotype and another, the existence of an intermediate haplotype can be assumed, which is represented by a small white circle (Figure 1) between the connecting axes). The haplotypes network was constructed using ss sequences from 127 *H. pylori* strains of different subtypes (s1a, s1b, s1c and s2) deposited in GenBank of the National Center of Biotechnology Information (NCBI) and the ss of our 5 sequenced strains. Geographic distribution of *vacA* allelic types of *H. pylori* was also included in this analysis.

The population structure analysis was made using the linkage model of Bayesian clustering algorithm "STRUCTURE" (45). Analysis was carried out in order to explore how local haplotypes were grouped into subpopulations, and shows the ancestry proportion of each of the subpopulation formed. This technique employed a Bayesian approach to establish the probability of the number of populations in a sample of sequences. This algorithm

Figure 1: Genealogic relationship of the *vacA* signal sequence from 132 strains of *Helicobacter pylori* isolated from different parts of world.
a. It shows the network of haplotypes that form 5 well defined populations (s1a, s1b, s1c, s1d and s2). The numbers between bars show the mutational steps that diverge between one subpopulation and other; the small empty circles indicate a hypothetical intermediate haplotype. The subpopulation s2 type diverges about 14 mutational steps from subpopulations s1b / s1d. The subpopulations s1a and s1c (phenotypically cytotoxic) diverge from subpopulations s1b / s1d (less cytotoxic) to 4 mutational steps.
b. It shows two clades, which diverge from s1b to a mutational step. Clade I groups all s1d haplotypes of *H. pylori* strains isolated from children with recurrent abdominal pain in Mexico. Clade II corresponds to strains isolated from other Latin American countries.
c. Results of Structure analysis assuming 3 subpopulations, each color represent one subpopulation: blue for s1a, orange for s1c and green for s1b/d.

was run assuming a K value between 2 and 7 populations (15 replicates per value of K) with a burning of 50,000 iterations and 100,000 MCMC subsequent iterations. We found an optimal K value of 3, which defined 3 subpopulations (s1a, s1c and s1b/s1d) very well.

Assessment of vacuolating activity: Each strain was cultured as described earlier. A swab of plate-grown bacteria was then used to inoculate 25 ml of brucella broth with 10% fetal calf serum. The culture was grown for 48 h under microaerobic conditions (as described above) with gentle agitation. Culture

supernatants were concentrated 40-fold by ultrafiltration as describe previously [17]. The concentrate supernatants were incubated in AGS cells in two-fold serial dilutions. Cells were observed for up to 48 h to identify the development of vacuoles. The maximum dilution of the sample that produced vacuolation in more than 30% of the AGS cells was defined as the cytotoxin activity score for a sample. Non-inoculated broth was used as a negative control, and broth culture concentrates from strains 60190 and Tx30a were used as positive and negative vacuolating controls, respectively.

Results

In a previous study, we reported on the characterization of the *vacA* genotypes from *H. pylori* strains isolated from patients with different gastric pathologies, where the s1b/m1 *vacA* allele was the most frequently observed in *H. pylori* strains found in both the antrum and corpus [11,13]. However, using Atherton's characterization, the *vacA* signal sequence was non-typeable in approximately 10% of the strains, with the majority of typing being achieved in strains isolated from children with recurrent abdominal pain [8].

The characterization of all strains by PCR using the conserved primers VA1-F and VA1-R were typed as s1. All the strains were characterized as m1 in the *vacA* mid-region using the primers VA3-F and VA3-R [8].

Sequence analysis: Sequence analysis of the 623 bp fragment of the 5' end of the *vacA* gene showed the substitution of two bases in the nucleotide sequence corresponding to the 3' end of the complementary sequence of recognition for alignment of the forward primer (SS3-F). The substitutions prevented the binding of this primer and resulted in a lack of amplification of the PCR product, while the complementary nucleotide sequence for the binding site of the VA1-R reverse primer was completely conserved (Figure 2). All the sequences of the 5 selected strains showed the same nucleotide substitution at the 3' end of the complementary sequence of recognition for alignment of the forward primer (SS3-F).

The alignment of the remaining nucleotide sequences was more similar to s1b than s1a or s1c. In order to find out if these nucleotide changes were present in all childhood strains (previously not characterized), one new primer SS5-F was designed and new PCRs were performed. We observed PCR products characterizing the signal sequence in all non-subtypeable strains. To determine the specificity of this new primer, we performed individual PCR using SS5F and VA1-R primers with DNA of 60190, 26690, 84183 and TX30a strains. This primer set did not amplify the *vacA* signal sequence of 60190 and 26690 (s1a), 84183 (s1b) and TX30a (s2) strains, indicating that the primer SS5-F is specific for the signal sequence of our strains, which we named as s1d subtype.

