Inhibition of *H. pylori* colonization and prevention of gastritis in murine model

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**Abstract** *Helicobacter pylori* is a Gram-negative spiral bacterium that colonizes human gastric mucosa causing infection. In this study aiming at inhibition of *H. pylori* infection we made an attempt to evaluate immunogenicity of the total (UreC) and C-terminal (UreCc) fragments of *H. pylori* urease. Total UreC and its C-terminal fragment were expressed in *E. coli*. Recombinant proteins were analyzed by SDS-PAGE and western blot and then purified by Ni–NTA affinity chromatography. Female C57BL6/j mice were immunized with the purified proteins (UreC and UreCc). Antibody titers from isolated sera were measured by ELISA. Immunized mice were then challenged by oral gavage with live *H. pylori* Sydney strain SS1. Total of 109 CFU were inoculated into stomach of immunized and unimmunized healthy mice three times each at one day interval. Eight weeks after the last inoculation, the blood sample was collected and the serum antibody titer was estimated by ELISA. Stomach tissues from control and experimental animal groups were studied histopathologically. UreC and UreCc yielded recombinant proteins of 61 and 31 kDa respectively. ELIZA confirmed establishment of immunity and the antibodies produced thereby efficiently recognized *H. pylori* and inhibited its colonization in vivo. Pathological analysis did not reveal established infection in immunized mice challenged with *H. pylori*. The results support the idea that UreC and UreCc specific antibodies contribute to protection against *H. pylori* infections.

**Keywords** UreC · UreCc · C57BL6/j mice · *H. pylori* · Immunity

**Introduction**

*Helicobacter pylori* was originally isolated from the stomachs of patients with active chronic gastritis and gastric ulcers by Marshall & Warren in 1984. *H. pylori* is a Gram-negative spiral bacterium (Brooks et al. 2007; Nagai et al. 2007) which infects about 50 % of the world’s population and is associated with gastritis, peptic ulcer, and gastric cancer (Malfertheiner et al. 2009; Tarkhashvili et al. 2009; Chung et al. 2010). Infection with *H. pylori* may lead to gastroduodenal diseases. Ammonia generated by the hydrolysis of urea neutralizes gastric acidity and forms a neutral microenvironment surrounding the bacterium within the gastric lumen. Treatment of patients suffering from peptic ulcer usually includes acid suppression and antimicrobial therapy. The urease from *H. pylori* is presented in both the cytoplasmic form and as a surface protein, and is necessary for neutralization of stomach acidity during colonization of the bacterium (Scott et al. 2010; Guo et al. 2012; Sachs et al. 2009). Among various antigens introduced as potential vaccine candidates, urease protein is the most promising (Malfertheiner et al. 2008; Bégué and Sadowska-Krowicka 2010). This protein plays an important role in survival and pathogenicity of the organism. The urease-deficient mutant strains of *H. pylori*
are unable to colonize the gastric mucosa (Bégué et al. 2007; Elliott 2009). *Helicobacter pylori* urease enzyme is a multimeric protein with high molecular mass (530 kDa) and it composed of \( \alpha \) (26.5 kDa) and \( \beta \) (61.7 kDa) subunits (Morihara et al. 2007). *H. pylori* urease shows different structure from most microbial ureases which contain three different subunits, and also from the jack bean urease, which contains a single polypeptide (Morihara et al. 2007; Koletzko et al. 2006) and the high cost of therapy are significant drawbacks (Fuccio et al. 2008). Recently, many studies have been published on *H. pylori* vaccine candidates (Gong et al. 2010; Rupnow et al. 2009; Zhang et al. 2010; Hoffelner et al. 2008; Agarwal and Agarwal 2008). Vaccine development is an effective method to battle diseases rather than treating them. Based on these facts, present study was designed to evaluate recombinant UreC and UreCc proteins as vaccine candidates against *H. pylori* infections. Recombinant UreC protein and its C-Terminal fragment were expressed and used separately as antigens to raise immune responses in C57BL/6j mice. Antibodies were then analyzed for their specificity and affinity toward *H. pylori*. Mice challenged with live *H. pylori* were subjected to serological and pathological analyses.

**Materials and methods**

Expression and purification of UreC and UreCc proteins:

