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Immune Responses of Conserved 32 KD fragment from N-terminal of *H. pylori* cagA gene

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H. pylori **is gram-negative, microaerophilic and motile bacterium that has been associated with gastritis, peptic ulcer and adenocarcinoma. Aim of this research was study of conserved rCagA 32 KD fragment immunogenicity characterizations from amino end of cagA gene.** ***CagA*** **gene was amplified by PCR and target 841 bp fragment was cloned into proper vectors and was approved with enzymatic digestion and PCR.**

We showed that 32 KDa our designed recombinant CagA proteins from amino ends of *H. pylori* was immunogenic and enable to induce proper immune Th1 responses in mice. The results showed that designed rCagA had the same antigenicity as native CagA and also unlike whole cagA is completely conserved.

We recommended this conserved and immunogenic fragment of CagA for developing immune-detecting method for CagA+HP and proper candidate for formulation of multi-component vaccine.

Key words: *Helicobacter pylori*, Cloning, 32 KD rcagA.

H. pylori is a gram-negative, microaerophilic spiral bacterium[1]. It plays a central role in the development of gastritis and chronic gastric cancer. Despite the immune response that induced by *H. pylori* but infected hosts are unable to clear the infection[1]. The cytotoxin-associated gene A (cagA) is part of the cag pathogeneicity island (cagPAI), which is found in disease-causing strains of *H. pylori* [2]. Once *H. pylori* cagA injected into gastric epithelial cells, it enable alter gene expression, cytokines release, host cells structure and cycle[3,4]. CagA protein exists in many different forms and the main forms are East-Asian and Western CagA[5]. Both forms of cagA will be included in immunization formulation. Inactive CagA enable to induce antibodies. The *H .pylori* CagA in immunization formulation would result in decreased *H. pylori*-related gastric carcinogenesis and chronic gastritis. About 60 to 80% of *H. pylori*

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strains express CagA protein with molecular mass of 120 to 128-kDa[7,6].

In this study we cloned an 841 bp fragment of amino end of cagA gene. This 32 KD recombinant protein of *H. pylori* may be of value for diagnostic, therapeutic and prophylactic (vaccine development) purposes[8]. Research data showed our 32 KD rCagA was immunogenic. In the present study, we report proper yield expression of recombinant CagA. We also report the recognition of recombinant CagA by hyper immune sera raised in animals immunized with this protein.

MATERIAL AND METHODS

Bacterial culture

H. pylori ATCC 700392 were grown on brucella agar or Brucella broth (Merck, Germany) with 5% frozen-melting sheep blood, 10% fetal calf serum (Gipco), and Skirrow's antibiotic supplement (MAST, U.K) in a microaerophilic atmosphere for 5 to 10 days at 37°C. *H. pylori* morphology was determined by light microscopy after Gram staining and cultures were approved with catalase, oxidase, rapid urease tests (Chem Enzyme) then preserved and stored at -20°C.

Extraction of genomic DNA

H. pylori ATCC 700392 were added to a 1.5 ml micro centrifuge tube, washed once with phosphate-buffered saline (pH 7.2), and pellted by centrifugation at 8 000 *g*. Genomic DNA was extracted by DNA Extraction Kit (Bioneer, Korea), the DNA pellet was suspended in buffer TE (10 mmol/L Tris-HCL, 1 mmol/L EDTA pH: 8.0) and stored at -20°C .

Synthetic primers

Single primer pair was used to amplify *H. pylori* cagA gene target fragment based on Gene Bank. The primers had a *Bam* HI site incorporated into the 5/ end and a *Sac* I site at the 3/ end and their sequences as follows:

F: 5/- *aa**ggatcc**actaacgaaaccattgacca*-3 and R: 5/-aa**gagctc**actccctcaactctaacatt-3/ that enable amplify fragment to length of 841bp. Primers in early 841 bp of cagA fragment were candidated and designed with use of DNA star program, consider conserved fragment, epitopes with induce Th1 and Th2 immune responses.This region be absent EPIYA motif. Also a single primer pair was used to amplify *H. pylori* ureC gene based on Gene Bank to approve of *H. pylori* genus.

