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Full Length Research Paper

Identification of single chain Fv antibody fragment against *Helicobacter pylori*

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***Helicobacter pylori* is one of the most common causes of infectious diseases around the world. Although combined antibiotic treatment for the infection exist, the development of therapies such as immunotherapy or an effective vaccine can be helpful for containment and prevention of the infection. Phage display technologies offer a simple way for selecting specific antibody fragments against a specific target. In this work, we report the construction of a phage display antibody library against *H. pylori* strain N2. The library is constituted of 2.4 × 10^4 transformants. A total of 94 clones were randomly selected and screened against the N2 strain, where 22% of them recognized whole-cell extracts of *H. pylori*. Moreover, other 94 clones were screened against the J99 strain, where 12% of the clones recognized this strain. The scFv-E1N2 was selected and it showed high reactivity in ELISA experiments. Furthermore, it reacted with an antigen of 27 kDa in both strains of *H. pylori* tested in our study. In conclusion, this scFv library may be used for the selection of antibodies for the development of an immunodiagnostic test. Also, it may provide insights in the identification of antigens for the development of a new vaccine or immunotherapy.**

Key words: Phage display libraries, scFv antibodies, *Helicobacter pylori*.

INTRODUCTION

Helicobacter pylori is one of the most common causes of infectious diseases around the world (Aguilar et al., 2001). Besides causing gastric and peptic ulcers, the presence of *H. pylori* in the human stomach also represents a risk of developing gastric cancer and lymphoma MALT (mucosa-associated lymphoid tissue) (Owens and Smith, 2011). Studies on patients with low degree of lymphoma MALT have confirmed a high occurrence of *H. pylori* (Wotherspoon, 2000). The bacteria is associated with the proliferation of gastric epithelial cells and this epithelial cells and this proliferation can be reverted through its complete eradication, suggesting that tumor cells remain in response to the infection (Maruta et al., 2005). Although combined antibiotic therapy for treating the infection exist, the development of therapies such as immunetherapy or an effective vaccine can be helpful for containment and prevention of the infection. Cao J. and coworkers demonstrated that phage-displayed scFv exhibited a bactericidal effect and inhibited the growth of *H. pylori*; additionally, pretreatment of the bacteria with these recombinant phages before oral inoculation prevented colonization of the mouse stomachs (Cao et al., 2000). On the other hand, as it has been seen with other pathogens, the development of an effective vaccine against *H. pylori* would be of enormous benefits, especially in developing countries where the infection reaches up to 90% among population (Frenck and Clemens,

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2003). However, some vaccine candidates against *H. pylori* tested in mouse models did not demonstrate protection in human clinical trials (Czinn and Blanchard, 2011). Meanwhile, many important efforts have been made for the identification of proteins and epitopes related to protection against these bacteria. Because of this, several methods have been used for helping in the development of vaccines such as bioinformatic tools, proteomics (Chakravarti et al., 2000), and phage display technology. Using the last technology, Li et al. (2010). identified two different mimotopes which induced humoral immune response against the protein urease B derived from *H. pylori* after immunization in mice. The antiserum induced by these mimotopes clearly inhibited the enzymatic activity of the urease B (Li et al., 2010). In addition, this protein was identified by antibody phage display as a potential target for the immune system and this protein has been used as a vaccine component in human clinical trials (Czinn and Blanchard, 2011).

A more in-depth understanding about the process of infection and its relation with the immune systems is necessary. The identification of potential epitopes or proteins with the capacity to stimulate an effective immune response is critical to eliminate the infection. Furthermore, the identification of new potential antibodies might be particularly relevant as a complement to current therapies against *H. pylori*. In the current report, we describe the construction of a scFv antibody library against *H. pylori* strains J99 and N2. From this library, we isolated and characterized a monoclonal scFv fragment E1N2 which demonstrated high reactivity against *H. pylori*. This library can be helpful for the identification of antibodies for its use as immunotherapy or immunodiagnostics; moreover, it would be useful for the identification of potential epitopes/mimotopes which might be included as a component in future immunogens or vaccines.

MATERIALS AND METHODS

Helicobacter pylori culture conditions

The *Helicobacter pylori* strains J99 and N2 were a kind gift of Dr. Guadalupe Ayala (Centro de enfermedades infecciosas, Instituto Nacional de Salud Publica de México). Bacteria were grown in tryptic soy agar supplemented with 5% sheep blood and 20 µg/ml of vancomycin under a microaerobic atmosphere (10% CO_2) at 37°C for 48 h. The bacteria were checked for the presence of urease, catalase, and oxidase activities in addition with stain characteristic as previously described (Oliva et al., 2000).