Figure 2: Alignment of nucleotide sequences (A) and amino acid sequences (B) of *vacA* signal sequence. A. The red, green, blue and pink rectangles show the alignment site of each specific forward primer for each signal sequence subtype characterization. B. Amino acid substitutions between s1b and s1d, and the change of glutamic acid (E) by glutamine (Q) in position 30 is constant among all sequenced strains.

In order to demonstrate that this was indeed a new subtype of signal sequences for the *vacA* gene, we performed phylogenetic analysis of 132 strains with different ss subtypes (s1a, s1b and s1c) including our own (s1d). The analyzed sequences were edited to ensure that all had the same size (132 nt). The editions were performed using the Seqman (DNASTAR Lasergene 7) and alignment was performed using the ClustalW2 program from Bioedit 7.0.9.0. The genealogy was reconstructed for the *vacA* ss of *H. pylori* using TCS software related to the Phylogenetic network estimation with statistical parsimony. Genealogic analysis of all the s1 signal sequence subtypes of 132 strains from different geographic origin was included (Figure 1). The haplotype network showed the formation of four main clades with a specific subtype grouping in each one, in which the s1d subtype formed an homogenous and compact cluster that diverged from the s1b signal sequence at two mutational steps and from s2 at 14 mutational steps (Figure 1a). This analysis revealed that there is recombination into the subpopulation between members of the same subtype but not between members of different subtypes. Considering the analysis of all the signal sequences available in the database, we can see that strains of s1d genotype derive from Colombia, Argentina, Peru and Venezuela, as well as one from Italy, indicating that this genotype has a distribution among Latin American populations predominantly (Figure 1b). This result supports previous reports in which the geographic distribution of *H. pylori* is linked to specific genotypes. This is the first study to report the presence of a new lineage (named here as s1d subtype) in strains isolated from children with recurrent abdominal pain.

Structure analysis also supported the presence of three s1 subtypes (s1a, s1c, s1b/s1d), which were well defined and completely independent, each with specific polymorphic changes for each subpopulation (Figure 1c). However, the close genetic relationship between the s1b and s1d subtypes suggests that they share a common ancestor, with one giving rise to the other following very few mutational steps, as shown in Figure 1b.

With respect to the analysis of the middle region, the amplified alleles indicate a clear separation between m1 and m2 sequences (data not shown).

Vacuolating cytotoxin activity: From the 23 *H. pylori* isolates tested for vacuolating cytotoxin activity, broth culture supernatants of 13 strains caused vacuolation in AGS cells, while the remaining 10 strains did not produce vacuolating activity.

Discussion

H. pylori exhibits pronounced genetic diversity at both the chromosome and nucleotide sequence level in genes of the same locus from different strains [18-21]. This variability strongly impacts the virulence of the bacterium. Specific genotypes have been associated with the development of gastritis, peptic ulcers and stomach cancer [5-7,9-15,22]. The difference between genotypes of this bacterium is given by the presence or lack of gene blocks in the accessory genome within the plasticity zones of the chromosome and by the polymorphism of the nucleotide sequences in structural genes, such as the *vacA* gene, which encodes for the vacuolating cytotoxin [19-21]. *H. pylori* genotype

1 strains present the *cag*-PAI island and produce vacuolating cytotoxin with high activity. However, this association is only obvious in certain populations in which the infection is caused by only one type of strain, as seen in the United States and countries in Western Europe [5-7]. In contrast, there are other populations, in Japan and Korea for example, in which the characterization of strains isolated from both children and adults have shown the prevalence of *cagA*, *vacA* s1, *iceA1* genotype to be very high but with little or no relationship to gastro duodenal disease [22]. In Mexico, the relationship between the genotype of the strain and gastric pathology is not so clear. Several studies show that infection by this microorganism is really a multicolonization of the gastric mucosa by different *H. pylori* strains with different genotypes. Therefore, it is not possible to attribute a particular genotype to disease outcome [11-15]. These same studies also indicate the presence of strains that were not characterized according to their signal sequence, referring to these strains as being non-typeable. The relationship between recurrent abdominal pain and *H. pylori* infection is controversial. Some studies show that *H. pylori* is more frequent in patients with RAP compared to asymptomatic children, while others do not find an association between RAP and *H. pylori* infection [23-30]. *H. pylori* infection has also been reported with non-specific symptoms, such as epigastric pain, dyspepsia, halitosis, nausea, vomiting and retrosternal pain. It is known that the prevalence of *H. pylori* infection among populations in different parts of the world varies with the highest levels being seen in developing countries where the infection is acquired during childhood. Epidemiological studies show that *H. pylori* infection is of mixed origin with strains of different genotypes of *vacA*, *cag* and *iceA* [11-15]. The coexistence of different strains in a bacterial population sharing only one niche leads to high rates of recombination, generating new genotypes among strains of the same species or different genders, which are better adapted to micro-environmental conditions [2,18,31,32]. The exchange of genetic material between bacteria is a common phenomenon in which the horizontal transference of genes participates through mobile elements, enriching the accessory genome that is suitable for the bacterium even more [33-37]. It is known that *H. pylori* is a natural transformant, which makes it genome dynamic. This process is necessary in response to stress conditions or selective pressures exerted by biochemical, physiological and anatomical factors in each region of the stomach [38,39].