F: 5′-CCCTCACGCCATCAGTCC CAAAAA-3′
R: 5′-AAGAAGTCAAAAAC GCCCCAAAAC-3′ that enable amplify fragment to length of 417bp. PCR was performed in a standard enzyme expand high fidelity PCR system (Roche company, Germany) concentration 2.6 U/50ul reaction, Mg^{2+} 1.5mM, dNTP 200 uM each primer 10uM , template DNA 50 ng. The mixtures were incubated for 2 min at 94°C for primary denaturation, 15 sec at 94°C for secondary denaturation of the target DNA and then, annealing at 53°C for 1 min, and extension at 72°C for 1 min that 35 cycle was performed. The amplified products were analyzed by electrophoresis on 1% agarose gel (Sigma LTD) containing 0.1 g of ethidium bromide per ml in TAE buffer. The PCR products was visualized under UV light and photographed.

Cloning and construction of recombinant plamids

The PCR product was purified by Bioneer PCR Fragment Recovery Kit. The purified products was ligated into thecompatible sites of the T-vector p^{GEM-T} (fermentas) by using T4 DNA ligase at a molar ratio of 5:1 at 4°C for 12 h. After the above product was transformed into *E. coli* DH5± (Novagen), p^{GEM-T}/cagA was selected and identified by PCR and restriction enzyme digestion.

Extraction of recombinant plasmid

The single bacterial colony (*E. coli* DH5 ± /p^{GEM-T}/cagA) was picked, and cultivated in 5ml LB broth containing 100 mg/L of ampicillin, at 37°C overnight, then recombinant plasmids were extracted according to manufacturer's instructions (Bioneer DNA Purification Kit), identified by PCR and restriction endonuclease digestion (Roche, Germany).

Isolation of cagA from T-vector and sub cloning cagA fragment in pET-28a vector

Plasmids contain cagA fragment was purified according to manufacturer's instructions (Bioneer DNA Purification Kit), and then restriction enzyme digestion was performed and ligated with pET-28a (Novagen) at a molar ratio of 6:1 at 4°C for 12h.

After the above product was transformed into *E. coli* DH5± and PET-28a/cagA was selected and identified by PCR and restriction enzyme digestion.

Cloning and construction of expression plasmid

Plasmids contain cagA fragment was purified according to manufacturer's instructions (Bioneer DNA Purification Kit), after enzyme digestion, was transformed into competence *E. coli* BL21 and transformants pET-28a/cagA were selected on LB agar plates containing 30ug/ml kanamycin[9] and identified by PCR and restriction enzyme digestion. *E. coli* cells harboring expression vector pET-28a/cagA were grown in LB medium supplemented with kanamycin (30 ug/ml) and chloramphenicol (34 ug/ml) at 37°C to an OD600= 0.6 - 0.8 for induction, IPTG (Sigma, USA) was added to a final concentration of 1 mM and the culture was grown at 37°C for 2h. The bacterial cells were harvested and suspended in lysis buffer (NaCl 0.5 M, PMSF 1 mM, EDTA 10 mM,1%(v/v), Triton X-100, 20mM Tris-HCl, pH: 7.5) then was freeze and thawed and sonicated on ice in presence of PMSF 1mM (Sigma). Recombinant CagA proteins were centrifuged in 14000 × g for 20 min and were collected in supernatant phase.

Production of Polyclonal antibodies in rabbit

Female New Zealand rabbits (purchased from Razi vaccine & serum research institute, Iran) were challenged with 1×108 CFU/ml *H. pylori ss1* and after of three months, rabbits exsanguinated by cardiac puncture under anesthesia. Rabbit serums were collected and stored at -70°C until of using. All of working with animal model was carried out accordance of Institutional and national ethical guidelines.

Immunizations and experimental study

Three groups of mice (*N*=5/group) were immunized three times intramuscularly (IM) at 10 day intervals. IM immunizations were performed with 10ug rCagA (Group 1) or 10ug rCagA and 10 ug CPG oligonucleotides (Group 2). IM immunizations were performed into the right thigh. Control animals (Group 3) received PBS using the same volume, route and schedule (Table 1). Serums were collected 7 days after IM immunizations. Serum IgG1, and IgG2± antibodies specific to *H. pylori rCagA* were measured by ELISA.

