World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i3.320

World J Gastroenterol 2010 January 21; 16(3): 320-329
ISSN 1007-9327 (print)

ORIGINAL ARTICLE

Helicobacter pylori HopE and HopV porins present scarce expression among clinical isolates

Maritza Lienlaf, Juan Pablo Morales, María Inés Díaz, Rodrigo Díaz, Elsa Bruce, Freddy Siegel, Gloria León, Paul R Harris, Alejandro Venegas

Maritza Lienlaf, Juan Pablo Morales, María Inés Díaz, Rodrigo Díaz, Elsa Bruce, Alejandro Venegas, Microbial Pathogenesis and Vaccine Biotechnology Laboratory, Department of Molecular Genetics and Microbiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Alameda 340, Santiago 651-3492, Chile
Freddy Siegel, Gastroenterology Unit, Faculty of Medicine, Universidad Austral de Chile, Independencia 641, Valdivia 511-0566, Chile
Gloria León, Institute of Biochemistry, Faculty of Sciences, Universidad Austral de Chile, Independencia 641, Valdivia 511-0566, Chile
Paul R Harris, Department of Pediatrics, School of Medicine, Pontificia Universidad Católica de Chile, Lira 85, Santiago 833-0074, Chile
Author contributions: Lienlaf M and Morales JP performed most of the cloning and immunoblotting experiments; Díaz MI was in charge of strains stock and growth; Díaz R and Bruce E collected biopsies and isolated strains; Harris PR, Siegel F and León G provided the collection of human material and were involved in editing the manuscript; Venegas A designed the study, provided financial support for this work and wrote the manuscript.
Supported by Grants FONDECYT #1030894 and 1085232 and FONDEF DO2I-1067 from CONICYT, Chile
Correspondence to: Dr. Alejandro Venegas, Microbial Pathogenesis and Vaccine Biotechnology Laboratory, Department of Molecular Genetics and Microbiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Alameda 340, Santiago 651-3492, Chile. avenegas@bio.puc.cl
Telephone: +56-2-6862661 Fax: +56-2-2225515
Received: September 15, 2009 Revised: November 11, 2009
Accepted: November 18, 2009
Published online: January 21, 2010

Abstract

AIM: To evaluate how widely *Helicobacter pylori* (*H. pylori*) HopE and HopV porins are expressed among Chilean isolates and how seroprevalent they are among infected patients in Chile.

METHODS: *H. pylori hopE* and *hopV* genes derived from strain CHCTX-1 were cloned by polymerase chain reaction (PCR), sequenced and expressed in *Escherichia coli* AD494 (DE3). Gel-purified porins were used to prepare polyclonal antibodies. The presence of both genes was tested by PCR in a collection of *H. pylori* clinical isolates and their expression was detected in lysates by immunoblotting. Immune responses against HopE, HopV and other *H. pylori* antigens in sera from infected and non-infected patients were tested by Western blotting using these sera as first antibody on recombinant *H. pylori* antigens.

RESULTS: PCR and Western blotting assays revealed that 60 and 82 out of 130 Chilean isolates carried *hopE* and *hopV* genes, respectively, but only 16 and 9, respectively, expressed these porins. IgG serum immunoreactivity evaluation of 69 *H. pylori*-infected patients revealed that HopE and HopV were infrequently recognized (8.7% and 10.1% respectively) compared to *H. pylori* VacA (68.1%) and CagA (59.5%) antigens. Similar values were detected for IgA serum immunoreactivity against HopE (11.6%) and HopV (10.5%) although lower values for VacA (42%) and CagA (17.4%) were obtained when compared to the IgG response.

CONCLUSION: A scarce expression of HopE and HopV among Chilean isolates was found, in agreement with the infrequent seroconversion against these antigens when tested in infected Chilean patients.

Key words: *Helicobacter pylori*; Gene expression; HopE and HopV porins; Antigens; Immune response

Peer reviewers: Mario M D'Elios, Professor, University of Florence, viale Morgagni 85, Florence 50134, Italy; Andrew Day, Assistant Professor, University of Otago, Christchurch Hospital, Christchurch 8140, New Zealand

Lienlaf M, Morales JP, Díaz MI, Díaz R, Bruce E, Siegel F, León G, Harris PR, Venegas A. *Helicobacter pylori* HopE and HopV porins present scarce expression among clinical isolates. *World J Gastroenterol* 2010; 16(3): 320-329 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i3/320.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i3.320

INTRODUCTION

Helicobacter pylori (*H. pylori*) are Gram-negative, microaerophilic, spiral-shaped bacteria isolated from human gastric biopsies in 1983[1]. In order to survive in this aggressive environment, *H. pylori* are able to neutralize their close surrounding space by production of urease, which catalyzes the conversion of urea into ammonium and CO_2, raising pH close to neutral. In addition, to colonize the epithelium, this bacterium is able to bind to the epithelial cell surface, partially avoiding its removal by natural peristalsis or mucus renewal. These characteristics allow *H. pylori* to persist for decades.

H. pylori infection affects one half of the world population, roughly 73% in Chile[2], with higher prevalence as age increases. After several years of chronic gastric infection, approximately 10%-15% of infected patients develop severe gastrointestinal diseases such as chronic gastritis, peptic ulcer and gastric carcinoma[3,4]. In Chile, 5% of the infected population develops gastric cancer[2] and this malignancy is the second cause of death by cancer in the country.

