

EXPERIMENTAL WORKS

Chitosan Nanoparticles-Mediated pCDNA3.1(–)-*hcpD* DNA Vaccine against *Helicobacter pylori* in BALB/c Mice

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Received May 18, 2018; revised May 26, 2018; accepted January 26, 2019

Abstract—The gastric cancer is one of the most common carcinomas and the second cancer-related death in the world. The risk factors for this cancer include genetic factors and environmental factors such as *Helicobacter pylori* infection. The protein HcpD (HP0160) of *H. pylori* is a member of the cysteine-rich protein family that interacts with host immune systems. One of the modern approaches to stimulate the humoral and cellular immune systems against diseases is utilization of DNA vaccines. Using qPCR method, this study aimed to evaluate the expression level of cytokines genes including *IL17*, *IL4*, and *interferon gamma* (*IFNγ*) in BALB/c mice vaccinated with pCDNA3.1(–)-*hcpD* DNA vaccine against *H. pylori*. In this study, pCDNA3.1(–)-*hcpD* recombinant vector was prepared and transformed into *E. coli* to obtain a large number of plasmids. After plasmid purification and confirmation of the transformation by digestion and PCR, the chitosan nanoparticles were synthesized using ionic gelation method. The injectable solutions containing pCDNA3.1(–)-*hcpD*, pCDNA3.1(–)-*hcpD* + nanoparticles or pCDNA3.1 (empty vector as control group) were injected into BALB/c mice, separately. Then, the blood and tissues samples from each animal were collected and the expression levels of cytokine genes were examined by a qRT-PCR method. The *IL-4* expression level was significantly decreased in pCDNA3.1(–)-*hcpD* + nanoparticle and pCDNA3.1(–)-*hcpD* groups ($p < 0.001$). Conversely, the expression level of *IFNγ* gene in both groups was increased significantly ($p < 0.001$). The expression level of *IL17* gene showed no significant difference between DNA vaccine containing nanoparticle compare with pCDNA3.1(–)-*hcpD* ($p > 0.05$). During 15, 30 and 45 post-injection days, the expression level of *hcpD* decreased in hip tissue of mice vaccinated with pCDNA3.1(–)-*hcpD* and pCDNA3.1(–)-*hcpD* + nanoparticle although no significant difference found between the vaccinated groups ($p > 0.05$). pCDNA3.1(–)-*hcpD* vaccine can stimulate the immune system in vaccinated mice either as the sole agent or combined with chitosan nanoparticles. Therefore this method can be an effective way for immunization against *H. pylori* infection.

Keywords: *Helicobacter pylori*, cytokine genes, DNA vaccine, *hcpD*, BALB/c mice

DOI: 10.3103/S0891416819020083

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, microaerophilic bacterium known as an inducer of peptic ulcers, chronic inflammation, gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT), and gastric atrophy [1, 2]. In more than 50% of the adults *H. pylori* infection may be acquired at an earlier age [3, 4]. Depending on the level of health, the epidemiology of *H. pylori* infection varies in different populations as this pathogen may infect up to 90% of the population in developing countries [3, 5]. Urease enzyme helps this dominant human pathogen to persist for a long time in the stomach and its motility help it to enter to the gastric epithelium [6].

H. pylori secrete several protein and toxins that cause tissue damage in the host. In addition to the

motility features which play important roles in its pathogenicity, the virulence factors of this bacterium include the cag Pathogenicity Island (cag PAI), vacuolating cytotoxin (Vac), and urease [7, 8]. The genome of *H. pylori* encodes a family of eight or nine secreted proteins known as eukaryotic Scl1 regulatory proteins containing a large number of disulfide bridges [9]. For instance, *Helicobacter* and *Campylobacter* genera express *Helicobacter* cysteine-rich proteins (Hcp) [10, 11] from HcpD interacts with host immune systems and play a role in *H. pylori* pathogenicity [12, 13].

Seven days antibiotic treatment is usually suggested for patients who are positive for *H. pylori* infection. This treatment is typically a combination of anti-acid agents (proton-like pump inhibitors group such as omeprazole, lansoprazole, pantoprazole or H₂ inhibi-

tors including ranitidine, cimetidine, roxatidine, and famotidine) with one or two antibiotics (clarithromycin (LAC), amoxicillin or metronidazole) [14, 15].

During recent decades, vaccination is the most effective strategy for controlling the infectious diseases. In order to achieve effective vaccine against *H. pylori* a number of efforts have been made to use the entire cell (dead, live, and attenuated vaccines), subunit vaccines or recombinant strains (oral or injectable) as vaccines [13, 16]. Although the production of recombinant proteins are more affordable comparing to natural proteins, the production and purification of the recombinant antibodies are still costly [17].