Ethics statement

The Local Ethics Committee, (Instituto de Ciencias Biomédicas, UACJ) approved the study.

Chicken immunization

Two chickens White Leghorn were immunized with lysed *H. pylori* N2 strain three times at 14-day intervals. The immunogen (20 and 200 µg, respectively) was mixed with Freund´s incomplete adjuvant (IFA) for priming and with complete Freund´s adjuvant (CFA) for boost injections in the pectoral area. Pre- and post-immunization sera were tested in ELISA for the presence of specific anti-*H. pylori* antibodies. Briefly, a 96-well plate (Nunc, Roskilde, Denmark) was coated with 2 µg/ml of lysed *H. pylori* strain N2 in phosphate buffer (pH 7.2) overnight at 4°C. The plate was washed with PBS containing 0.2% Tween 20 and blocked with PBS containing 2% of serum albumin. After washing, chicken sera diluted 1:100 in PBS-BSA 1% was added, and the plate was incubated for 1 h at 37°C. After washing, goat anti-chicken IgY conjugated with HRP (Abcam, USA). After incubation of 1 h at 37°C, the plate was washed and 100 µl of ABTS single solution (Zymed) was added. The OD reading at 405 nm was registered using a microplate reader.

Construction of anti-*Helicobacter pylori* scFv antibody phage display library

The mRNA was extracted from the splenocytes of immunized chickens using RNA STAT-60 (Tel-Test Inc, USA). The kit RT-PCR Superscript II (Invitrogen, USA) was used for cDNA synthesis. All procedures were done according to the manufacturer´s instructions. Oligonucleotides used for the amplification of the heavy (V_H), light (V_L) chain and ScFv construction were described previously (Andris-Widhopf et al., 2000). Briefly, the reaction mixture for V_L amplification was prepared with 10 µl of PCR buffer 10x, 8 µl of dNTP´s (2.5 mM), 3 µl of the oligonucleotide CSVK and CKJo-B (20 pmol/µl), 2 µl of cDNA and 5 µl of Taq DNA polymerase (1U/µl). The primers CSCVHo-F and CSCG-B were used for V_H amplification in the same conditions. Assembly reaction to join V_L and V_H genes were done using the primers CSC-F and CSC-B. PCR products were gel purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA). The PCR products were digested using Sfi I (New England Biolabs, USA) for 12 h and ligated into the similarly digested vector pComb 3X (Andris-Widhopf et al., 2000). The ligation was precipitated with isopropanol and then it was introduced into 50 µl *Escherichia coli* X1-blue competent cells by heating and chilling. Transformed cells were cultured in 10 ml of super broth media with ampicillin (50 µg/ml) and tetracycline (10 µg/ml). Next, phagemid-containing bacterial colonies were infected with the helper phage VCSM13 (6×10^{11} PFU/ml) (Invitrogen, USA) and the volume of the culture media adjusted at 100 ml with ampicillin and kanamycin (50 µg/ml). The culture was grown overnight in agitation.

Selection of scFv antibodies against *H. pylori* by biopanning

Selection and amplification procedures for the scFv library were described previously (Pedroza-Roldan et al., 2009). Briefly, phages were recovered by double precipitation with PEG/NaCl. The precipitated phages were resuspended in 400 µl of PBS-BSA 1%. The panning was done as reported previously (Solorzano-Vargas et al., 2008). For this, a 96-well plate was coated overnight with 2 µg/ml of lysed *H. pylori* strains J99 and N2 separately. Plates were washed and blocked with BSA 3% and incubated for 1 h at 37°C, and then the phages were added at a concentration of 3×10^{11}/ml. After washing, bound phages were eluted using 50 µl of trypsin (10 mg/ml). The eluted phages (individually for J99 and N2) were used to repeat another round of selection. Four rounds of selection were performed.