H. pylori carries various virulence factors, and some may have potential as vaccine antigens. These factors may be grouped as: (1) colonization factors, which allow bacterial residence; (2) persistence factors which enable bacteria to accomplish an effective and lasting survival; and (3) disease inducing factors which cause adverse pathological effects on the gastric mucosa[5].

Based on a bioinformatics analysis of the *H. pylori* genome, a family of outer membrane proteins (OMPs) composed of 33 members has been identified[6]. These proteins are assembled into the outer membrane exposing, on the bacterial surface, small peptide loops which may act as epitopes to induce an immune response. This feature may be useful when selecting appropriate antigens for vaccine design. All these members contain an N-terminal signal peptide (processed by signal peptidase type I or II) that allows these proteins to cross the inner membrane on their way towards the outer membrane. The *H. pylori* OMPs form 2 families: the Hop members (21 proteins) and the Hor members (12 proteins). Hor proteins lack a characteristic N-terminal Hop motif[7]. Hop proteins have structural homology with the *Escherichia coli* (*E. coli*) outer membrane protein F (OmpF) porin[8]. Currently, 5 *H. pylori* Hop members (HopA, HopB, HopC, HopD and HopE) from strain 26 695 have been characterized as porins using planar bilayer techniques[7,9] and some also behave as adhesins[10,11]. These properties make them attractive candidates as vaccine antigens. In fact, other bacterial porins from *Salmonella*, *Pseudomonas*, *Chlamydia* and *Neisseria*, have been found to be strong immunogens[12-15]. However, in the case of *H. pylori,* it has been suggested that not all the genes encoding OMPs may be functional at a given time. Some of these genes are under a control mechanism that operates by strand slippage during DNA replication or DNA repair. DNA polymerase slippage may easily add or remove nucleotides when DNA synthesis occurs in front of a homopolymeric tract or dinucleotide repeats at the template strand (i.e. *polyG* or *polyCA* gene segments) causing mutations either at the promoter or at the coding region. This type of mutation may turn off or on some *hop* genes that may include these polynucleotide features. For instance, the *hopC* gene has been reported to carry a *polyT* tract (13 Ts in length) near the 5' end but *hopA*, *hopB* and *hopE* do not have such long *polyT* tracts either at their coding regions or 5' upstream at the promoter regions. Gene switching will produce a change in the antigenic bacterial surface, a strategy that will distract the host immune system. For this reason, whether any *H. pylori* OMP would be considered as a vaccine antigen, *omp* genes containing long homopolymeric tracts or dinucleotide repeats should be avoided.

Regarding HopV and HopW, genetic heterogeneity in orthologous members of the Hop family among *H. pylori* strains has been described[16]. These new members were defined as part of the HopA/HopE family, because of their homology at the N-terminal sequence and the presence of 7 homologous domains in the C-terminus region. Regarding functional aspects, HopV and HopW have pore sizes similar to that of the *E. coli* OmpF porin[16] and HopE has been defined as the homolog to the *E. coli* OmpF porin[17].

Since the use of porins as antigens has been reported as successful[12-15], we decided to evaluate members of the *H. pylori* Hop family as putative antigen candidates for vaccine development by determining how widely they are expressed among Chilean *H. pylori* isolates and how often Chilean patients develop antibodies against them. A brief bioinformatic survey indicated that some genes of the Hop family had homopolymeric tracts or dinucleotide repeats in their coding sequences and promoter regions, with potential capability to promote strand slippage which may affect stability of gene expression[18]. Considering this aspect, only porin genes having single homopolymeric tracts or dinucleotide repeats no longer than 6 bases in their coding sequences were chosen as source of putative useful antigens for a vaccine. For this reason, among several OMP genes, only *hopE* and *hopV* sequences were selected for the present study.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors

E. coli DH5α was used for polymerase chain reaction

Table 1 Primer sequences used for amplification and sequencing of *H. pylori hopE* and *hopV* genes

Name	5'→3' sequence	Restriction sites[1]
HopV1[2]	GGGCCCATATGCTCAATTTTATGACAAAGAAGAAAAATAGAATGC	*Nde* I
HopV2[2]	GGATCCCATGGTTAAAAATCCCTCAAGTAACTGATTTG	*Bam*H I
HopE1[2]	GGCGCCATGGAATTTATGAAAAAGTTTGTAGCTTTAGG	*Nco* I
HopE2[2]	CGCGAAGCTTTTAAAAAGTGTAGTTATACCCTAAATAAAG	*Hind*Ⅲ
HopE11[3]	GCAAGTGGTTTGGTTTTAGAG	-
HopE22[3]	ACCATATCCAACTGGATTTT	-
HopV11[3]	GGCGTGGGGTTAGATACGCTG	-
HopV22[3]	ACCATGTTTTCTTTATTCAC	-
HopVint[3]	ATGCGTTATTATGGGTTTTTTGACT	-
pETT7d[4]	TAATACGACTCACTATAGGG	-
pETT7r[4]	GCTAGTTATTGCTCAGCGG	-

[1]The restriction sites included in primer sequences and used for ligation to plasmid vectors are shown underlined; [2]External primers used to clone porin genes; [3]Internal primers used for gene sequencing and also for confirmatory PCR reactions for those cases in which primers derived from 5′ and 3′ gene ends failed to raise PCR products; [4]Vector primers for 5′ and 3′ end gene sequencing.