In recent years, DNA immunization has attracted considerable attention as a promising approach for stimulation of both humoral and cellular immune responses against bacterial pathogens [18]. Typically, a DNA vaccine is a genetically engineered DNA with several advantages compared to traditional vaccines [19, 20]. Eliminating the risk of using actual infectious organisms, DNA vaccines stimulate both the antibody and cell-mediated components of the immune system. Furthermore, they are highly specific, stable at room temperature, inexpensive and easy to prepare [19, 21].

Considering the lack of appropriate and affordable therapeutic method for controlling *H. pylori* infections especially in developing countries and the fact that the expression of *hcpD* gene of *H. pylori* leads to stimulate the immune system of the mammalian hosts, establishing a method to produce an efficient vaccine against *H. pylori* seems to be necessary. The present work aimed to utilize pCDNA3.1(-)-*hcpD* expression construct as a DNA vaccine (either alone or in combination to chitosan nanoparticles) and evaluate the immunization efficiency against *H. pylori* in BALB/c mice via qPCR analyses of cytokine genes expression levels.

MATERIALS AND METHODS

Vector Preparation and Bacterial Culture

The pCDNA3.1(-)-*hcpD* that obtained from the previous study of Eslami and Doosti, 2017 were used as a recombinant DNA vaccine in this study [13]. Also, the pCDNA3.1(-) (Invitrogen, San Diego, CA) with 5428 bp length was used as control plasmid (without gene) for injection into BALB/c mice. The lyophilized stock of *E. coli* strain Top10F' was purchased from Pasteur Institute of Iran and was cultured in 5 mL of Luria-Bertani (LB) broth and then incubated for overnight at 37°C with shaking and were used for transformation and plasmid preparation.

Transformation of pCDNA3.1(-)-hcpD Recombinant Vector

For the preparation of enough pCDNA3.1(-)-*hcpD* for animal injection, the recombinant vector was

transformed into *E. coli* Top10F' using CaCl₂ and heat shock (42°C for 90 s) treatment. Colony-PCR was done on each colony for identification of inserted recombinant plasmid into *E. coli*. After culture of the selected colony on LB broth containing 100 µg/mL of ampicillin antibiotic for overnight at 37°C, the plasmid purification was done using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Freiburg, Germany) according to the recommendations of the manufacturer. The quality and quantity of the extracted plasmid were determined from the ratio of the absorbance at a wavelength of 260/280 nm using Thermo Scientific™ NanoDrop 2000 (Wilmington, DE, USA) according to the method described by Sambrook and Russell, 2001 [22]. The sequencing, PCR, and digestion with *Bam*HI and *Eco*RV restriction enzymes were done on purified plasmids for confirmation of transformation. The digested products, pCDNA3.1(-)-*hcpD* recombinant plasmid and empty vector were electrophoresed at constant voltage (80 V) on 2% agarose gel electrophoresis and their molecular weight was approximately determined using 100 b DNA ladder. The gel was stained with 2 µg/mL of ethidium bromide and examined under UV light and a photograph was obtained under UVIDoc gel documentation systems (Uvitec, UK).

Amplification of hcpD Gene

The following specific oligonucleotide primer pairs with the accession number: FM991728 including *hcpD*-F: 5'-ATGATAAGAATTGGACTAAAAAG-3' *hcpD*-R: 5'-TTATTGCGTATCATCTTGCGTGTC-3' was obtained from Eslami and Doosti, 2017 study and was used for amplification of *hcpD* gene. The polymerase chain reaction (PCR) was performed in a final volume of 25 µL in 0.2 mL tubes containing 50 ng of purified plasmid, 1 µM of each forward and reverse primer, 2 mM MgCl₂, 200 mM dNTPs, 2.5 µL of 10× reaction buffer, and 1 unit of *Taq* DNA polymerase (all Thermo Fisher Scientific, Freiburg, Germany). The amplifications were carried out in a Thermal Cycler (Mastercycler Gradient, Eppendorf, Germany). The temperature profiles were included an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of a denaturation step at 94°C for 1 min, a primer-annealing step at 62°C for 1 min, extension step at 72°C for 1 min, and then a final step at 72°C for 5 min. The amplified products were detected on 2% agarose gel electrophoresis as mentioned above.

Synthesis of Chitosan Nanoparticles Using Ionic Gelation Method

100 mg of chitosan powder (Sigma-Aldrich, USA) was dissolved into 50 mL of the acetic acid (2 mg/mL), and then the solution was kept at room temperature for 24 h on a magnetic stirrer at 1000 rpm. The pH of the solution was adjusted to 5.5 using 0.5 M NaOH. The

entire solution was filtered through a 0.45 μm filter to filter unblemished chitosans. The sodium tri-phosphate (TPP) solution was prepared at a concentration of 0.7 mg/mL and passed through a 0.45 μm filter. 50 mL of acetic acid solution containing chitosan was placed on a magnetic stirrer at room temperature and 20 mL of TPP (produced in the previous step) was dropped very slowly (every 7 s, 1 drop) to the rotary solution on the stirrer.