Lymphocyte proliferation test

The mice were immunized five times with 10 µg of rcagA in 100µl sterile PBS with 10 days interval, three times by oral administration and twice by I.M. immunization. Six weeks after last immunization, spleens were removed and suspended in cold PBS (4°C) under sterile condition from immunized and non-immunized mice; RBCs were lysed using NH_4Cl buffer. Cell suspension was prepared in complete RPMI 1640 (Gibco) and adjusted to 2.5 ×10 6 cells per milliliters. 100µl of cell suspension was added to each well of flat bottom 96 well plates and rcagA at concentration of 1µg/ml was added to each one and as negative controls some wells were not added antigen. Volume of all wells adjusted to 200 µl and as positive we used PHA at final concentration of 5µg/ml. After incubation for 72 h at 37°C in 5% CO_2 humid incubator, cell proliferation was measured by using 3[4, 5-dimethyl thiazol-2-ll]-2,5-diphenyl tetrazolium bromide; thiazolyl-blue (MTT) dye assay. Briefly, 20 µl MTT was added to each well and plates were further incubated at 37 °C for 4 h. Following incubation, the plates were centrifuged at 300 *g* for 10 min and then supernatant was aspirated carefully and formazan crystals were solubilized by adding 100 µl dimethyl sulfoxide into each well. The absorbance of each well was then determined at a wavelength of 540nm. Stimulation Index (SI) was calculated according to formula: SI= OD of the wells stimulated with antigen / OD of the wells containing only the cells without antigen stimulation.

Statistical analysis

Comparison of responses between groups of mice was done with t-student test. Two-way analysis of variance and LSD, using SPSS V13 statistical software, compared the levels of proliferative responses to antigen. Probability values below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

PCR amplification of *H. pylori* cagA fragment

H. pylori with cagA fragment was amplified by PCR from the above primers and the PCR products was electrophoresed and visualized by 1% agarose gel (Fig. 1). It revealed that the size of cagA DNA fragment amplified by

PCR was 841 bp and UreC fragment was 417bp (Fig. 1).

Identification of recombinant vector by PCR

The recombinant plasmid was extracted from recombinant bacteria and conducted as template to amplify by PCR under the conditions of mentioned above. The PCR products were visualized by 1% agarose gel electrophoresis (Fig. 2).

It indicated that recombinant plasmid contained the objective gene. At the same time, it was successful in transforming recombinant plasmid into *E. coli* DH5±.

Purification of recombinant CagA protein

Recombinant CagA expressed *by E. coli* BL-21(DE3) pLysS was purified by nickel resins (Invitrogen, USA). Recombinant CagA was detected by western blot with polyvinyliden diflouride (PVDF) membrane (Hi-bond Amersham Bioscience USA). Recombinant CagA eluted under the same conditions as those observed previously for the native enzyme, suggesting that the recombinant CagA was indeed assembled in *E. coli* BL-21(DE3) pLysS. Protein samples (10ug) from each purification step were electrophoresed on SDS-polyacrylamide gel and stained with Coomassie blue (Fig. 3). SDS-PAGE analysis was done for purified *H. pylori* protein with separating gel contained 12% acrylamide.

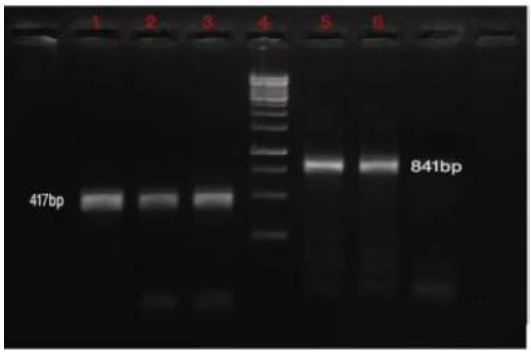


Fig. 1. 1% agarose gel electrophoresis of UreC and CagADNA fragment amplified by PCR from *H. pylori SS1*.Lanes 1, 2, 3: *H. pylori amplified Urec.* Lane 4: 1 Kb Marker. Lanes 5, 6: *H. pylori SS1* amplified CagA

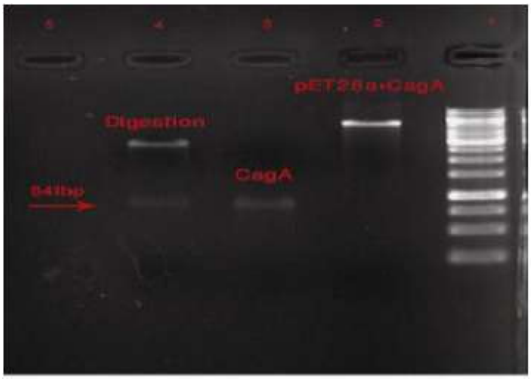
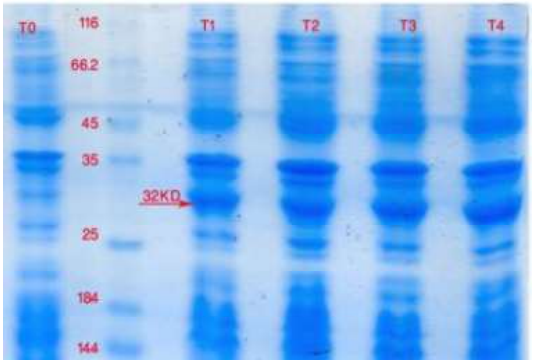


Fig. 2. The identification of the pET-28a/cagA by PCR and digestion with restriction endonucleases. Lane1: 1Kb marker, Lane 2: pET28a /cagA, lane 3: amplification cagA of recombinant plasmid, Lane4: digestion with restriction enzyme by Bam HI and *Sac I.*



Lane T1, T2, T3 and T4: induced bacterial pellet with IPTG, Lane T0: non- induced bacterial pellet.