(PCR) cloning, and *E. coli* BL21 (DE3), JM109 (DE3) and AD494(DE3)pLysS as host for porin expression assays. For cloning of PCR fragments plasmid pGEM-T Easy from Promega was used. For expression studies pET21a and pET21d (Novagen) were selected. *H. pylori* Chilean strain CHCTX-1 was used as DNA source for gene amplifications[19]. A collection of 130 *H. pylori* strains isolated from infected patients living in different Chilean cities was already available[20].

Bacterial cultures

E. coli cells were grown overnight in LB media at 37℃ with shaking. *H. pylori* strains were grown under 50 mL/L CO_2 and 80% humidity in *Brucella* agar plates enriched with 5% horse blood cells and grown at 37℃ for 2-3 d. *E. coli* strains were kept for short periods in LB plates at 4℃. Bacterial strains containing 14% glycerol were stored frozen for longer periods at -70℃.

Plasmid purification, DNA manipulation and bacterial transformation

Plasmids were usually detected by the "one step" method[21], and purified by alkaline lysis method[22]. Restriction digestions, DNA ligations and plasmid dephosphorylations were done according to standard procedures[22]. Electroporation in 0.2 cm electrode separation cuvettes was performed as previously described[23], in a Gene Pulser™ apparatus. Electrocompetent cells were prepared according to described protocols[23] with a yield of 1×10^9 to 1×10^{10} cells/mL.

PCR assays

Primers corresponding to the 5' and 3' ends as well as the internal sequences of the *hopE* and *hopV* genes (Table 1) were designed based on *H. pylori* 26695 GenBank sequences. As templates, chromosomal DNA from the CHCTX-1 strain[19] and from clinical isolates was prepared according to described procedures[24]. PCR reactions were carried out in a BioRad Mastercycler Ⅱ thermocycler, with *Pfu* polymerase (Stratagene, CA, USA) or *Taq* polymerase (Promega, Madison WI, USA). Assays were done in 25 μL final volume following the manufacturer's instructions. Gene amplicons were detected by 1% agarose gel electrophoresis. Other conditions were as previously described[25]. VacA alleles were determined by using primers and assay conditions described by Atherton *et al*[26].

Polyclonal antibodies against *H. pylori* OMPs

According to standard procedures[27], anti-HopE and anti-HopV rabbit antibodies were prepared. Proteins were obtained from *E. coli* clones expressing the *H. pylori* porins after separation by SDS-PAGE and purification from gel slices by electroelution as previously described[28]. Pathogen-free New Zealand adult female rabbits (approximately 1400 g in weight) were immunized with 250 μg of each porin dissolved in 2 mL of Tris-glycine buffer mixed (1:1) with complete Freund's adjuvant. Two animals were used for each porin inoculation and 100 μL aliquots were applied subcutaneously in the back. This was followed by 3 boosters every 15 d.

SDS-PAGE and Western blotting

Lysates from clones expressing HopE and HopV were separated by polyacrylamide gels (12% or 15%) and run in minichambers according to Laemmli[29]. Western blotting were done as previously described[30]. As first antibody, patient serum (1:100 dilution) or rabbit anti-porin antibodies against HopE or HopV (1:1000 dilution) were used. As a second antibody for patient assays, goat anti-human IgA or anti-human IgG conjugated to peroxidase, were incubated (1:1000) overnight at 4℃. For anti-porin rabbit sera, a goat anti-rabbit peroxidase-conjugated antibody (1:1000) second antibody was used. To reduce cross reactions, rabbit antisera and human antisera were adsorbed with sonicated lysates from *E. coli* AD494(DE3)pLysS/pET21d and BL21 (DE3), respectively. Human sera immunoblotting were done with lysates expressing HopE or HopV porins and products

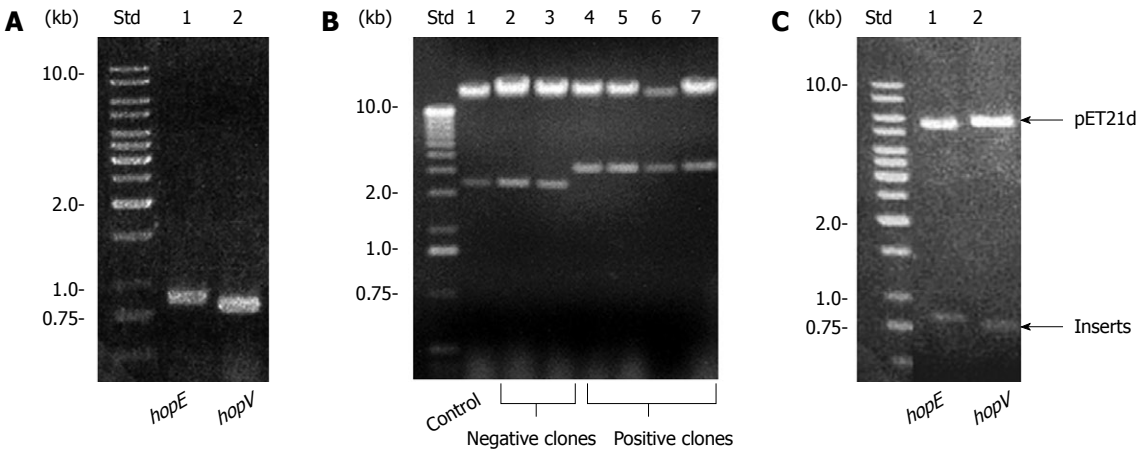

Figure 1 Cloning of *H. pylori hopE* and *hopV* genes. A: Polymerase chain reaction (PCR) amplification of *hopE* and *hopV* genes. Amplicons and plasmids were separated by 1% agarose gel electrophoresis. Lane Std: 1 kb DNA ladder standard; B: Detection of plasmids carrying the *hopE* gene. Lane 1: Strain AD494(DE3)pLysS with pET21d as control. Lanes 2, 3: Negative clones, lanes 4-7 plasmids carrying the *hopE* gene; C: Release of inserts carrying the *hopE* and *hopV* genes after *Nco* I /*Hind* Ⅲ and *Nde* I /*Bam*H I digestions respectively. Lane Std: 1 kb DNA ladder standard (Fermentas).