Selection and production of monoclonal scFv antibodies

Phagemid DNA was extracted from the third (strain N2) and fourth (strain J99) selection rounds and introduced into *E. coli* TOP10F competent cells. Individual colonies (94 for N2 and 94 for J99, respectively) were randomly selected and cultured in a 96-well plate

and incubated overnight at 37°C. Next day, a 5 µl aliquot of each sample from the overnight culture were added into another 96-well plate and incubated for 6 h at 37°C. After this time, 5 µl (0.5 M) of Isopropyl β-D-1-thiogalactopyranoside (IPTG) were added to each well and cultured for 22 h at 37°C. Supernatants were stored at -20°C. Protein extraction from bacteria used in this study was performed as follows: pellets were resuspended in PPD buffer (100 mM NaCl, 25 mM Tris HCl pH 8.0, 0.02% NaN_3 and 1% triton X-100). The resuspended pellet was sonicated in ice four times for 15 s each time.

The immunoassay was conducted as follows; two micrograms of lysed *Helicobacter pylori* strains N2 and J99 were coated separately in a 96-well plate overnight at 4°C. Plates were blocked with PBS/BSA 3% for 1 h at 37°C, after washing, 50 µl of the supernatant was added. After washing, goat anti-HA conjugated with HRP (Roche Diagnostics, USA) was added and incubated for 1 h at 37°C. After incubation for 1 h at 37°C, plates were washed and 100 ul of ABTS single solution (Zymed) were added. The OD reading at 405 nm was registered using a microplate reader.

Characterization of the monoclonal scFv antibody E1N2

To characterize the capacity of the scFv EIN2 to recognize *H. pylori*, the clone E1N2 was cultured in 30 ml of SB and induced by adding 300 µl of IPTG 0.5 M in the culture medium for 16 h at 37°C. The scFv antibody was purified from the supernatant and cell-associated fraction by a Ni-NTA agarose system (Qiagen, USA) according to the manufacturer´s instructions. Bradford´s assay was used to quantify proteins extracts.

A 96-well plate was coated with 2 µg/ml of lysed *Helicobacter pylori* strain N2 or *Klebsiella sp, E. coli, Enterobacter cloacae, Salmonella typhimurium, Proteus vulgaris, and Staphylococcus sp*. in PBS overnight at 4°C. The plate was washed with PBS containing 0.2% Tween 20 and blocked with PBS containing 2% of BSA. After washing, E1N2 scFv antibody (10 µg/ml) was added, and the plate was incubated for 1 h at 37°C. As a control, a non-related antibody fragment that binds a hapten was used. After washing, goat anti-HA conjugated with HRP (Roche Diagnostics, USA) was added and incubated for 1 h at 37°C. Next, the plate was washed and 100 µl of ABTS single solution (Zymed) was added. The OD reading at 405 nm was registered using a microplate reader. The supernatant and sonicated bacteria were separated in SDS-PAGE under reducing conditions and transferred onto PVDF membrane (Osmonics 0.45µM). The membrane was blocked with PBS-BSA 3% for 1 h at 4°C. Goat anti-HA conjugated with HRP diluted 1:5000 in PBS-Tween-BSA 0.2% was added and incubated for 2 h at 4°C. On the other hand, to identify the molecular weight of the antigen recognized by the scFv antibody E1N2; 10 µg of lysed *H. pylori* strains J99 and N2 were separated in SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with PBS-BSA 3% for 1 h at 4°C and then 2 µg/ml of the purified antibody was added and incubated for 2 h at 4°C. After washing, goat anti-HA conjugated with HRP diluted 1:5000 in PBS-Tween-BSA 0.2% was added and incubated for 1 h at 4°C. The membranes were developed using the ECL western blotting detection system (Amersham Biosciences, UK).

DNA sequencing

Four clones were selected for DNA sequencing. The sequencing process was made in an ABI PRISM 3100 sequencer (Applied Biosystems, USA) using the primers g-back and OMPSEQ described previously (Andris-Widhopf et al., 2000).

Statistical analysis

All the experiments were reproduced at least 2 times. Data were expressed as mean ± standard deviations of the means (SD). Tukey's multiple comparison test was performed using GraphPad Prism v.5.0 for Windows. P-value less than 0.05 was considered significant.

