

ARTÍCULO ORIGINAL

Cloning and expression of a recombinant *CagA*-gene fragment of *Helicobacter pylori* and its preliminary evaluation in serodiagnosis

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Introduction: *Helicobacter pylori* strains expressing cytotoxic CagA protein are more commonly associated with peptic ulceration, atrophic gastritis and gastric adenocarcinoma than those lacking CagA. Determination of anti-CagA antibodies, therefore, acquires a relevant clinical significance in the serological detection of *H. pylori* infection and disease risk prediction. However, the CagA-serology has been questioned due to the differences found in their performance evaluations in different populations.

Objective: To obtain a recombinant CagA fragment useful for serodiagnosis of *H. pylori* infection

Methods: A fragment of the *cagA* gene was cloned into a prokaryotic T7 RNA polymerase expression vector. A recombinant C-terminal His₆-tagged CagA was expressed, subsequently solubilized with urea and purified by immobilized metal affinity chromatography. The performance of the recombinant protein was evaluated using 180 human serum samples with an in-house Western blot assay compared to the Helicoblot 2.1 reference test.

Results: The expressed His₆-tagged CagA showed an immunoreactive 80kDa band as was revealed by SDS-PAGE and Western blot analysis using two different specific anti-CagA polyclonal antibodies. The recombinant protein was successfully purified obtaining a 93% of purity. The performance analysis of the purified recombinant antigen showed good immunoreactivity and exhibited values of sensitivity, specificity and accuracy of 88.1%, 100% and 92.7%, respectively.

Conclusion: The CagA fragment of the study may constitute a useful tool for serological diagnosis of CagA-positive *H. pylori* infection.

Keywords: *Helicobacter pylori*; serological test

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Clonación y expresión de un fragmento recombinante del gen *cagA* de *Helicobacter pylori* y su evaluación preliminar en el serodiagnóstico

Introducción. Las cepas de *Helicobacter pylori* que expresan la citotoxina CagA, se asocian más frecuentemente con úlcera péptica, gastritis atrófica y adenocarcinoma gástrico que las que carecen de esta citotoxina. Por lo anterior, el determinar la presencia de anticuerpos anti-CagA adquiere gran importancia clínica en la detección serológica de la infección por *H. pylori* y la predicción del riesgo de enfermedades. Sin embargo, los métodos serológicos que emplean CagA han sido cuestionados debido a las diferencias encontradas en las evaluaciones de su desempeño en diversas poblaciones.

Objetivo. Obtener un fragmento recombinante de la proteína CagA para el serodiagnóstico de la infección por *H. pylori*.

Materiales y métodos. Un fragmento del gen *cagA* fue clonado en un vector de expresión procariota que contenía el promotor de la T7 ARN polimerasa. El fragmento de la proteína CagA con seis histidinas en la región C-terminal, se expresó, se solubilizó con urea y se purificó por cromatografía de afinidad con iones metálicos inmovilizados. El desempeño de la proteína recombinante se evaluó empleando un método *in house* de Western Blot y 180 sueros humanos. Los resultados se compararon con la prueba de referencia Helicoblot 2.1.

Author contributions:

L. González and B. L. Rodríguez: contributed equally to this work. Designed the research, analyzed the data and wrote the paper.

K. Marrero: designed the research

L. Martínez, O. Reyes and E. Rodríguez: collected the samples and managed the patient data.

L. González and E. Rodríguez: performed the research.

Resultados. La proteína CagA expresada mostró una banda inmunorreactiva de 80 kDa en el Western Blot al emplear dos anticuerpos policlonales anti-CagA específicos. La proteína recombinante fue purificada hasta un 93 % de pureza y el análisis de desempeño del antígeno recombinante purificado mostró buena inmunorreacción y exhibió valores de sensibilidad, especificidad y exactitud de 88,1 %, 100 % y 92,7 %, respectivamente.

Conclusiones. El fragmento de la proteína CagA del estudio puede constituir una herramienta útil para el diagnóstico serológico de la infección por cepas de *H. pylori* positivas para CagA.

Palabras clave: *Helicobacter pylori*, pruebas serológicas.

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Introduction

Helicobacter pylori is a Gram-negative spiral-shaped bacterium that persistently colonizes the stomach of more than half of the human population. This persistent colonization plays an important role in the appearance of gastroduodenal pathologies, such as peptic ulcers, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (1).