derived from expression of *cagA* and *vacA* gene fragments cloned from strain CHCTX-1[19].

Patient sera

A sera panel from 69 infected patients (63 with gastritis, 6 with ulcers) recruited from the Universidad Católica de Chile Medical Center in Santiago, with signed consent, was available. Each patient's infected condition was defined by endoscopy, positive urease rapid test and detection of hematoxylin/eosin-stained curved bacteria on gastric tissue biopsies. Also, 8 non-infected patients were included in this study. The local ethics committee approved the protocol for this study.

Immunoprecipitation of IgG from patient sera

In order to obtain a cleaner IgA reaction in Western blotting assays using patient serum antibodies, protein G-plus-Agarose (Santa Cruz Biotechnology, catalogue #sc-2002) was utilized to first remove IgG from serum by immunoprecipitation. One hundred microliters of each serum without pre-adsorption treatment were incubated overnight with 200 μL of protein G-plus-agarose at 4℃ with mild shaking. After sedimentation for 5 min at 2500 r/min and 4℃, the supernatant of each sample was used as a source of IgG-free serum.

DNA sequencing

DNA samples were previously purified by a commercial kit, and sequenced at our Sequencing Core Facility. T7 and internal primers for DNA sequencing are listed in Table 1.

RESULTS

Cloning of porin genes derived from a Chilean *H. pylori* strain as putative antigens

Selection for cloning and expression studies of the *hopE* and *hopV* genes were based on known gene sequences from strain *H. pylori* 26695. Since our study was focused on antigens obtained from local strains, *H. pylori* CHCTX-1 strain, a clinical isolate obtained from a Chilean patient[19] was selected as the DNA source for gene cloning in this study.

Cloning of HopE and HopV porin genes, including their signal peptide regions, was done by PCR. Primers and assay conditions are described in Table 1 and Methods, respectively. Amplicons from *hopE,* and *hopV* genes were separated in a 1% agarose gel (Figure 1A), purified and treated with Taq polymerase and dATP to be ligated to pGEM-T. Recombinant plasmids were detected by insert release after *Eco*R I digestion and separation in 1% agarose gel electrophoresis. The *hopE* and *hopV* cloned inserts were subjected to *Nco* I -*Hind*Ⅲ and *Nde*I-*Bam*HI double digestions and ligated to pET21d and pET21a, respectively. As expected, fragments with sizes corresponding to these genes were observed. For expression purposes, plasmids were transferred to *E. coli* AD494(DE3)pLysS cells and visualized by the "one step method"[21]. Some clones containing plasmids with the *hopE* gene are displayed in Figure 1B. Purified plasmids were used for restriction digestions and also as DNA templates for PCR gene detection. *Nco* I -*Hind*Ⅲ and *Nde* I -*Bam*H I double digestions of plasmid DNA isolated from single clones were analyzed by agarose gel electrophoresis, and released inserts with sizes close to the expected ones (819 bp for *hopE* and 735 bp for *hopV*) were observed (Figure 1C).

Expression of *H. pylori* HopE and HopV porin genes in *E. coli*

E. coli AD494(DE3)pLysS was able to express detectable amounts of HopE and HopV porins, as seen after SDS-PAGE and Coomassie blue staining (Figure 2A and B) and Western blotting assays (Figure 2C and D).

Expression conditions were optimized by 5 h induction with 1 mmol/L isopropyl β-D-thiogalactoside