RESULTS

Library construction

To achieve the construction of the library, two white leghorn chickens were immunized separately with 20 and 200 µg of whole-cell lysate of *H. pylori* strain N2. Chicken two which received 200 µg had a better response in comparison with chicken 1 (20 µg) as shown in Figure 1. As shown in Figure 1, chicken were immunized three times with 20 and 200 µg of lysed *H. pylori* in the pectoral area in the presence of CFA. Sera were tested in immunoassays experiments for the presence of anti-*H. pylori* antibodies. Chicken 2, which presented high levels of specific anti-*H. pylori* antibodies, was sacrificed and the splenocytes extracted for cDNA synthesis. The V genes were amplified from mRNA. A molecular weight of 400 pb and 350 pb were found in an agarose gel for V_H and V_L, respectively (Figure 2). The V_H and V_L were spliced with each other (Figure 2) and the obtained scFv gene repertories were cloned into pComb 3X phagemid to generate a phage-displayed scFv antibody library constituted of 2.4×10^4 transformants. PCR analysis of 24 randomly selected clones showed that all carried full length inserts (data not shown).

Selection of scFv antibodies by biopanning and monoclonal scFv screening

In order to identify specific monoclonal scFv antibodies against *H. pylori*, phagemid DNA from the eluted phages of the third round for J99 and fourth round for N2 were used to transform *E. coli* TOP10F cells. The transformed cells express the scFv antibody without fusion to the coat protein III of the phage. A total of 188 clones (94 for N2 and 94 for J99) were screened to evaluate their reactivity. For the N2 scFv library, 22% of the selected clones recognized the antigen in ELISA assays (Figure 3a), compared to 12% for the J99 scFv library (Figure 3b). As a consequence of the screening, 10 clones were selected, 5 from the N2 library (10GN2, E1N2, 9EN2, 11FN2, and 11AN2) and 5 from the J99 library (10HJ99, 11CJ99, 11DJ99, 10EJ99, and 6HJ99). Next, we decided to determine whether the selected scFv antibodies might be the same or were different. To this end, we employed restriction fragment length polymorphism procedure. We isolated plasmid DNA and amplified the scFv by PCR; the PCR products were digested with Bst N1 and separated in an agarose gel. The scFv antibody genes 6HJ99,

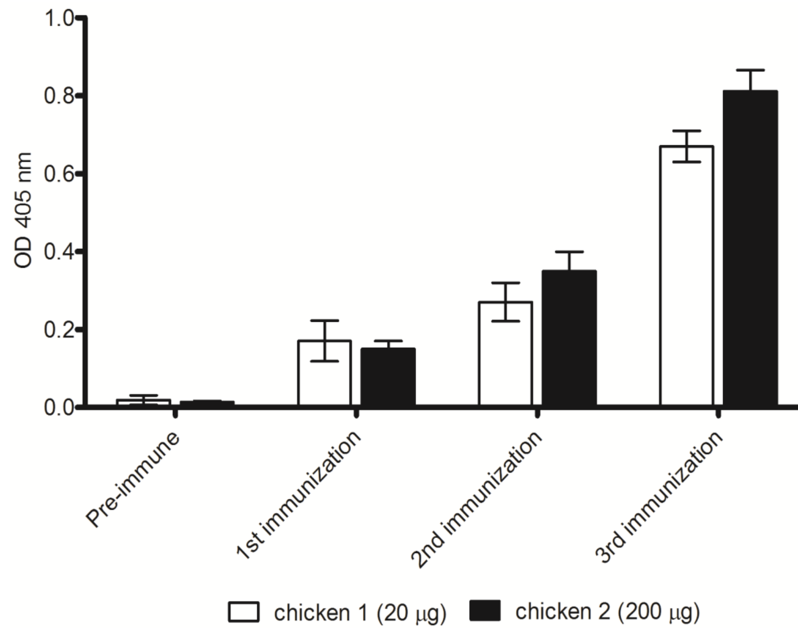

Figure 1. Immune response induced in chickens by immunization with whole-cell lysate of *Helicobacter pylori* N2 strain. Chicken were immunized three times with 20 and 200 µg of lysed *H. pylori* in the pectoral area in the presence of CFA. Sera were tested in immunoassays experiments for the presence of anti-*H. pylori* antibodies. Data shown are the mean ± SD of two independent experiments performed by triplicate.