This solution was kept on the magnetic stirrer for 1 h at 1000 rpm. The solution was centrifuged at 14000 rpm for 15 min and the supernatant containing chitosan nanoparticles was collected. The solution was dried and powdered using freeze-dried and the physico-chemical properties of chitosan nanoparticles were determined using Zeta Analyzer (Malvern Instruments, UK).

Preparation of Plasmid and Nanoparticles

Chitosan nanoparticles (1%) and plasmid solutions (2000 $\mu\text{g}/\text{mL}$ in PBS) were prepared separately and then they (nanoparticle + plasmid) were mixed equally and placed at 55°C for 1 h.

Mice Grouping and Injection Method

In the present study, the ethical approval was obtained from the Ethical Committee and Research Deputy of the Islamic Azad University of Shahrekord Branch, Iran on July 22, 2016. The 6 weeks old female BALB/c mice were classified into three groups (25 mice in each group) according to different injections. These groups included injected mice by chitosan nanoparticles recombinant plasmids (DNA vaccine + nanoparticles), recombinant plasmids (DNA vaccine), and the target-free plasmids (control group). 100 μL of each solution including DNA vaccine + nanoparticles (100 μg of DNA mixed with nanoparticles), DNA vaccine (1000 μg per mL of recombinant vector in PBS), and control group (1000 μg per mL of target-free plasmids) was injected into mice's hip muscles. The injections were carried out at intervals of 7 days during three weeks. 15, 30, and 45 days after the last injection, 7 mice from each group were killed and sampled. After anesthesia, at each sampling step, 1.5 mL of complete blood samples was obtained from the heart of the mice (the area under the jaw) and was transferred into anticoagulant (EDTA). Then, the mice's hips were removed and the tissues at the injection site were isolated.

Quantitative Real-Time PCR (q-PCR)

For RNA isolation a total of 100 mg of each tissue or 100 μL of blood samples (buffy coat) were subjected. RNA was extracted immediately from blood and tissue samples using TRIzol reagent (Invitrogen, USA), according to manufacturer's instructions. The quality

and quantity of isolated RNA were measured by the A260/280 ratio using NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). 1 μg of each RNA sample was used for reverse transcription with a cDNA synthesis kit (Takara, Kyoto) containing oligo (dT) and specific primers according to the manufacturer's protocol. The cDNA synthesis reaction was done at 85°C for 5 s, 42°C for 15 min, and finally the reverse transcriptase was inactivated for 5 min at 85°C.

To investigate the expression profiles of *IL-17*, *IL-4*, *IFN γ* cytokine genes and compared with *GAPDH* (as an internal control) and *hcpD* the specific oligonucleotide primers were designed using Gene Runner software version 3.05, Oligo Primer Analysis Software v.7 (Molecular Biology Insights Inc., Cascade, USA) and basic local alignment search tool (BLAST) of GenBank data (Table 1). After serial dilution of cDNA samples, 1 : 10 proportion was selected as appropriate. The Real-time PCR was carried out by Rotor-Gene 6000 (Corbett Research, Australia) in a final volume of 20 μL , containing 1 \times SYBR Green master mix (Toyobo, Japan), 1 μL of each of the primers (each 3 μM), 50 ng cDNA template, and 1 μL of cDNA sample. Amplification was performed using the following parameters: a denaturation step at 95°C for 5 min followed by 40 cycles of 15 s at 95°C (denaturation), 20 s at an annealing temperature of each primer, and 30 s at 72°C (extension). All reactions were performed at least in triplicate and the relative standard curve method was used to calculate the relative gene expression. The cycle of threshold (Ct) values was analyzed using the comparative Ct ($\Delta\Delta$ Ct) method. The melting curve was generated by holding the reaction mixtures at 95°C for 60 s and then lowering the temperature to 45°C at a transition rate of 0.1°C/s and maintaining for 120 s. Then, the reaction mixture of the samples was heated slowly at a transition rate of 0.05 to 80°C with continuous collection of fluorescence at 640 nm. The melting curve and quantitative analyses were conducted in a Corbett Rotor-Gene 6000 software program (version 1.7).

Statistical Analysis

All data were collected in Statistics programs for the Social Sciences software (SPSS, Inc., Chicago, IL, USA) version 20 and variance between groups was calculated by T-test. A *p*-value less than 0.05 were considered as statistical significance.