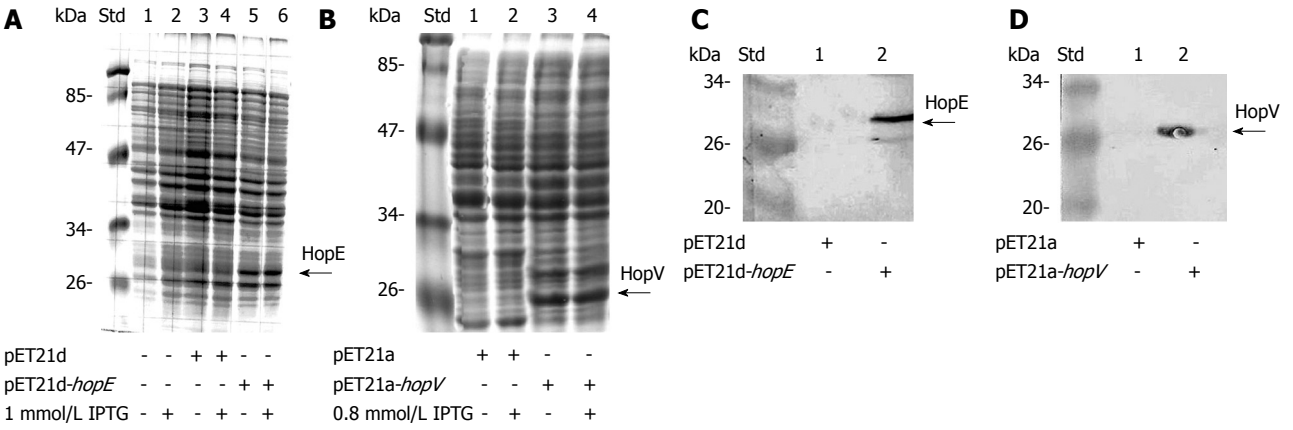

Figure 2 Expression of *H. pylori hopE* and *hopV* genes in *Escherichia coli* AD494(DE3)pLysS. Bacterial lysates were separated in 12%-15% PAGE-SDS gel, stained with Coomassie blue (A and B) or analyzed by Western blotting (C and D). Conditions for HopE expression are indicated below the figure (A, lanes 5 and 6; C, lane 2). Conditions for HopV expression are indicated under the figure (B, lanes 3 and 4; D, lane 2). Arrows indicate electrophoretic migration of these proteins. Lane Std: Prestained molecular weight marker in kDa (Fermentas).

(IPTG) for *hopE* and 0.8 mmol/L for *hopV* on previously saturated cultures. Protein sizes of 32 kDa for HopE and 28 kDa for HopV were observed. These porins displayed a certain amount of expression without IPTG induction, partially explained by the fact that the induction procedure was done on saturated cultures.

Sequence analysis of *H. pylori hopE* and *hopV* genes

The *hopE* (clone 1) and *hopV* (clone 13) gene sequences from the CHCTX-1 strain were obtained using external (T7 promoter and T7 terminator) as well as internal primers (Table 1) as described in Methods. Both *hopE* and *hopV* sequences were deposited at GenBank (accession numbers #EF635415 and #EF635416, respectively). As expected, these genes did not contain nonsense or frameshift mutations at their coding regions. Also, neither homopolymeric nor dinucleotide tracts longer than 6 nucleotides were found.

Detection of *hopE* and *hopV* genes in Chilean clinical isolates and their expression

From a collection of 240 clinical strains previously isolated[20], we selected 130 colonies (1 to 5 isolates per patient) as representatives from 6 Chilean cities: Iquique (IQ) in the North, Valparaiso (VA) and Santiago (SA) in the central region, Los Angeles (LA) and Valdivia (VL) in the South, and Punta Arenas (PA), the Southernmost city, to evaluate the distribution of strains carrying *hopE* and *hopV* genes and their expression throughout the country.

Detection of the genes was done by standard PCR. The amplicons were almost identical in size to those expected for *hopE* and *hopV* genes from strain 26695. Representative groups of isolates carrying *hopE* and *hopV* genes are shown in Figure 3A and B, respectively.

The *hopE* and *hopV* genes were detected in 46.9% (61 out of 130 strains) and 63.1% (82 out of 130) of the studied strains, respectively, and 40% (52 out of 130) of the strains revealed the presence of both genes simultaneously (Table 2). Among different cities, *hopE* and *hopV* gene contents varied between 30% and 69%. Curiously, *hopV* was frequently detected (69.2%) in strains from infected patients living in PA, the southernmost city. Patients from VL (mostly descendants from ancient aborigines) carried strains with a lower content (42.8%) of *hopV* gene. All PCR reactions were done at least twice using a pair of primers which bound to the gene ends. For those cases with negative amplification, additional assays using 2 primer combinations, including in each pair of primers one of the internal primers (Table 1), were performed. In most cases negative PCR reactions were confirmed and just a few strains showed positive PCR amplification only with pairs including internal primers, indicating that our initial estimation about the reduced presence of these genes in Chilean isolates was valid.

The positive results of HopE and HopV Western blotting expression assays in these isolates revealed no protein size variation, and selected results are displayed in Figure 3C.

Regarding porin expression, results showed that only 13.1% of the 130 strains expressed HopE and 6.9% expressed HopV. Altogether, 83.8% (109 out of 130 strains) did not express HopE or HopV porins either because of a lack of these genes, random inactivating gene mutations or gene silencing by the strand slippage mechanism (Table 2).

Recognition of HopE and HopV porins by sera from infected patients

In order to evaluate the capability of sera from Chilean *H. pylori*-infected patients to recognize recombinant *H. pylori* HopE and HopV porins, sera from 69 infected and 8 non-infected patients were tested. IgG and IgA serum antibodies against HopE and HopV antigens expressed as recombinant proteins in *E. coli* clones were tested using Western blotting assays on these bacterial lysates. VacA and CagA expressed similarly were used as immunodominant controls. The number of infected