The persistence of this infection, which is crucial for developing chronic disease phenotypes, mainly depends on specific characteristics of the bacterium. The most important virulence factor of *H. pylori* is the cytotoxin-associated antigen CagA. This protein is coded by the *cagA* gene that is part of the *cag*-pathogenicity island which encodes the components of a type IV secretion system (T4SS). *H. pylori* uses this T4SS to inject CagA into human gastric cells, where it perturbs several host signaling pathways to provide a local environment that is more suitable for the pathogen survival and thereby promoting severe damages of gastric epithelial cells and mucosa (2,3,4).

The CagA protein is an immunodominant antigen (5,6) and the *cagA*-positive strains are commonly associated with more severe gastroduodenal diseases than *cagA*-negative strains (7,8). Several studies in cell culture and animal models indicate the likely role of CagA in human cancer associated with *H. pylori* infection (3). Transgenic expression of CagA in mice led to the development of gastric epithelial hyperplasia and adenocarcinoma of the stomach and the small intestine, providing the first direct evidence of the potential oncogenicity of CagA *in vivo* (9). Thus, CagA is the first bacterial

oncoprotein to be identified in relation to human cancer. Therefore, determination of the CagA-status in *H. pylori* infection is an important and an informative approach in disease risk prediction.

Different methods have been used to detect *H. pylori* infection with CagA-positive strains. Among all, the serologic methods have been successfully used due to the immunogenicity of this protein and the non-invasivity of the method (10). In addition, it has been observed that CagA seropositivity is able to predict the development of atrophy and constitute a risk factor for intestinal metaplasia (11). However, the accuracy of CagA-serology has been challenged due to the differences found in their evaluations. It has been suggested that this variability is mainly related with the differences in the antigens or methodologies used to detect the anti-CagA antibodies, or the use of different populations (12). Here we described a new recombinant fragment of CagA and evaluated its efficacy for the serodiagnosis of the CagA status of *H. pylori* infection.

Materials and methods

Serum samples

Serum samples were obtained from 180 consecutive Cuban dyspeptic patients with an average age of 36.5 years (range, 18 to 55) who attended the Department of Gastroenterology of Carlos J. Finlay Hospital. Ninety-two of the patients presented gastritis (51.1 %), 34 gastric ulcer (18.8 %) and 54 duodenal ulcer (30.1 %). All patients gave their written informed consent to participate in the study and the Carlos J. Finlay Hospital ethic committee approved the protocol by which the specimens were obtained.

Bacterial strains, plasmids and reagents

H. pylori strain 26695 was used for genomic DNA extraction. *E. coli* Mach1 (Invitrogen, USA) was used as a host strain for cloning manipulation of *cagA* gene. *E. coli* BL21(DE3) (Invitrogen, USA) was used as CagA expression strain. The pET22b(+)

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plasmid (Novagen, USA) was used as cloning and expression vector. This vector contains the T7 promoter and allows expression of the recombinant protein fused to histidine tail at the C-terminus. The restriction enzymes and PCR reagents used in cloning procedures were purchased from Roche, USA.

Molecular techniques and construction of the expression plasmid

Genomic DNA from *H. pylori* was extracted by CTAB methodology with phenol/chloroform and isopropanol precipitation as previously described (13). A fragment of the *cagA* gene was amplified by PCR using forward primer *cagA.F* [5'-CATGCCA TGGGGGATAACAGGCAAGCTTTTGA-3'] which created the NcoI site (underlined), and reverse primer *cagA.R* [5'-GAATTCGAGGTCGCTTTTGC-3'] XhoI site. These primers were designed based on the DNA sequences of 26695 *cagA* gene obtained from the GenBank database and using the Gene Runner software version 3.05.

PCR reactions were performed in final volumes of 50 µl containing 100 ng of *H. pylori* genomic DNA, 0.2 mM dNTP, 2.5 mM MgCl₂, 0.6 µM of each primer, 1 unit of Taq DNA polymerase and 1× reaction buffer. For amplification, a Thermal Cycler, Mastercycler® personal (Eppendorf, Germany) was used with the following settings: 1 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 61 °C and 1 min at 72 °C and then a final extension of 5 min at 72 °C. The PCR products were analyzed by agarose (2%) gel electrophoresis using a 100 bp DNA molecular size marker (Roche, USA) and then purified using the High Pure PCR Product Purification Kit (Roche, USA).