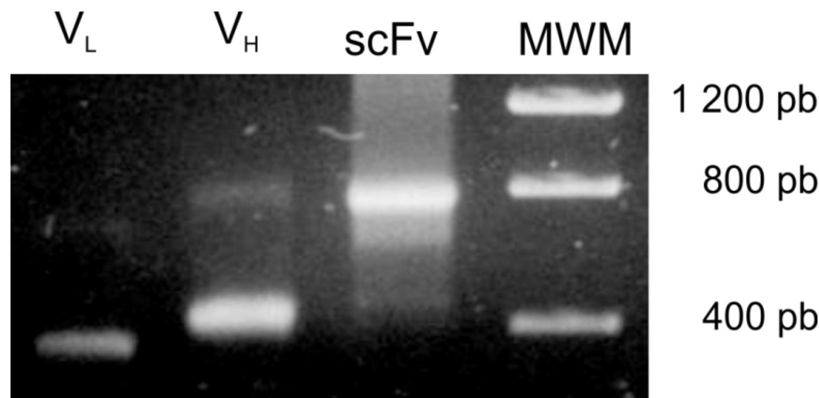

Figure 2. Amplification of the V genes repertories by PCR. V_L and V_H genes were amplified from the splenocytes of the chicken 2. V_H and V_L were spliced in order to obtain the scFv gene repertories. MWM, Low mass ladder.

11DJ99, 10EJ99 and E1N2 presented different restriction patterns; the other scFv genes presented the E1N2 restriction pattern (data not shown).

Characterization of the scFv antibody E1N2

We tested the capacity of the scFv antibody E1N2 to react against *H. pylori*. To this end, we expressed and purified the scFv antibody. The scFv E1N2 demonstrated a significant sensitivity on the detection of the bacteria with respect with the control ($P < 0.05$) (Figure 4a). Moreover, the recognition of *H. pylori* by the scFv antibody E1N2 in ELISA was higher in comparison with non-related bacteria strains ($P < 0.05$) (Figure 4b). Next, we purified the antibody fragment from culture supernatant and from cell-associated fraction; these extractions were separated by SDS-PAGE and transferred onto membranes for Western blot analysis. A great proportion of the antibody was found accumulated in the bacterial extract