In Mexico, infection by the bacterium is a chronic process with infection often being acquired during childhood. Colonization over an indefinite period of time is the result of well-developed strategies to avoid the immune response, competition for nutrients and to avoid over population in a specific niche [31,40,41]. We do not know why multiple infection in our patients is frequent. Two theories that could explain this phenomenon are: a) the strains that colonize the stomach present genetic exchange between them over years, thereby generating new strains, which are different from the original ones, or b) through the life (child-adult) of an individual, repeated infections with strains of different genotypes occur. Whatever the cause of mixed infection by *H. pylori*, certain questions arise, such as what is the role of

each genotype in the development of disease when infection is caused by strains of different genotypes? Does synergism exist between different genotypic strains to produce more severe disease? Is there competition between different genotypic strains with the eventual abatement of a specific genotype? Does mixed infection lead to balance, controlling the development of disease? What is the result if this equilibrium is broken? Which genotype allows *H. pylori* to adapt in a better way? The predominance of which genotype allows it to be detected by gastric biopsy culture?

The phylogenetic and genealogic analysis of the nucleotide signal sequences of *vacA* showed that s1 and s2 genotypes diverge over 14 mutational steps (Figure 1a). Additionally, analysis of genotype s1 showed that each subtype (s1a, s1b, s1c and s1d) forms a coherent, robust and separate lineage, while s1d shows a close relationship to s1b, suggesting a common ancestry for both these subtypes. The geographical distribution of the different *vacA* subtypes is well known with the s1b subtype being predominant in Hispanic countries. Meanwhile, s1d subtype distribution is almost exclusively seen in Latin America. Interestingly, the s1d Mexican haplotypes were isolated from children with recurrent abdominal pain. These results support the hypothesis suggested by Van Doorn, et al. that the distribution of the different *vacA* genotypes suggests a particular adaptation of the microorganism to specific host populations, as seen with the s1a genotype that is widely distributed in Northern and Eastern Europe, as well as in Canada and Australia, while the s1c subtype is more commonly found in East Asia.

According to the nucleotide sequence analysis, we can infer that the lineages of s2, s1b and s1d were the ones who settled first in the stomach. It would be interesting to perform a genetic study and analysis in a single stomach following co-evolution of different genotypes over a long period of time in order to establish the original genotype and deduce its genetic changes until new subtypes are observed.

The results from this present study suggest that the first *vacA* genotypes of *H. pylori* established in the human stomach are not cytotoxic. Probably, the bacterium modified its genome generating cytotoxic genotypes (s1a, s1c), changing its status from non-pathogenic to pathogenic. This tends to support the hypothesis formulated by Blaser that “*Helicobacter pylori* is a commensal bacterium” and the acquisition of genetic material and mutations into its genome and individual genes make it a pathogenic bacterium [2, 31,40-42].

The present study does contain some limiting factors. The study group was small, limited to just a strains that could not be typed in a previous study. This made it impossible to determine any association between s1d type and RAP at a level with statistical significance. Although it is true that all the strains were isolated from children with RAP, it is necessary to study a larger number of strains isolated from children with RAP together with a larger control population (asymptomatic children) to find a real association with the s1d *vacA* signal sequence type.

In conclusion, our results show evidence of recombination between members of a population with the same subtype (Figure

1). However, recombination between members of different subtypes is rare, suggesting that each population of each subtype continues to colonize the stomach independently, which would explain the persistence of mixed infections. This phenomenon makes it difficult to associate one specific genotype to a given gastric pathology. A novel *vacA* s1d signal sequence type was found in Mexican children with recurrent abdominal pain. This type of signal sequence presented variable vacuolating activity. Further studies will be needed to determine if this *vacA* sequence signal subtype has important implications on the pathogenesis of digestive diseases. This s1d subtype appears to be almost exclusively from Latin America. Finally, sequence analysis of each *vacA* signal sequence (s1a, s1b, s1c, and s1d) subtype demonstrated that each subtype forms a coherent, robust and separate subgroup.

Conflict of interest

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