Fig. 3. Detection of expressed and purified recombinant CagA on SDS-PAGE (12% w/v), stained with coomassie blue G-250

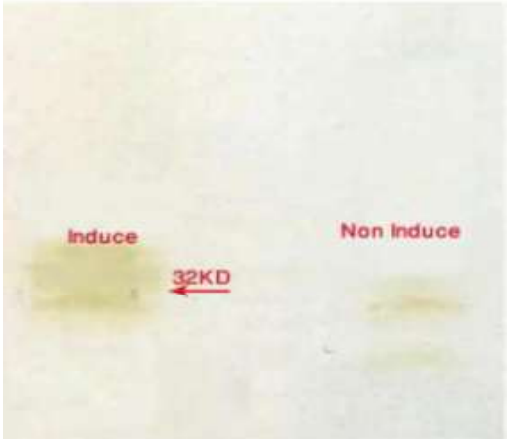


Fig. 4. Analysis of western blots of rCagA protein with IgG *H. pylori* human anti- cagA

Western-blot analysis

Western-blot was performed according to Bumatte[10]. Following protein separation by SDS-PAGE, those were transferred to nitrocellulose paper by electroblotting for 12 hours at 20V. At room temperature after non specific binding was blocked with twine 20, the nitrocellulose papers were incubated for 2 hours with 1:5000 dilutions of anti rabbit poly clonal sera. After being washed and incubated with goat anti-IgG rabbit (heavy and light chain) horseradish peroxidase (HRP) conjugate antibody (Bio-Rad) at 1:2000 dilutions, the nitrocellulose papers were stained with diamino-benzidine (Sigma, USA) (Figs. 6,7,8).

Immunization and proliferation test

The IgG1/IgG2a ratio in the mice immunized with rCagA and rCagA plus CpG was <1, while the ratio of control group was >1 (Table 1). Mice were immunized 3 times orally and 2 times intramuscularly. Ten days after last immunization, mice of each group were scarified and spleen cells were stimulated 72h in vitro with rCagA and proliferation were detected with MTT assay as described in materials and methods. Data presented as mean ± SEM of 5 mice per groups (Fig. 7).