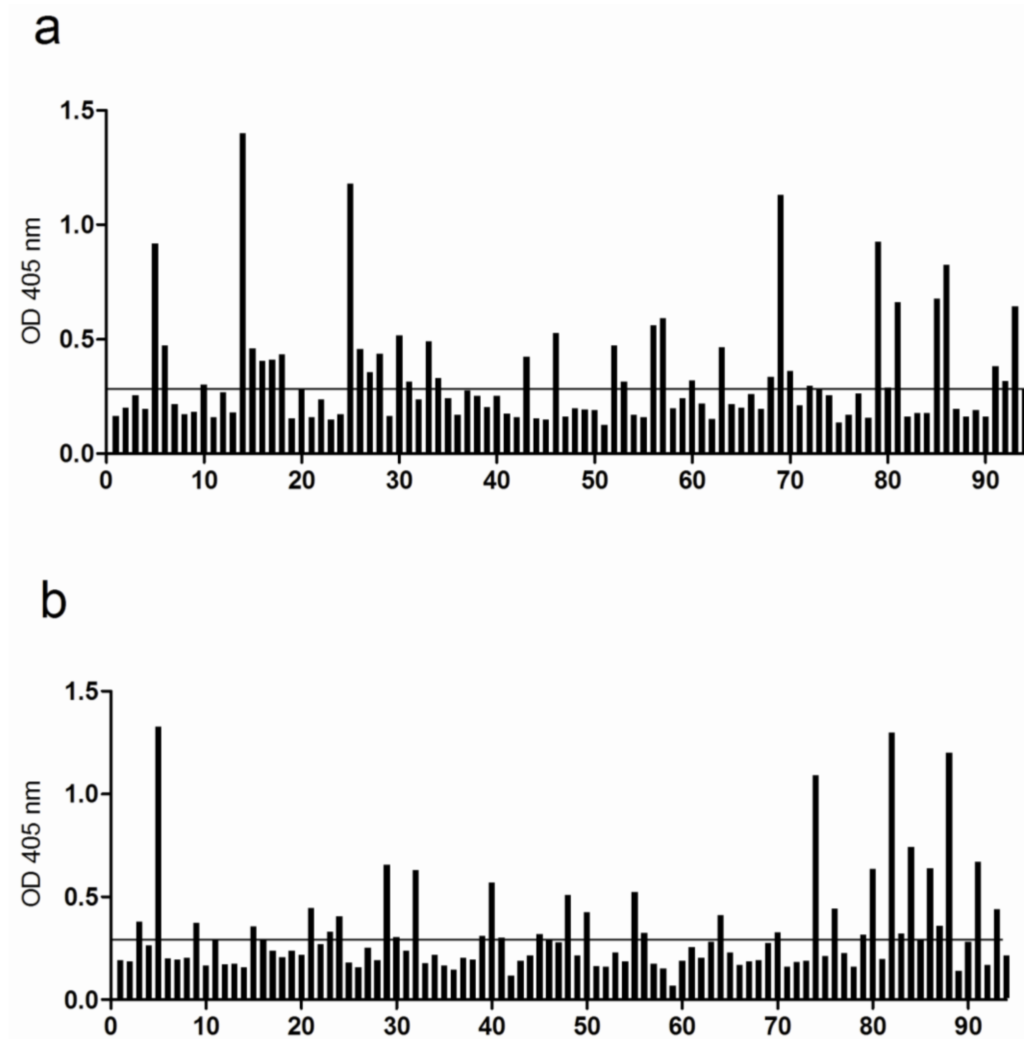

Figure 3. Isolated scFv clones from enriched phages after 3 and 4 rounds of selection against *H. pylori* J99 and N2 strain, respectively. Monoclonal scFvs were expressed without phage fusion and screened for binding activity in ELISA. a) 22% of the clones bound to the *H. pylori* strain N2 lysate and 12% bound to the *H. pylori* strain J99 lysate.

in the bacterial extract as a dimer with a MW of 46 kDa, whereas most of the monomer conformation was found with a MW of 26 kDa in the supernatant (Figure 5a). On the other hand, to determine the molecular weight of the antigen recognized by the scFv antibody E1N2, whole protein from *H. pylori* (J99 and N2) were separated by SDS-PAGE and transferred onto PVDF membranes. The scFv antibody E1N2 recognized an antigen with a MW of 27 kDa. Interestingly, the identified protein was shared by both strains as shown in Figure 5b. No reactivity was found with proteins from *E. coli*. The four clones selected by restriction patterns (6HJ99, 11DJ99, 10EJ99, and E1N2) were sequenced. They showed a high diversity in the complementary determining regions (Figure 6). The sequences presented a 93% of identity with the germline sequence of the chicken.

DISCUSSION

H. pylori is one of the major challenges in the field of public health; therefore, the appropriate diagnosis and treatment can diminish the risk of development of gastric ulcers, lymphoma malt and eventually cancer. With this in mind, we constructed a scFv library against *H. pylori* from which we isolated and characterized the scFv-E1N2 antibody. This antibody demonstrated a significant reactivity against *H. pylori* strains N2 and J99 (Figure 4a). Furthermore, the antibody fragment demonstrated a specific recognition of the bacteria (Figure 4b). We produced the scFv E1N2 antibody in *E. coli*, and found it as a dimer in sonicated bacteria (Figure 5a). On the other hand, protein purification from the supernatant showed that the majority of the antibody could be found as a