RESULTS AND DISCUSSION

Confirmation of Transformation by Enzymatic Digestion

The enzymatic digestion using BamHI and EcoRV restriction enzymes was done on the pcDNA3.1(-)-*hcpD* recombinant expression vector (resulting in 5428 and 933 bp fragment of pcDNA3.1(-) vector and the *hcpD*

Table 1. The primers details used for investigation of gene expression in this study

Primers	Sequence	Annealing temperature, °C	Product length, bp	Accession number
GAPDH	F: 5'-TCCCGTAGACAAAATGGTGAAGG-3' R: 5'-ATGTTAGTGGGGTCTCGCTCCTG-3'	65	261	XM_017321385
IL-17	F: 5'-CTACAGTGAAGGCAGCAGCGATC-3' R: 5'-CTTCCCTCCGCATTGACACAG-3'	66	263	NM_010552
IL-4	F: 5'-TCACAGGAGAAGGGACGCCATG-3' R: 5'-TGGACTTGGACTCATTGATGCTGC-3'	67	246	NM_021283
IFN γ	F: 5'-GCCTAGCTCTGAGACAATGAACG-3' R: 5'-GCCAGTTCCTCCAGATATCCAAG-3'	64	188	M28621
hcpD	F: 5'-GAGTTGTGGTAGTTTGGGTTTTATG-3' R: 5'-TGCTCTCCATTTTGCTTGACATC-3'	63	279	EF372717

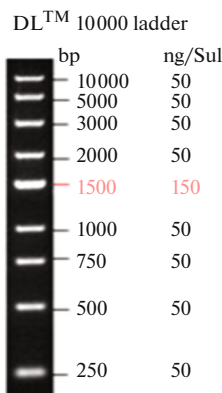
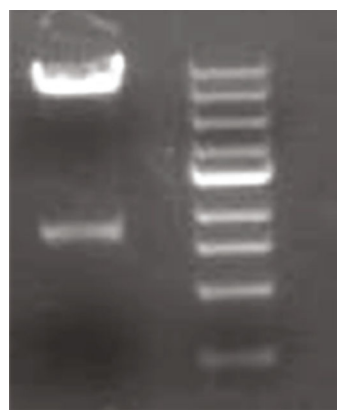
gene respectively) and confirmed the formation of the final construct (Fig. 1).

Chitosan Nanoparticles Characteristics

The size of chitosan particles with a low molecular weight was verified by dynamic light scattering (DLS) method. The results showed that 98% of these particles had a diameter of 134.3 nm (Fig. 2).

Zeta-potential measurements were performed on chitosan nanoparticles, placed in polystyrene, using Malvern Zetasizer Nano-ZS (ZEN3600) and the dispersion intensity was measured at 25°C with an incident wavelength of 633 nm (Fig. 3).

Scanning electron microscope (SEM) (Nikon S530 Hitachi, Japan) was used to determine the appearance of chitosan nanoparticles. They exhibited a homogeneous structure with almost half-spherical shape and smooth edges (Fig. 4).

**Fig. 1.** The enzymatic digestion on the recombinant plasmid pcDNA3.1(-)-hcpD.

Real-Time PCR Analysis

Quantitative real-time PCR analyses showed that the expression level of cytokine genes in the blood and tissue samples of vaccinated mice (the pcDNA3.1(-)-hcpD group) was changed according to details below: *IFN γ* gene expression was increased significantly (p -value < 0.001), *IL17* gene expression was not significantly altered (p -value > 0.05), and *IL4* gene expression was decreased significantly (p -value < 0.001) (Fig. 5).

In the pcDNA3.1(-)-hcpD + nanoparticle group, the expression of *IFN γ* and *IL17* genes were increased significantly (p -value < 0.001 and p -value < 0.05, respectively). Also, the *IL4* gene expression was decreased significantly (p -value < 0.001) (Fig. 6).

The comparison of the expression level of cytokine genes in the vaccinated mice by pcDNA3.1(-)-hcpD and pcDNA3.1(-)-hcpD + nanoparticle showed that *IFN γ* gene expression in pcDNA3.1(-)-hcpD + nanoparticle compared with pcDNA3.1(-)-hcpD group was different (statistically significant, p -value < 0.001). Moreover, the expression level of *IL17* and *IL4*

	Diam., nm	% Intensity	Width, nm
Z-Average (<i>d</i> , nm): 111.7	Peak 1: 134.3	98.4	68.84
PdI: 0.241	Peak 2: 5137	1.6	518.2
Intercept: 0.696	Peak 3: 0	0	0

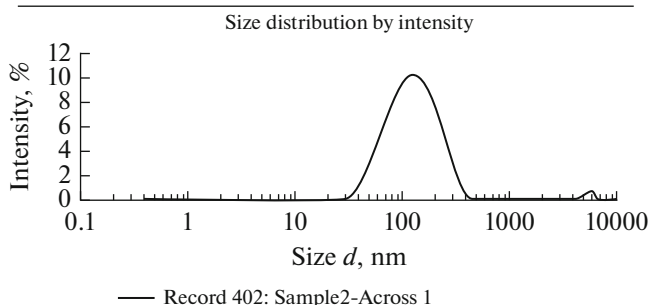
Result quality: **Good**

Fig. 2. The size of low molecular weight chitosan nanoparticles.