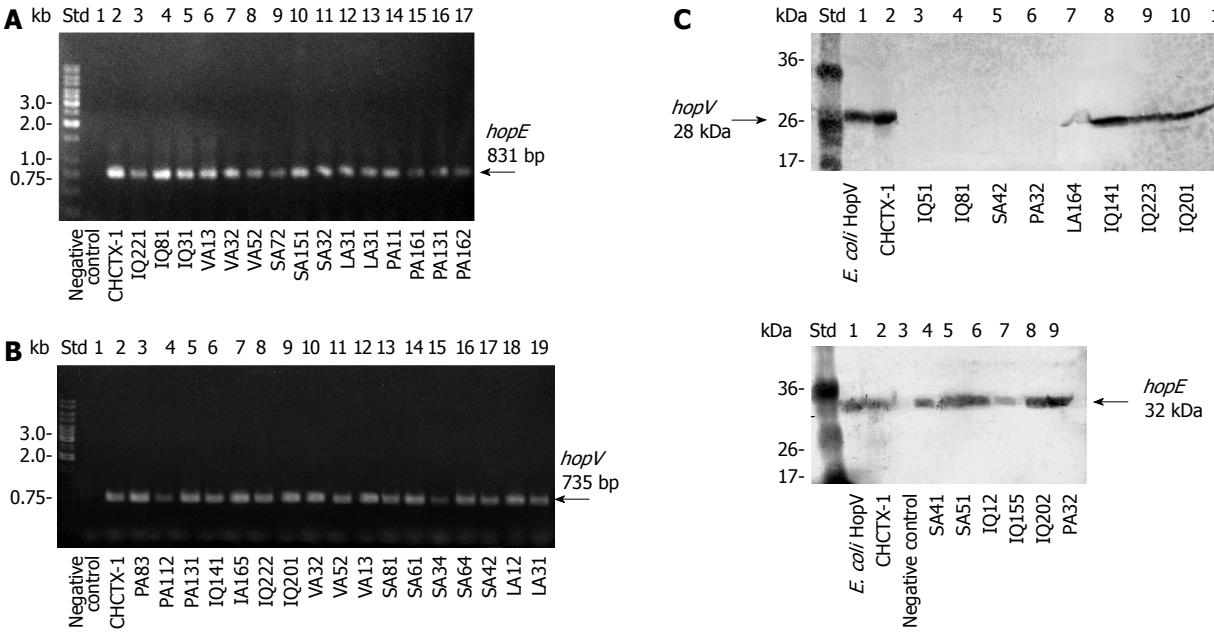

Figure 3 Detection of *hopE* and *hopV* genes and their expression in *H. pylori* clinical isolates from different Chilean cities. Amplicons were separated in 1% agarose gels. A: PCR detection of the *hopE* gene; B: PCR detection of the *hopV* gene. Arrows indicate migration of the respective gene fragments. Lane Std: 1 kb DNA ladder standard (Fermentas); Lane Std2: Lambda DNA/HindIII marker (Fermentas); C: Expression of HopV and HopE porins in *H. pylori* Chilean strains separated by 12% SDS-PAGE gels and detected by Western blots with respective polyclonal antibodies. Clinical isolates are indicated under the respective lanes. Std: Prestained molecular weight marker (Fermentas). The CHCTX-1 strain was included as a positive control.

Table 2 Number of strains presenting genotypes and corresponding phenotypes indicating the presence of *hopE* (E) and *hopV* (V) genes and their expression in 130 *H. pylori* strains isolated from infected patients from 6 Chilean cities

Genotypes[1] (E/V)	No. of strains	Phenotypes[2] (E/V)	No. of strains	No. of strains with different E/V phenotypes per city[3]					
				IQ	VA	SA	LA	VL	PA
(+/+)	52	(+/+)	5	4	-	1	-	-	-
		(+/-)	12	4	-	5	1	-	2
		(-/+)	-	-	-	-	-	-	-
		(-/-)	35	15	3	3	1	7	6
(+/-)	9	(+/-)	-	-	-	-	-	-	-
		(-/-)	9	2	-	-	-	5	2
(-/+)	30	(-/+)	4	1	-	2	-	-	1
		(-/-)	26	11	-	4	-	2	9
(-/-)	39	(-/-)	39	11	2	11	2	7	6
Totals	130	Totals	130	48	5	26	4	21	26

[1]Presence of *hopE* (E) and/or *hopV* (V) genes in Chilean *H. pylori* isolates determined by PCR amplifications as described in Methods using purified DNA templates from single colonies collected from 69 patients of the indicated cities. (+): gene presence; (-): no detection; [2]HopE and or HopV expression assayed by Western blotting (see Methods). (+): detection; (-): no detection; [3]Total number of strains with the assigned phenotype per city. IQ: Iquique; VA: Valparaiso; SA: Santiago; LA: Los Angeles; VL: Valdivia; PA: Punta Arenas. The number of patients per city was IQ = 21, VA = 4, SA = 12, LA = 3, VL = 5, PA = 15. Number of strains isolated per patient ranged between 1 and 5.

patient sera able to recognize these antigens are shown in Figure 4A. It was found that, as expected, IgG human antibodies more frequently recognized VacA (68.1% or 47 out of 69) and CagA (59.4%), but rarely recognized HopE (8.7%) and HopV (10.1%) porins. A similar distribution for HopE (11.6%) and HopV (10.5%), but lower distribution values for VacA (42%) and CagA (17.4%) were found for IgA antibodies. The lower number of anti-HopE and anti-HopV reactive sera can be explained by the low proportion of *H. pylori* strains able to express these genes, being 13.1% (17/130) for HopE and 6.9% (9/130) for HopV (Table 2). Taken together, these results strongly suggest that *H. pylori* possesses a mechanism to switch on/off these OMP genes as a strategy to evade the host immune response.