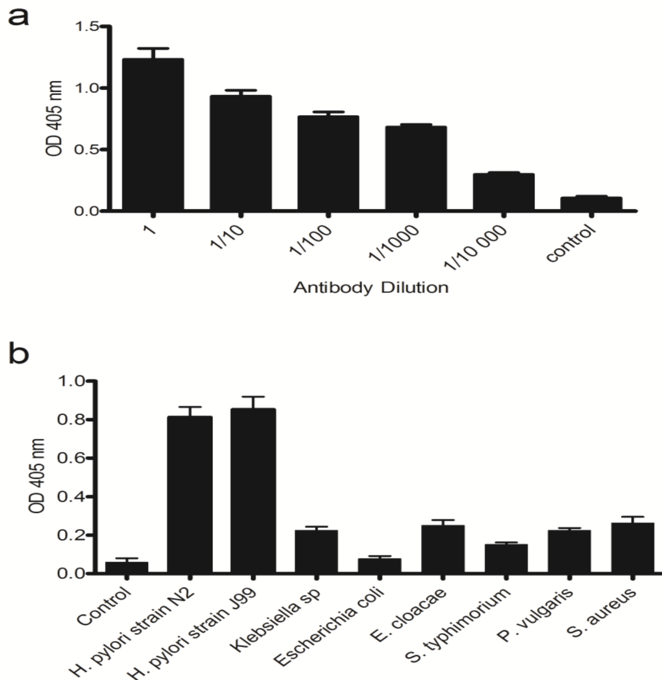

Figure 4. Reactivity of the E1N2 scFv antibody. (a) E1N2 antibody fragment was serially diluted from a stock with a concentration of 10 µg/ml, the dilutions were added in a previously coated 96-well plate with *H. pylori* N2 strain; after washing, goat anti-HA HRP was added. (b) For testing specificity, a 96-well plate was coated with sonicated *H. pylori* strain N2, J99 and other non-related bacteria. ScFv-E1N2 was added to each well; after washing goat anti-HA HRP was added. ABTS single solution was added and the OD reading at 405 nm was registered. As a control, a non-related antibody fragment that binds a hapten was used. Data shown are the mean ± SD of three independent experiments by triplicate.

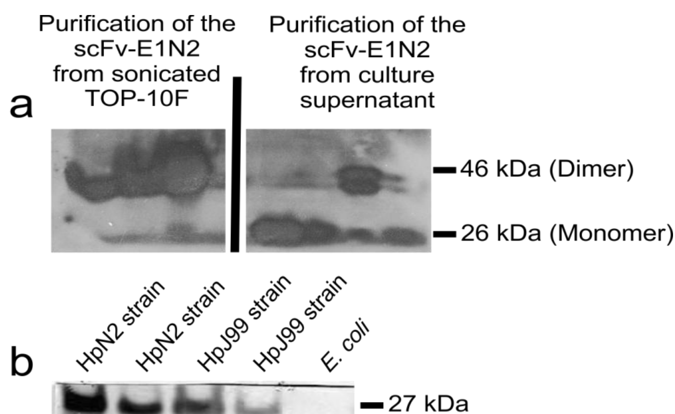

Figure 5. SDS-Page and Inmunoblotting analysis of purified scFv-E1N2 antibody and sonicated extracts of *H. pylori*. a) scFv-E1N2 antibody was recovered and purified from sonicated TOP-10F and culture supernatant by Ni-NTA agarose system. The antibody was separated and transferred; after washing, the membrane was incubated with anti-HA HRP. b) Sonicated *H. pylori* N2 and J99 strains were separated and transferred. Membrane was incubated with Ni-NTA purified E1N2 antibody; after washing, anti-HA HRP antibody was added. All the membranes were revealed with the ECL western blotting detection. *Escherichia coli* was used as a control.

monomer (Figure 5a). This conformation taken by the antibody fragment is because of the length of the linker that joins V_L and V_H chains; in this study the linker used was composed of 7 aminoacids (GQSSRSS) (Andris-Widhopf et al., 2000). Previous reports have shown that shortening of the linker (1 to 7 aminoacids) decrement stability of monomers, increasing the capacity of the scFv to form dimers, trimers, tetramers, and higher molecular mass multimers (Dolezal et al., 2000). Moreover, reduction of the linker could be a strategy to increment the avidity and stability of the antibody fragment; however, these types of modification do not increment affinity for the target (Cai-Qun et al., 2010; Dolezal et al., 2000).

H. pylori employ urease protein for living in a highly acidic gastric milieu (Sachs et al, 2006). The native urease of *H. pylori* is a hexameric molecule with a molecular mass of approximately 540 kDa. This enzyme is composed of two subunits, one of them called UreB with a molecular mass of 60 kDa and a small one called UreA with a molecular mass of 26.5 kDa (Olivera-Severo, 2006;Berdoz and Corthesy, 2004). Species identified to date, produce large amounts of urease, which may account for 15% of the bacterial protein composition and is highly immunogenic (Czinn and Blanchard, 2011). The scFv-E1N2 antibody had reactivity against an antigen of approximately 27 kDa in both strains of *H. pylori* (Figure 5b). Because of this, we may speculate that the antigen recognized by the scFv-E1N2 antibody is related with the subunit A of ureasa protein; however, more experiments are needed to characterize the antigen.