	Mean, mV	Area, %	Width, mV
Zeta potential, mV: 20.8	Peak 1: 20.8	100	10.4
Zeta deviation, mV: 10.4	Peak 2: 0	0	0
Conductivity, mS/cm: 2.69	Peak 3: 0	0	0

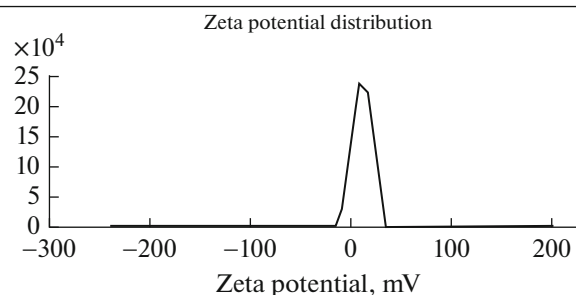
Result quality: **Good**

Fig. 3. Zeta-potential measurements on low molecular weight chitosan nanoparticles.

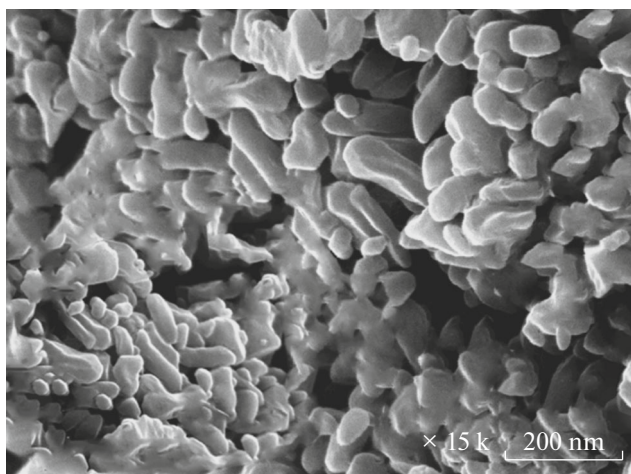


Fig. 4. SEM picture of chitosan nanoparticles morphology.

genes in pcDNA3.1(-)-hcpD + nanoparticle group compared with pcDNA3.1(-)-hcpD were not significantly different (p -value > 0.05) (Fig. 7).

The comparison of *hcpD* gene expression level in tissue samples of vaccinated mice by pcDNA3.1(-)-hcpD showed significant differences in day 15 compared with day 30, day 15 compared with day 45, and day 30 compared with day 45 (p -value < 0.001) (Fig. 8). Moreover, the comparison of the target gene expression in tissue samples of vaccinated mice by pcDNA3.1(-)-hcpD + nanoparticle indicated the significant difference of *hcpD* gene expression level in day 15 compared with day 30, day 15 compared with day 45, and day 30 compared with day 45 (p -value < 0.001) (Fig. 8). The *hcpD* gene expression level in pcDNA3.1(-)-hcpD group and pcDNA3.1(-)-hcpD + nanoparticle group in each sampling time (day 15, 30, and 45 after the last injection) were not significantly different (p -value > 0.05) (Fig. 9).

pcDNA3.1(-)-hcpD

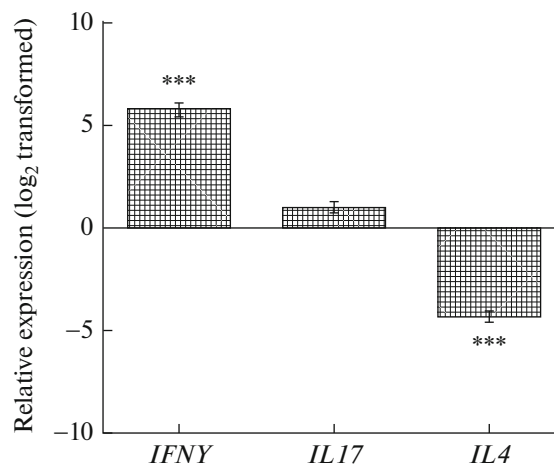


Fig. 5. The expression level of cytokines in the blood samples of vaccinated mice by pcDNA3.1(-)-hcpD recombinant plasmid.