H. pylori express surface protein CagA with a MW of 120-128 KD. A dominant characteristic of this protein is high

Fig. 5. Analysis of Western blot with *H. pylori rabbit* polyclonal antibody

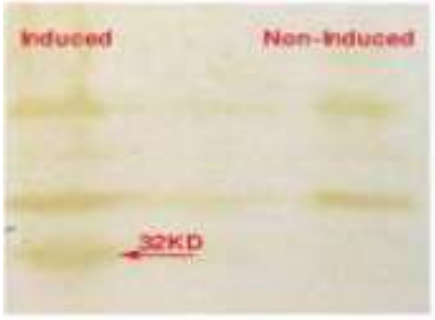


Fig. 6. Western blot with rabbit non purified *H. pylori* polyclonal antibody

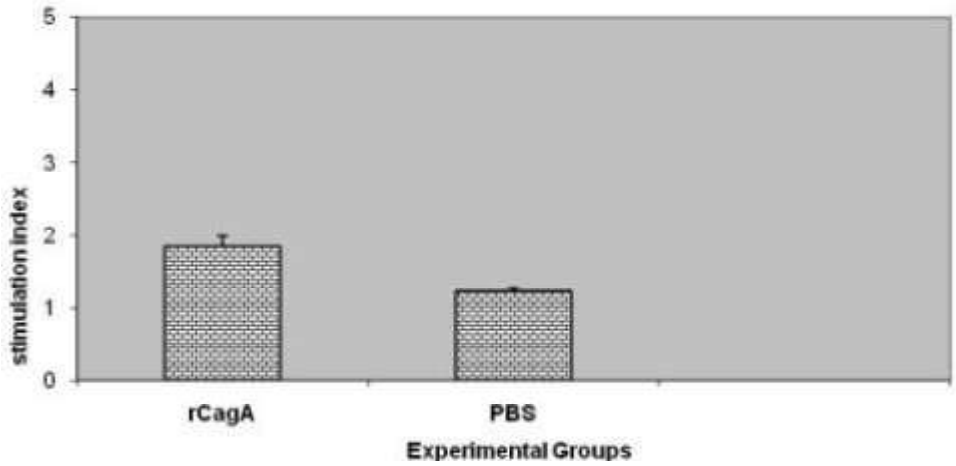


Fig. 7. Lymphocyte proliferation test following stimulation with rCagA , PBS. Each bar represents the mean ±SE from 3-4 animals. Statistically difference between control and treated animals were assayed with student t- test.

immunogenicity and antigenicity[11]. Numerous rCagA were expressed at its carbonic end and the antigenicity of its amino end is necessary for study. The repeat units and variable regions in C-terminal end affect the host immune response. The 5/ end of cagA had no usable restriction endonuclease sites and also absent of EPIYA motif and contain sequence with properties of conserved region[10]. In this study we designed the 841bp fragment with DNA star program and used PCR to amplify the 5/end of cagA and cloned into p^{GEM-T} and pET28a vectors. Subsequently the *E. coli* DH5± and BL-21(DE3) pLysS were transformed by pET28a/cagA, and a 32KD recombinant CagA protein was induced by heat when temperature reached 37°C. The new protein

Table 1. Experimental groups of immunized mice and immune responses

Group	Immunization	IgG1/IgG2α
1	rCagA	0.98±0.5
2	rCagA+CpG	0.73±0.2
3	Control	1.37±0.4

Mean (±SEM) IgG1 and IgG2± rCagA specific serum titer ratios measured by ELISA

was combined with human *H. pylori* IgG anti-cagA, indicating that designed recombinant protein had the antigenicity of cagA. This designed fragment is conserved in all *H. pylori* strains and will enable induced Th1 and IgG responses that essential for infection clearance[12]. The results showed that designed rCagA had the same antigenicity as native CagA. Several studies have shown that anti-CagA antibodies can be detected in patients infected with a *cagA* positive *H. pylori* strain[9]. The presence of anti-CagA antibodies correlates with the presence of many cag pathogeneicity island encoded virulence factors, and patients infected with a *cagA* positive strain were shown to be more prone for the development of clinically significant *H. pylori*-related disease. A significant association between the presence of *cagA* and PUD and gastric cancer has been reported[11, 9, 13-15]. Approximately 60-80% of *H. pylori* isolates in the world possess the cytotoxin-associated gene A (cagA). As a result screening for anti-CagA antibodies might be useful to identify patients with an increased risk for development of pathology, thus allowing selective and/or differential treatment regimes[16]. The relation between the presence of *cagA* and the clinical outcome of *H. pylori* infection is primarily based on studies using the detection of anti-CagA antibodies to determine the *cagA* status[9,17-21]. The balance between Th1 / Th2 responses is essential for *H. pylori* clearance. Th2 responses are needed for sufficient antibody production. However, Th1 responses are seen to occur more often in natural cases of *H. pylori* infections[12]. Antibodies against each antigen will allow for neutralization and opsonization of the pathogen. IgG will also play a role in immunity, especially if the pathogen is not neutralized or opsonized before it is able to breach the epithelial lining of the gastrointestinal tract that will allow neutralization of the CagA toxin. Usually, antigens are selected as potential imminization candidates because they are surface exposed, thus easily was attacked by the immune response; because they are abundant and, supposedly more immunogenic; because they are well conserved in all microbial strains, thus suitable for wide protection; and because they represent key virulence factors important in the pathogenesis of the infection.The *H. pylori* antigens selected as potential immunization candidates meet one or more of these criteria. In this study the IgG1/IgG2a ratio in the mice immunized with rCagA and rCagA plus CpG was <1, indicating a Th1 type response, while the ratio of control group was >1, indicating a strong Th2 response. These data suggest that immunization with rCagA promoted a Th1 immune response that is essential for infection clearance. Analysis of lymphocyte proliferation showed that rCagA increase lymphocyte proliferation compared to control group (Fig. 7).

In conclusion, we recommended designed 32 KD rCagA as a conserved and immunogenic antigen for combination of multi-component formulation of *H. pylori* vaccine.

Conflict of interest

This work was supported by Vice-Chancelor Research from Tarbiat Modares University for the Promotion of Sciences and we did not any relationship with other people or organizations that could bias this study.

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