Because this pathogen is able to acquire resistance to pharmacological treatment (Benhar, 2001;Weeks et al., 2011), there is a special interest in the development of immunotherapies, as alternatives for the treatment of infectious microorganisms as viruses and bacteria. In previous reports, phage displayed scFv antibodies recognized a 60 kDa protein which was related with the urease protein B (UreB; 60 kDa) (Houimel et al., 2001; Reiche et al., 2002). On the other hand, Cao J and co-workers found a scFv antibody that recognized an antigen not identified with a molecular weight of 30 kDa. Interestingly, an *in vivo* experiment with these recombinant phages exerts a bactericidal effect and inhibited bacterial growth in six different strains. Moreover, previous treatment of the bacteria with these phages reduced the colonization of the bacteria in mouse stomachs (Cao et al., 2000). Even though the main mechanism by which there was an inhibition of the bacteria was not identified in that work, other reports have shown that is possible to reduce the capacity of replication by antibody neutralization of the proteins urease B (60 kDa) and heat-shock protein 60 (Li et al., 2010;Yamaguchi et al., 1997). It would be interesting to determine if the scFv-E1N2 antibody can be able to diminish the capacity of colonization of the bacteria in mouse stomachs. Because *H. pylori* colonizes under the mucus layer of the gastric mucosa, it is well protected against conventional antibodies (Cao et al., 2000).

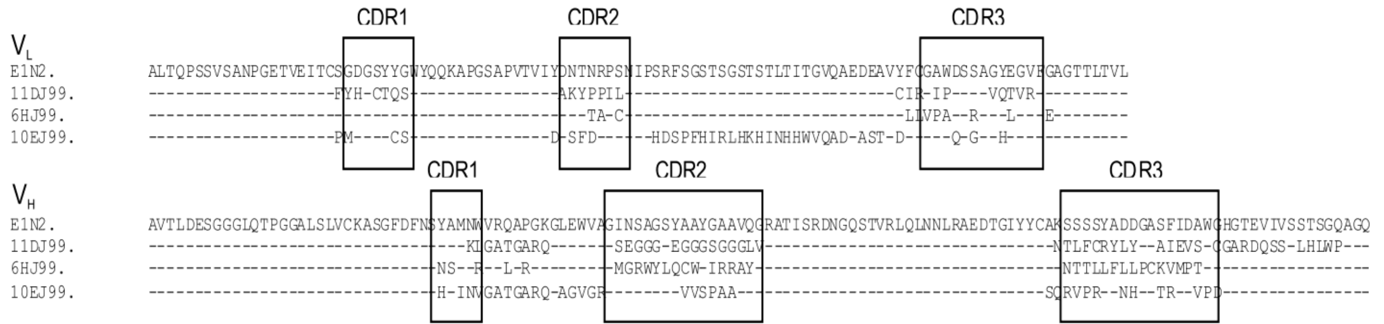

Figure 6. Sequence comparison of the four binders scFv antibody fragments. Clones E1N2, 11DJ99, 6HJ99, and 10EJ99 that showed different pattern of restriction with the enzyme *Bst*N I, were sequenced. The figure shows the V genes (V_L and V_H) and complementary determining regions (CDRs). Dashes denote sequence identity.

Nystrom J and coworkers demonstrated that albeit high levels of *H. pylori* specific serum antibody titers, there is not a significant reduction of the colonization until the development of a Th1 profile simultaneously with an IgA specific antibody response (Nystrom and Svennerholm, 2007). This and other reports suggest the importance of IgA antibody for blocking bacterial adhesion to the mucus layer (Berdoz and Corthesy, 2004). For this reason, it might be possible to convert the scFv-E1N2 into a polymeric secretory IgA as it has been reported previously (Berdoz and Corthesy, 2004). This modification might give the antibody more stability into the gastric environment.

Currently, the detection of *H. pylori* antigens in feces is a valid method to identify whether a person is infected or not. Some diagnostic tests as ImmunoCard STAT, HpSA and Diagnostec *H. pylori* antigen EIA Kit has a sensibility and specificity comparable with other invasive and non-invasive methods (Calvet et al., 2010). The scFv-E1N2 or another scFv antibody selected from the library might be used for the development of a low cost immunodiagnostic with the primary approach to detect in challenging conditions as human feces and biopsies. Anti-*H. pylori* scFvs might be also being used for the identification of potential proteins, epitopes or mimotopes to being incurporated in future vaccines as it was reported previously (Gevorkian et al., 2004; Manoutcharian et al., 2004). Because this scFv library was constructed from immunized chickens with whole lysate of *H. pylori*, it might be possible to find scFv antibodies against many *H. pylori*-related proteins. In conclusion, these scFv antibodies might be used for the progressive development of complementary immunotherapies and immunodiagnostics, in addition to its use for the identification of additional *H. pylori*-related antigens.

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