H. pylori is the main cause of chronic and acute gastritis, gastric and duodenal ulcers, and prolonged inflammation of the gastric mucosa due to neutrophils, lymphocytes and plasma cells activity [23, 24]. Benefitting from the urease enzyme, this bacterium grows in the deep mucus layer of the gut epithelial cells and forms colonies. Therefore, endoscopic approaches usually fail to provide clear clinical diagnosis [25, 26]. Many studies have shown the role of this pathogen in the development of malignancy and gastric cancer; hence the World Health Organization's (WHO) Cancer Research Agency nominated this bacterium as a carcinogen in the first class [27, 28]. Due to the antibiotic resistance and lack of a suitable vaccine against *H. pylori* infection, it is important to find an appropriate vaccine (especially DNA vaccine) against *H. pylori* for the treatment and prevention of gastrointestinal infections [28, 29]. Gene vaccines compared to the other vaccines have numerous advantages like simple

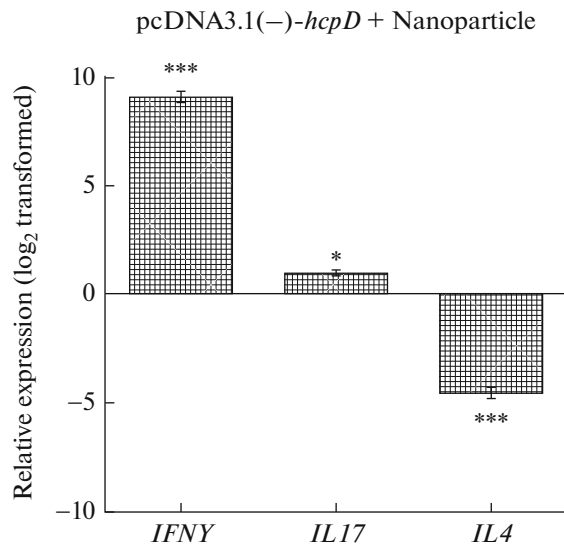


Fig. 6. The expression level of cytokines genes in the blood specimens of vaccinated mice by pcDNA3.1(-)-hcpD + nanoparticle.

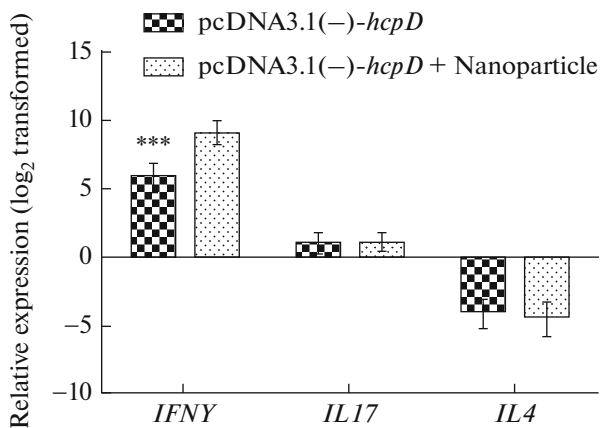


Fig. 7. The comparison between the expression level of cytokine genes in the blood samples of vaccinated mice by pcDNA3.1(-)-hcpD + nanoparticle and pcDNA3.1(-)-hcpD.

production, product purity, easy storage, stimulation of both cellular and humoral immune responses, and simple maintenance at room temperature.

In the present study, after successful preparation of pcDNA3.1(-)-hcpD, this recombinant vector was used as a DNA vaccine for vaccination of BALB/c mice either alone or combined with chitosan nanoparticles and their efficiency against *H. pylori* was evaluated via investigation of cytokine genes expression level by q-real-time RT-PCR. The DNA vaccines including pcDNA3.1(-)-hcpD and pcDNA3.1(-)-hcpD + nanoparticle were injected into mice hip muscles. The pcDNA3.1(-) (empty vector) was used as a control group. After 15, 30, and 45 days from the last vaccination, tissues and blood samples from vaccinated mice

were collected and the expression level of cytokine genes including *IL-17*, *IL-4* and *IFN γ* were evaluated using real-time qPCR. The expression of the *hcpD* gene as a vaccine gene against *H. pylori* was also investigated by this method.