The purified PCR product was cloned into the pET22b(+) expression vector following the manufacturer instructions. The cloning product was transformed into *E. coli* Mach1 electrocompetent cells and recombinant clones were identified by plasmid purification using the High Pure Plasmid Isolation Kit (Roche, USA) and confirmed by restriction enzyme analysis. The identity of the insert was determined by DNA sequencing (Macrogen Inc., Korea). Sequencing reactions were performed for both DNA strands, and the ClustalW2 multiple-sequence alignment software was used to analyze sequencing results.

Expression and localization of recombinant CagA

Recombinant plasmid pET22b(+)-CagA was transformed into *E. coli* BL21(DE3) electrocompetent

cells and recombinant clones were identified as described above. One recombinant clone was used to inoculate 5 ml of Luria–Bertani medium containing 100 µg/mL ampicillin which was incubated in an orbital shaker (37 °C, 200 rpm) until OD_{600nm} 0.6. At this moment, expression of the recombinant protein was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG), with a final concentration of 0.1 mM, and incubated for additional 4 h in the same conditions. Cells were harvested by centrifugation (10 000 g, 4 °C, 5 min) and resuspended in 0.1 M phosphate-buffered saline, pH 7.4 (PBS) and later the 5× SDS-PAGE loading buffer was added (250 mM Tris–HCl pH 6.8, 50% glycerol, 1 M 2-mercaptoethanol and 1% SDS). After boiling for 10 min, the sample was submitted to 12% SDS-PAGE according to Laemmli (14). Expression of the recombinant protein was visualized by Coomassie Blue staining. To confirm the presence and immunoreactivity of the recombinant protein, Western blot analysis was carried out essentially as Towbin and Gordon (15) and two different anti-CagA polyclonal antibodies, kindly provided by professors Guillermo Pérez-Pérez (New York University, School of Medicine, New York, USA) and Rainer Haas (Ludwig Maximilian University, Munich, Germany), were used.

In order to improve the CagA expression, several experimental parameters were optimized: temperature, IPTG concentration, OD_{600nm} at induction, and incubation time after induction. This protein expression analysis was monitored by SDS-PAGE and using a Gene Genius gel documentation system (Syngene, UK).

To localize the recombinant CagA protein (rCagA) into the cell extract, the clone expressing the rCagA was tested regarding the solubility of the protein. Cells were lysed by freeze-thawed three times in buffer A (20 mM Tris, pH 7, containing 0.5 M NaCl, 5 mM imidazole, and 1 mM PMSF) at a ratio of 1 g of wet cells/5 ml of buffer A, as previously described (16). The soluble and insoluble fractions were separated by centrifugation at 10 000 g for 5 min at 4 °C and applied in a 12% SDS–PAGE as above.

Purification of recombinant CagA

The cell lysis insoluble fraction, containing the rCagA, was suspended in buffer A and treated with different concentrations of Urea (2, 4, 6, and 8 M) employing an Ultra-Turrax homogenizer (IKA-WERKE, Germany) at 9500 rpm for 1 min.

After incubation overnight in gentle agitation at 4 °C, the samples were centrifuged at 14 000 g for 20 min at 4 °C and all soluble and insoluble fractions were analyzed by 12% SDS–PAGE to determine the minimal Urea concentration needed for the best rCagA solubilization. Then, the recombinant protein was purified by immobilized metal affinity chromatography (IMAC) using a Quelating Sepharose Fast Flow matrix (Amersham Pharmacia, Sweden) pre-charged with Cu²⁺. The column was equilibrated with buffer A containing 4 M Urea and the protein refolding was performed on-column using a linear gradient from 4 to 0 M Urea in buffer A. The recombinant protein was eluted with a linear gradient from 5 to 200 mM imidazole and fractions of 1 ml were collected and analyzed by 12% SDS–PAGE. The purified protein was dialyzed on Sephadex G-25 column equilibrated with PBS.

Determination of protein concentration and purity

The concentration and purity of the rCagA was determined by Bradford's assay (17) and SDS-PAGE analysis using the Syngene Gene Genius imaging system, UK, respectively. Bovine serum albumin was used as standards in both cases.