In addition, considering that the immune response in children[31,32] could be quite different from that in adults[33], the IgA (Figure 4B) and IgG (Figure 4C) immune responses of the infected patients were plotted for 2 age groups: under 18 years of age and adults. It was noted that the number of sera with IgA and IgG responses against CagA antigen was significantly higher

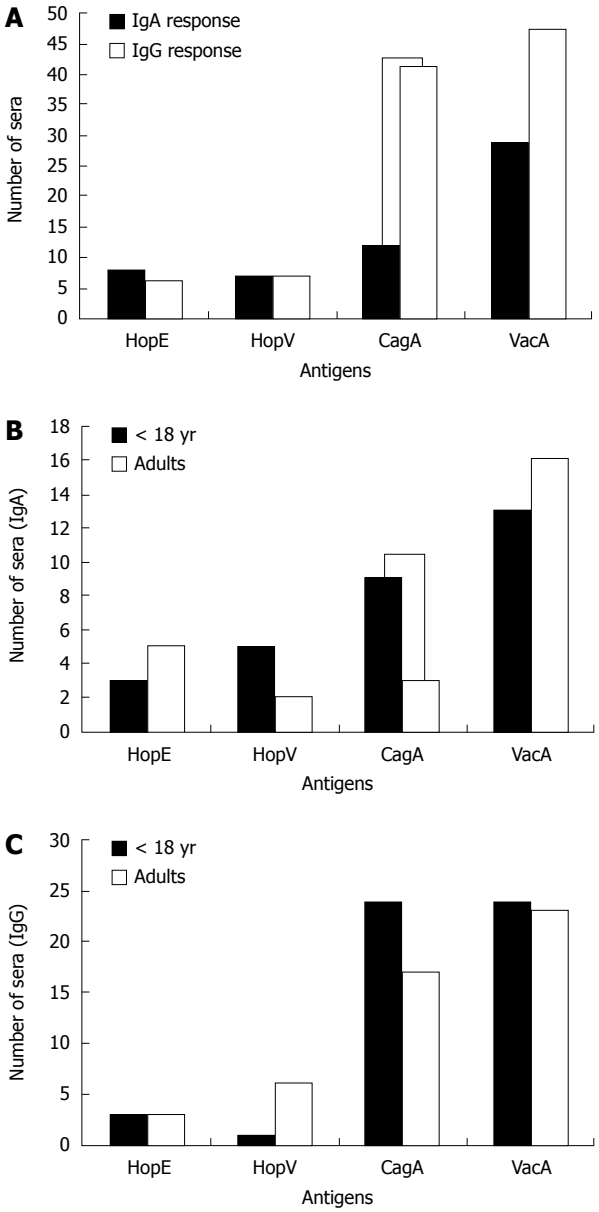

Figure 4 Frequency of recognition of 4 *H. pylori* antigens by human sera. Bars represent number of immunoreactive human sera from a panel of 69 *H. pylori*-infected patients showing IgG- and IgA-type immunoreactivity tested by Western blotting assays on *E. coli* lysates expressing separately HopE, HopV, CagA and VacA cloned antigens. A: Frequency of IgA- and IgG-type immune responses (as number of sera) which reacted with lysates containing one of these antigens; B: IgA response data taken from panel A, with patients separated by age into 2 groups: under 18 years old and adults; C: IgG response data taken from panel A, separated by age as above. As negative controls, sera from 8 non-infected patients did not display any immune response when tested with these antigens (not shown). Fisher's test was used for statistical analysis, and significance lower than 0.05 is indicated.

in patients under 18 than in adults. In contrast, IgA responses against HopE and VacA and the IgG response against HopV seemed to be more frequent in adults than in children.

DISCUSSION

H. pylori colonizes the human gastric epithelium in half of the world's population and induces strong serological and inflammatory responses in the host which persist during the entire life of the subject, rendering the host unable to eradicate infection. Knowledge of the most frequently recognized antigens in the infected population may contribute to an understanding of the bacterium survival strategy. In addition, this could also help to select appropriate antigens for vaccine design. The most extensively studied *H. pylori* virulence factors as potential vaccine antigens are urease subunits[34,35], VacA and CagA[36], *H. pylori* adhesin A[37] and neutrophil-activating protein[38,39]. However, results indicating reduction in colonization after oral vaccination of human subjects have been rather modest[40,41]. As new antigens are needed, and there are few studies comparing porin genes among different *H. pylori* strains, we have looked for *H. pylori* porins suitable for vaccine development. The present report provides new information, particularly about Chilean strains, regarding porin expression among clinical isolates.

Folded porins have exposed loops at the bacterial surface which may induce a strong immune response. Porins have been proposed for the design of oral vaccines to eradicate *H. pylori*[6]. This pathogen has an extensive collection of OMPs with defined pore characteristics, such as the Hop group. However, some may display a unique on/off mechanism affecting gene expression, based on DNA strand slippage during replication. The instability of expression status makes antigen selection a very important issue. Antigens that will not be permanently expressed in most of the strains should be avoided, since they will provide a limited protection.

PCR assays carried out on 130 Chilean isolates detected *hopE* and *hopV* genes in 46.9% and 63.1%, respectively. However porin expression was infrequently detected in these strains (HopE = 13.1%, HopV = 6.9%), concurring with low seroprevalence of these porins among sera of infected patients, suggesting that these genes may be under DNA strand slippage control.

Infrequent detection of expression cannot be explained by low immunoreactivity of the rabbit antisera resulting from amino acid sequence diversity among strains, since these 2 porin sequences seem to be conserved. HopE and HopV amino acid sequence identities among those from strain CHCTX-1 and the corresponding sequences from fully sequenced *H. pylori* genomes, were in the range of 98%-99% and 94%-96%, respectively.