The results of the qPCR on the expression levels of cytokine genes showed increased levels of *IFN γ* in both groups (pcDNA3.1(-)-hcpD + nanoparticle and pcDNA3.1(-)-hcpD) although the expression in DNA vaccine containing nanoparticle significantly differed ($p < 0.001$) compared to pcDNA3.1(-)-hcpD group (without using nanoparticle). In addition, the expression level of *IL17* and *IL4* genes in pcDNA3.1(-)-hcpD + nanoparticle group compared to a vaccinated group by pcDNA3.1(-)-hcpD were not significantly different ($p > 0.05$). Moreover, the comparison of the expression levels of *hcpD* gene in tissue samples of vaccinated mice in both pcDNA3.1(-)-hcpD and pcDNA3.1(-)-hcpD + nanoparticle groups in each sampling day (day 15, 30, and 45 after the last injection) were significantly different ($p < 0.001$). The comparison of the expression level of a target gene (*hcpD*) in tissue samples of vaccinated mice by pcDNA3.1(-)-hcpD and pcDNA3.1(-)-hcpD + nanoparticle in day 15, 30, and 45 after the last injection were not significantly different ($p > 0.05$).

There are many studies performed on treatment and prevention against *H. pylori* infection. Miyashita and colleagues in 2002 evaluated the immune response of mice after vaccination by catalase-encoding gene (pcDNA3.1-kat vaccine). They detected anti-IgG antibodies against catalase in the serum of mice which protected them against *H. pylori* infection and caused a severe reduction in the stomach inflammation (30). In our study, the pcDNA3.1(-)-hcpD and pcDNA3.1(-)-hcpD + nanoparticle vaccines were used and in pcDNA3.1(-)-hcpD group the *IFN γ* gene expression was increased and *IL4* gene expression was decreased significantly. In addition, in the pcDNA3.1(-)-hcpD + nanoparticle group, the expression level of *IFN γ* and *IL17* genes were increased and the *IL4* gene expression was decreased. The findings of the present work indicated that these DNA vaccines can induce the immune response against *H. pylori*.

In another study, the immunization of DNA vaccine expressing the *dysmotase coding* gene (SOD) was evaluated by intra-muscular injection of pcDNA-SOD in BALB/c mice [31]. Their findings showed that this DNA vaccine led to the humoral and cellular immune response through stimulation of Th-1 response and *IFN γ* production in mice and they suggested that this vaccine can be used against *Brucella abortus* infection. Bai and co-workers managed to clone the *Baba2* gene into pET-22b(+) expression vector and demonstrated that the recombinant BabA protein may be a potential vaccine to control and treat the *H. pylori* infection [32]. Another research focused on the creation of a

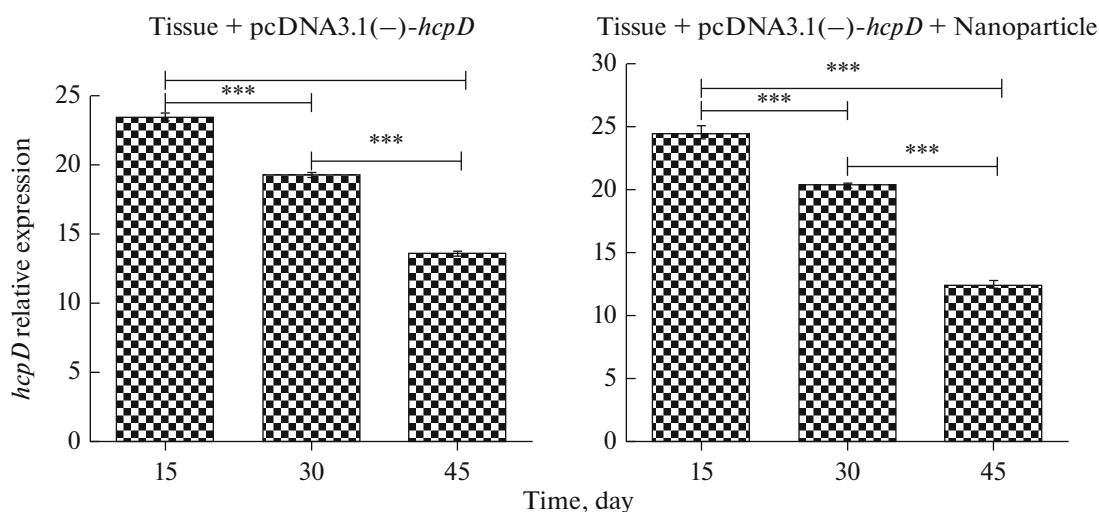


Fig. 8. The comparison of *hcpD* gene expression in tissue samples of vaccinated mice by pcDNA3.1(-)-*hcpD* (left) and pcDNA3.1(-)-*hcpD* + nanoparticle (right) in 15, 30, and 45 days after the last injection.

vaccine against *Brucella* by targeting the extracellular protein 31 (*omp31*) in *B. melitensis* and *B. ovis*. *Omp31* gene was cloned into a pCI expression vector and injected into BALB/c mice. The results showed that this vaccine produced cytotoxic reactions and could potentially affect the *Brucella* infection [33]. This research was similar to the present study, but in our study, the *hcpD* gene and pcDNA3.1(-) expression vector were used. Doosti and colleagues cloned *Omp31* gene of *Brucella melitensis* into pcDNA3.1 resulting to the construction of *Omp31*-pcDNA3.1 vector. The immunization using this construct was evaluated in BALB/c mice and their findings showed the Th-1 cells reaction in mice [34].