Evaluation of recombinant CagA antigen for serodiagnosis

In order to assess the efficacy of the rCagA in serodiagnostic, a panel of 180 human sera was simultaneously evaluated using an in-house Western blot assay and the commercial immunoblot assay Helicoblot 2.1 (HB 2.1) (Genelabs Diagnostics, Singapore), which was regarded as the reference assay in the study. The Western-rCagA was performed as follow: the proteins resolved on a 12% SDS-PAGE gel were transferred to nitrocellulose membrane (Amersham Pharmacia, Sweden) for 3 h at 36V using a wet tank transfer system (Hofer Inc., USA). The membranes were incubated with blocking reagent (5% skim milk in PBS), 1h at 4°C and subsequently incubated with the serum samples overnight in gentle agitation at room temperature. All sera were diluted 1/100 in PBS containing 0.05% Tween 20. After washing with distilled water containing 0.05% Tween 20, membranes were incubated 1 h at room temperature with a specific anti-human immunoglobulin antibody conjugated to horseradish peroxidase (Promega, USA) diluted 1/5000 in PBS containing 1% skim milk and 0.05% Tween 20. Reacting bands were detected using 3,3'-diaminobenzidine substrate 0.02% in buffer 10mM Tris, pH 7.6.

The HB 2.1 assay was developed in accordance with the manufacturer's instructions. Results from Western-rCagA test were compared with those from the HB 2.1 test to assess the performance of the recombinant antigen by determining the relative sensitivity, specificity and accuracy as described below,

Sensitivity = $a / (a + c) \times 100$, where 'a' is the number of sera positive by both, the Western-rCagA and HB 2.1 while 'c' is the number of sera positive by HB 2.1 but negative by Western-rCagA.

Specificity = $d / (b + d) \times 100$ where 'd' is the number of sera negative by both, the Western-rCagA and HB 2.1 while 'b' is the number of sera negative by HB 2.1 but positive by Western-rCagA.

Accuracy = $(a + d) / (a + b + c + d) \times 100$.

Results

Cloning of *H. pylori* CagA gene

The designed primers allowed effective amplification of the selected fragment of *cagA* gene. These PCR reactions generated DNA fragments of 2130 bp as it was expected. Amplified fragment digested with NcoI and XhoI, was purified and successfully cloned into the pET22b(+) expression vector as was corroborated by restriction enzyme analysis (Fig. 1). Sequencing of the pET22b(+) insert, compared to 26695 *cagA* gene, obtained from the GenBank database, confirmed the identity of the cloned *cagA* sequence (data not shown).

Expression of recombinant CagA

E. coli BL21(DE3) harbouring plasmid pET22b(+)-CagA, expressed a major protein of approximately 80kDa upon IPTG induction that could be detected by SDS–PAGE analysis (Fig. 2A, lane 5). This molecular size corresponds to the estimated molecular weight of the selected *cagA* fragment (81.5kDa). After SDS-PAGE analysis, the target protein was further analyzed by immunoblotting with two different anti-CagA polyclonal antibodies to corroborate the presence of the recombinant protein. The Western blot assay also showed a major reacting band of 80kDa approximately (Fig. 2B, lane 1). This band was not detected without IPTG induction (Fig. 2B, lane 2).

The optimization of the recombinant protein production showed that the best conditions were an expression at a OD_{600nm} 0.8 with 1mM IPTG at 37°C and 200 rpm during 4 h. The expressed protein under these conditions constituted 27%