As a complementary approach, 69 patient sera were tested by Western blotting on *E. coli* clones expressing CagA, VacA, HopE or HopV. It was revealed that HopE and HopV porins were not often recognized within the analyzed sample. Only 6 sera (8.7%) showed IgG-type immune reaction against HopE-containing lysates and 7 (10.1%) against HopV. Similar results were obtained analyzing the IgA response. These results agree with the fact that HopE and HopV porins are sporadically expressed. In contrast, CagA (its gene is present in

about 50% of the strains) and VacA (its gene is present in almost 100% of the strains) reacted with 59.4% and 68.1% of the IgG patient sera, respectively.

Regarding nucleotide sequence features, dinucleotide repeats in *hopE* and *hopV* sequences from the CHCTX-1 strain barely reached 5 nucleotides in length. However, they contained CCCCCC and TTTTTT tracts after codons 58 and 66, respectively. These findings, taken together with the low number of strains expressing these porins, and their low seroprevalence among Chilean patients, suggest that *hopE* and *hopV* may be under strand slippage gene control. Confirmation should be done by sequencing strains carrying silenced genes.

In *H. pylori,* at least 3 porin genes from the Hop family (*hopZ*, *hopP* and *hopO*) may be subjected to this on/off switching[11,42]. Another study[43] showed a similar case: 3 different *H. pylori* strains re-isolated after *Maccacus rhesus* infection lost expression of BabA adhesin which binds Lewis b antigen. These observations support the idea that *H. pylori* can modulate expression of some OMP genes. This feature provides an adaptive mechanism to avoid induction of a strong host immune response. This is supported by the large repertory of OMPs genes in the *H. pylori* genome. Functional redundancy of porins may explain emergence of mutations in these genes without affecting bacteria viability. It has been proposed that the role of such redundancy of outer membrane proteins is to sustain antigenic variation to support pathogen survival by evasion of the host immune response[44]. The strand slippage mechanism is not normally operating in *E. coli*, therefore, in most cases, lack of heterologous expression of *H. pylori* genes in *E. coli* should be due to mutations that previously affected the *H. pylori* genome.

In spite of the low content of homopolymeric and dinucleotide repeats found in CHCTX-1 *hopE* and *hopV* genes, some strains may have switched these genes off but, in a few cases, expression could be restored by the same mechanism. This may restrict the use of these *H. pylori* OMPs as a single source of antigens for vaccine design. However, in order to provide a wider and stronger immune response, vaccines based on a mixture of *H. pylori* antigens with the inclusion of HopE and HopV should be considered.

ACKNOWLEDGMENTS

We thank Marco Olmos (Universidad Católica de Chile) for his careful review of the manuscript.

COMMENTS

Background

Few studies have been done on HopE and HopV porins from *Helicobacter pylori* (*H. pylori*). These proteins have been described as part of a large family of outer membrane proteins having similar functions, mainly as gating for influx of nutrients. Compared to other enterobacteria, such as *Escherichia coli* (*E. coli*) with only 3 major porins, *H. pylori* have shown a remarkable redundancy in this kind of outer membrane protein. The explanation for such redundancy has not arisen. A study looking at how widely these porin genes are distributed and what proportion is actually active among clinical isolates should provide some answers.

Research frontiers

Switching on/off in outer membrane genes in a few bacteria has been described as a mechanism to distract the immune system during infection by changing the proteins displayed on the surface. The authors found that *H. pylori* HopE and HopV porin genes seemed to be absent in some isolates, and about half of those who carried them did not express them. In addition, sera from infected patients do not frequently recognize these antigens. This feature may contribute to the ability of these bacteria to avoid the host immune response allowing their persistence in humans for an extended period of time.

Innovations and breakthroughs

Recent reports have shown that some outer membrane protein genes from *H. pylori* could be turned on/off by random nucleotide insertions or deletions either at the promoter or within the coding region, through a mechanism called "strand slippage" during DNA replication. This is the first report proposing that this switching may also occur in the *hopE* and *hopV* genes, explaining why around 70%-90% of these genes are shut down in Chilean clinical isolates.

Applications

Determining whether a protein is subjected to on/off switching during its expression at the bacterial surface, together with the knowledge of its immunoreactivity will be useful to select potential antigen candidates to be used in the design and development of vaccines.

Terminology

H. pylori HopE and HopV proteins are part of a large family of outer membrane proteins and are located at the bacterial surface. They are defined as porins because they form a pore structure to allow the influx of small size nutrients and other compounds. They may constitute a target for the immune system. The "strand slippage" mechanism to control gene expression is a result of random errors during strand DNA replication consisting of nucleotide insertions or deletions that alter the genetic code of the protein or the functionality of elements (i.e. promoter) that control gene expression.

Peer review

The manuscript by Lienlaf *et al* assesses the patterns of 2 porin genes in *H. pylori*. *H. pylori* remains a significant problem in developing countries around the world. Although much is now known about the pathogenesis of this bacterium and about host responses to infection, the organism remains a clinical problem. Various investigators have focused upon the establishment of a vaccine for this pathogen. An appropriate selection of a conserved and widely expressed antigen of *H. pylori* clinical isolates will assure a suitable design of a protective vaccine. This work is promising.

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