In another study by Jie and co-workers in 2013, they isolated and cloned the *ureI* segment from the *urease* gene into a PCDNA3.1 expression vector to make the PCDNA3.1(+)-*ureI* construct. These researchers concluded that this construct was able to express the protein production of *ureI* and stimulate the cellular and humoral response of the immune system in the C57BL/6 mice and thus it can be used as a vaccine [35]. Their study is similar to the present study in several respects and supports our findings, although the type of the mouse and gene was different. In another research by Al-Mariri et al., 2014 the *B39* and *Sp41* genes from *Brucella melitensis* were cloned in the expression vector and used as a DNA vaccine concluding that this vaccine could be a successful method for the immunization of BALB/c mice against *Brucella* infection [36]. Mahmoudi Vashian and Doosti, 2016 created an *ureG*-pCI-neo recombinant vector and transformed it into the CHO cells by electroporation and analyzed the expression of the *ureG* gene on SDS-PAGE gel. Results showed that the *ureG* cloned gene was able to express and produce a specific protein in CHO animal cells. They showed in the animal model

that this genetic construct can be considered as a suitable candidate for vaccination against *H. pylori* [37].

In a study by Safarpour and colleagues, a *tagD* gene from *H. pylori* was cloned into a PFLAG-CMV-3 eukaryotic expression vector. They transferred PFLAG-CMV-3-*tagD* into the CHO cells by electroporation method and evaluated its expression level in this cell line. They concluded that this construct gene is useful to evaluate the immunogenicity as a DNA vaccine against *H. pylori* infection in animal models [28]. Their study in terms of work processes was similar to the present work.

According to the findings of the present study, the expression of *IFN γ* gene in pcDNA3.1(-)-*hcpD* +

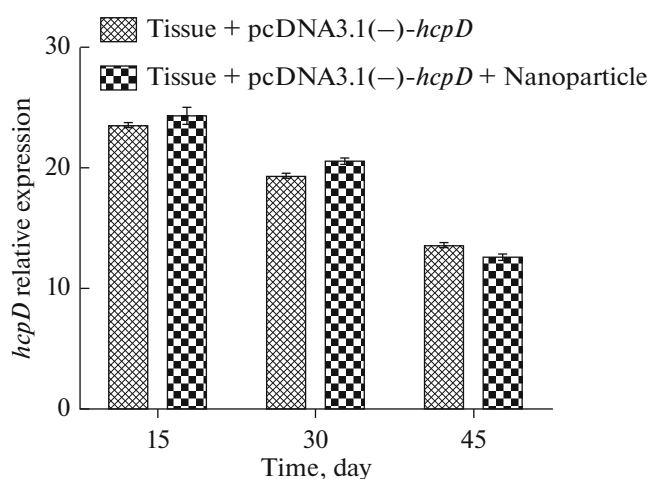


Fig. 9. The comparison of *hcpD* gene expression in tissue samples of vaccinated mice by pcDNA3.1(-)-*hcpD* and pcDNA3.1(-)-*hcpD* + nanoparticle in different days after the last injection.

nanoparticle and pcDNA3.1(-)-*hcpD* groups was increased, while *IL-4* expression was reduced in both groups. In addition, the expression level of *IL17* gene in pcDNA3.1(-)-*hcpD* + nanoparticle group were increased but in pcDNA3.1(-)-*hcpD* group was not changed significantly. Generally, these effects indicated the stimulation of the mice immune system by both vaccines with or without chitosan nanoparticles at a significant level. The comparison of the expression level of *hcpD* gene in the hip tissue of vaccinated mice in both pcDNA3.1(-)-*hcpD* and pcDNA3.1(-)-*hcpD* + nanoparticle groups during 15, 30 and 45 days after injection showed that the expression was decreased with the passage of time and no significant difference was observed in both vaccinated mice.

CONCLUSIONS

The pcDNA3.1(-)-*hcpD* along with nanoparticles produced in this study is useful for stimulation of the immune system and it can be used as a DNA vaccine against *H. pylori* infection in human studies in the future.

ACKNOWLEDGMENTS

This article was obtained from the MSc thesis. We would also like to sincere thanks from Research Deputy of Islamic Azad University of Shahrekord Branch and Biotechnology Research Center for their sincere cooperation.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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