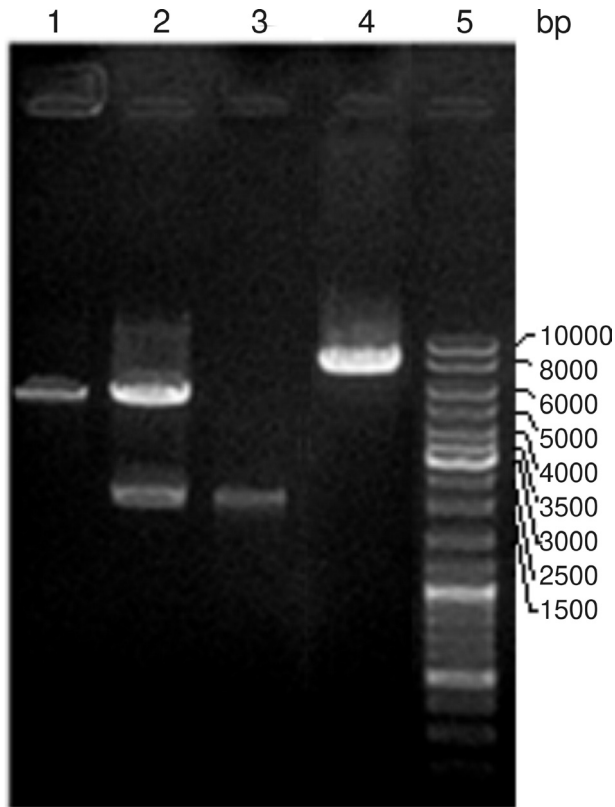


Figure 1. Agarose gel analysis of the *cagA* fragment cloning. Lane 1, pET22b(+) cloning vector digested with NcoI. Lane 2, pET22b(+)-*cagA* recombinant vector digested with NcoI and XhoI. Lane 3, PCR *cagA* gene product (2130bp). Lane 4, the recombinant vector pET22b(+)-*cagA* linearized with XhoI. Lane 5, DNA weight marker 1000bp (Roche, USA). DNA band sizes are in base pairs (bp)

of the total cellular protein as was determined by densitometry. The cells were successfully broken by the freeze-thaw procedure and the rCagA was found in the insoluble fraction after cell lysis (data not shown).

Purification of recombinant CagA

The recombinant protein accumulated in the insoluble fraction of cell lysate was solubilized in buffer containing urea. The solubilization assays allowed determining that 4 M urea was the minimal concentration needed to suspend the rCagA with the minimum presence of contaminants (data not shown).

The recombinant protein was successfully purified by IMAC and the rCagA was eluted from the column at 30 mM of imidazole with approximately 93% purity (Fig. 2D, lane 3).

Performance of the Western-rCagA

The performance of the rCagA in serodiagnostic was studied using a panel of 180 serum samples and the HB 2.1 as the reference test. Among all sera tested, the HB 2.1 identified 109 (60.6%) CagA positive and 71 (39.4%) CagA negative cases. On the other hand, 96 from the 109 CagA positive sera detected by HB 2.1 were also immunoreactive with the rCagA, while all the 71 CagA negative cases detected by HB 2.1 were also negative by the Western-rCagA. Thus, there were 13 false

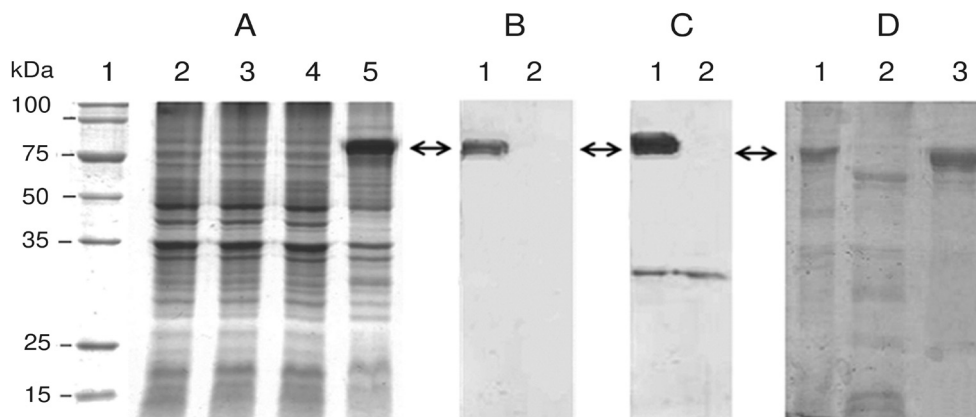


Figure 2. Expression and purification of the CagA recombinant protein. Panel A. 12% SDS-PAGE analysis of the expressed CagA protein. Lane 1, Protein weight marker (Promega, USA). Lane 2, *E. coli* BL21 DE3 transformed with the recombinant pET22b(+)-Cag plasmid without IPTG induction. Lane 3, *E. coli* BL21 DE3 transformed cells with the non expressing pET22b(+) vector. Lane 4, *E. coli* BL21 DE3 transformed cells with the recombinant pET22b(+)-CagA plasmid without IPTG induction. Lane 5, *E. coli* BL21 DE3 transformed cells with the recombinant pET22b(+)-CagA induced with 0.1 mM IPTG. Panel B and C. Western blot analysis of the recombinant CagA with two different polyclonal anti-CagA antibodies. Lane 1, *E. coli* BL21 DE3 transformed with the recombinant pET22b(+)-CagA plasmid with IPTG induction. Lane 2, Transformed BL21 DE3 cells induced without IPTG. Panel D. 12% SDS-PAGE analysis of the recombinant CagA purification. Lane 1, total cell protein extract of the BL21 DE3 with pET22b(+)-CagA. Lane 2, flowthrough of the IMAC column. Lane 3, recombinant CagA protein eluted with 30 mM Imidazole. Protein band sizes are in kilodalton (kDa).

negative and none false positive results when the Western-rCagA was used to detect anti-CagA antibodies. Therefore, the Western blot assay using the rCagA developed here showed a sensitivity, specificity, and accuracy of 88.1%, 100%, and 92.7%, respectively.

Discussion

Several studies using diagnostic methods to detect CagA antibodies have demonstrated that this antigen improve the sensitivity of *H. pylori* detection (18). Also, it has been observed that the positivity for CagA antibodies has the ability to predict the development of atrophy and appears related with young gastric cancer cases (10). However, the accuracy of the tests developed with this regard has been questioned because the results differed when they were used in different populations. It has been suggested that an important issue to this heterogeneity appeared to result from differences in the antigens used to detect anti-CagA antibodies (12). Consequently, new studies using the already existent CagA antigens, and/or some new design fragments of the cytotoxin, should be done to evaluate the reactivity of representative populations to different antigens.

In the present study a new recombinant fragment of CagA cytotoxin was expressed in *E. coli*. The goal of the study has been the production of a specific antigen that should be able to react with a broad range of human anti-CagA antibodies and could improve the serodiagnosis of the CagA-positive *H. pylori* infection.

The selected fragment corresponds to all conserved N-terminal and central conserved regions of the CagA protein, containing 716 aa and an estimated molecular size of 81.5 kDa. Previous studies have demonstrated that CagA is a challenging protein for recombinant expression, due to a propensity for degradation (6,19). Specifically, it has been suggested that the region preceding the first EPIYA motif contains an important cleavage site (19). We designed a region that excluded both, this site and the CagA variable region at the C-terminus (the EPIYA region) and also lacked the most proximal N-terminal variable region. The selected CagA sequence constitutes a suitable fragment for the recognition of antibodies due to contain several epitopes that are highly conserved and immunogenic (20,21). In agreement with the above data, the selected recombinant CagA fragment of this study showed a low degree of degradation and was highly immunoreactive (Fig. 2 and 3).

For large-scale production of recombinant proteins, the T7-Lac promoter based vectors containing histidine tails have been successfully used for hyper-expression of the selected proteins, which help in devising simple chromatography protocols for obtaining highly pure protein in high yields (22). The expression system used in this study produced a large amount of the heterologous protein (27% of the total cellular protein). The rCagA was expressed as C-terminally His₆-tagged protein and was purified by IMAC. The use of the histidine affinity tag facilitated the recovery of the recombinant protein, using one-step chromatography procedure, to obtain a 93% of purity (Fig. 2, panel D) without affecting its immunoreactive properties (Fig. 3, panel A).

The two polyclonal anti-CagA antibodies used here were generated using two different recombinant CagA fragments (20,23). These two recombinant proteins comprise different fragments, which are mostly included in the wide conserved sequence selected for our rCagA. Therefore, the good reactivity observed with both antibodies demonstrated the identity of the CagA produced fragment (Fig. 2, panel B and C).

The CagA seropositivity (60.6%) obtained with Helicoblot 2.1 test in this study was similar to the positivity observed in CagA genotyping of Western strains (24) and slightly lower than the *cagA* positive strains detected in Cuban *H. pylori* isolates (25). The Western blot assay with the rCagA developed here demonstrated adequate performance in comparison to HB 2.1 that contains the entire CagA antigen. This comparison showed sensitivity of 88.1%, specificity of 100% and accuracy of 92.7%. The sensitivity was the lowest performance parameter observed, but it is in the accepted range compared with others ELISA and immunoblotting tests developed to detect CagA antibodies (26,27). Interesting, among the 13 false negative samples obtained with the Western-rCagA, 9 were classified as *H. pylori* negative CagA positive by HB 2.1. It is though that CagA seropositivity in *H. pylori*-seronegative subjects may either be a sign of a past *H. pylori* infection due to the long life of anti-CagA antibodies (18,28) or represent a false positive reaction (29). Therefore, probably the rCagA of this study may not contain the epitopes causing either of the above mentioned events that should be, if demonstrated, an advantage of this recombinant fragment to diagnose CagA-positive *H. pylori* infection. In addition, our rCagA should

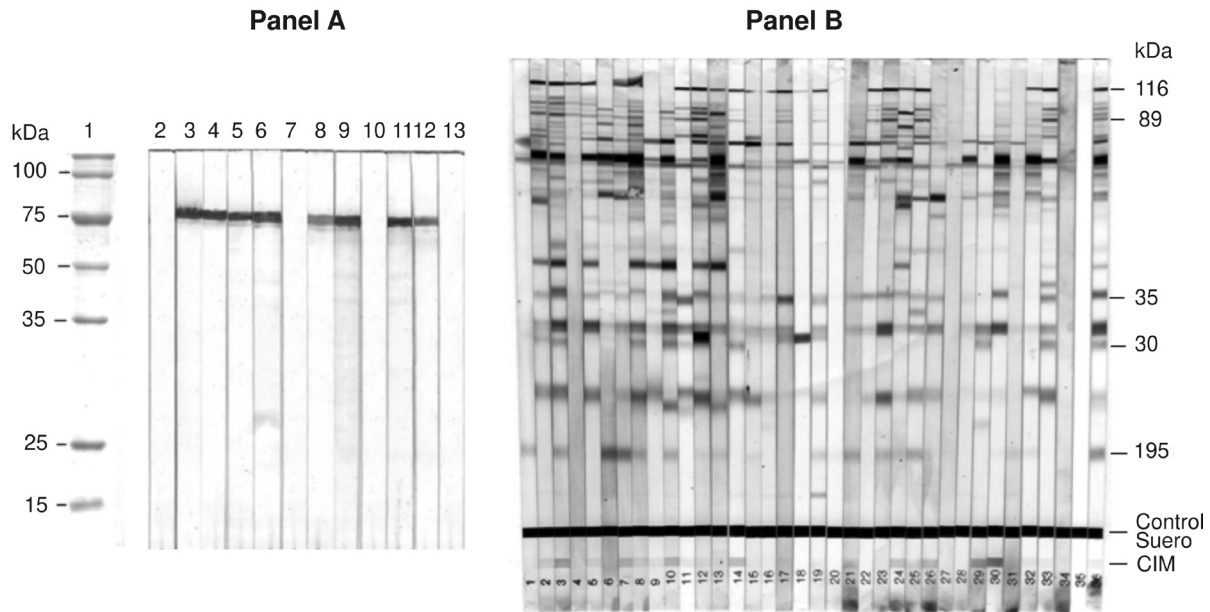


Figure 3. Representative results of the 180 serum samples evaluated by the Western-rCagA and the Helicoblot 2.1 (HB 2.1) reference test. Panel A. The Western-rCagA analysis. Lane 1, Molecular weight marker (Promega, USA). Lane 2, HB 2.1 negative control serum. Lane 3, HB 2.1 positive control serum. Lane 4 to 13, representative serum samples of this evaluation. Panel B. Helicoblot 2.1 assay. Lane 1 to 34, representative serum samples of this evaluation. Lane 35, HB 2.1 negative control serum. Lane 36, HB 2.1 positive control serum.

be sensitive enough due to the fact that contains the conserved part of the CagA cytotoxin, which has been recently shown to have the most reactive epitopes of the entire CagA protein (21). On the other hand, there were no false positive results and therefore the Western-rCagA showed 100% specificity. This is also a good result for the rCagA of this study because the lack of specificity of anti-CagA tests has been attributed in part to differences in the CagA antigens used (12,30).

Taken together, the data of this study suggest that the recombinant CagA fragment developed here should be a good reagent to be included in a CagA serodiagnostic assay to be standardized and validated in representative populations.

Conflicts of interest

The authors have declared no conflicts of interest.

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