UreC gene (GeneBank accession number GU942733) was optimized based on *E. coli* codon bias and synthesized by Shine gene company (China). A blast search was carried out on UreC gene and C-terminal fragment (610 bp) to determine gene specificity. UreC gene and C-Terminal fragment were amplified using primers as given in Table 1. PCR products of UreC and UreCc genes were separately cloned in pET28a vector using *EcoRI* and *SalI* restriction enzymes. Recombinant pET28a vectors were transferred into *E. coli* BL21 (DE3) followed by clone confirmation by plasmid digestion and sequencing using T7 universal primers. For expression of each gene, 100 μl of overnight culture of transformed bacteria in LB broth was used to inoculate 100 ml of LB broth containing 50 μg/ml Kanamycin. Expression was induced by 0.7 mM of isopropyl \( \beta \)-D-thiogalactoside (IPTG) at an optical density of 0.6 at 600nm. Protein expression was confirmed by western blotting with anti His-tag antibodies (Qiagen). The cells collected by centrifugation 6 h after IPTG induction and were suspended in lysis buffer (8 M Urea, 1 mM EDTA, 500 mM NaCl, 20 mM Tris, pH 8). The cells were disrupted by sonication and incubated at 37 °C for 2 h to dissolve the inclusion bodies. Cell debris were removed by centrifugation and 10 μl of supernatant was loaded in 12 % SDS-PAGE gels. Proteins were transferred on nitrocellulose paper by western blotting using Bio-Rad mini-protein tetra system (75 V, 60 min). Nitrocellulose paper was blocked with 5 % skim milk in PBS at 4 °C overnight with mild agitation. After washing with PBS-T [0.05 % Tween-20 in PBS [pH 7.2]] anti His-tag antibody was added at final concentration of 1/1,000 in PBS-T and incubated for 1 h at 37 °C with mild agitation. Nitrocellulose paper was washed and incubated in PBS-T for 15 min followed by addition of substrate buffer (50 mM Tris–HCl pH 7.2, 0.6 mg/ml DAB and 1 μl/ml H2O2). The reaction was stopped by addition of PBS upon appearance of the bands. The recombinant UreC and UreCc proteins were purified by nickel nitritolriacetic acid (Ni–NTA) affinity chromatography. The purified proteins were studied by SDS-PAGE. Concentration of purified UreC and UreCc was determined as described by Lowry et al. (Peterson 1977).

**Immunization of mice**

Total of 74 three to 4 weeks old female C57BL/6j mice weighing 15–20 g were purchased from Pasteur Institute, Tehran, Iran. Animals were divided into four groups. Groups I and II consisted of 18 mice each, were used for immunization tests. Eight mice were grouped as negative control and another 30 mice were kept as positive control group and used for *H. pylori* infection as described under “Mice infection and challenge”. Animals were kept in well aerated rooms and were fed on standard mice diet. The recombinant proteins were emulsified with complete Freund’s adjuvant. Total numbers of 36 C57BL/6j mice were immunized. Mice in groups I and II received 10 μg of UreC and UreCc respectively, 10 μg from each of the proteins with the same volume of incomplete Freund’s

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Restriction enzyme</th>
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<tbody>
<tr>
<td>UreC-forward</td>
<td>5'-GAGGAATTCGAAATGAAAAAGATTAG-3'</td>
<td><em>EcoRI</em></td>
</tr>
<tr>
<td>UreC-reverse</td>
<td>5'-ATCGTCTGCAGAAAAATGCTAAAG-3'</td>
<td><em>SalI</em></td>
</tr>
<tr>
<td>UreCc-forward</td>
<td>5'-ACGGAATTCGCGCGCCGTGGGAAGT-3'</td>
<td><em>EcoRI</em></td>
</tr>
<tr>
<td>UreCc-reverse</td>
<td>5'-ATCGTCTGCAGAAAAATGCTAAAG -3'</td>
<td><em>SalI</em></td>
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adjuvant were given to each mouse group on days 15, 30 and 45 as boosters. Blood samples were collected 10 days after each injection through infraorbital route. Negative control group did not receive any injection, and positive group was infected with *H. pylori*.

Analysis of antibody response

Sera were assayed for antibody response against the recombinant proteins by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtitre plates (JET BIOFILM) were coated with 2 µg purified recombinant UreC or UreCc proteins with 100 µl of 50 mM bicarbonate buffer (pH 9.8) per well and were incubated overnight at 4 °C. Wells were blocked for 30 min at 37 °C using 5 % skim milk in phosphate buffered saline (NaCl 0.8 %, KCl 0.02 %, Na2HPO4 0.29 % and KH2PO4 0.02 % in deionized distilled water, pH 7.2). Serial dilutions of mice sera from each group ranging from 1:100 to 1:51,200 were added to corresponding wells followed by incubation at 37 °C for 45 min. 100 µl anti-mouse IgG conjugated with horseradish peroxidase (HRP) enzyme (Bangalore Genei, India) at final dilution of 1:2,000 in PBS was added to each well and the plate was incubated at 37 °C for 30 min. 100 µl 8 % ortho-phenylenediamine (OPD) (W/V) and 8 % H2O2 (V/V) in citrate buffer (pH 5.2) was used as substrate for each well. The reaction was stopped after 15 min using 2 M H2SO4. Plates were then read with a microplate reader (Perlong New Technology, Beijing) at 492 nm. Washing was performed with 200 µl of PBS-T after each step.

*Helicobacter pylori* culture

Sydney strain SS1 *H. pylori* was cultured in Brucella agar medium (Difco). Colonies were transferred in brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) containing 5 % horse serum (JRH Biosciences), 0.02 % (V/V) Amphostat (Thermo-Electron Corporation, Melbourne, Australia) and skim’s selective supplements [containing vancomycin (10 mg/l), polymyxin (2,500 IU/l) and trimethoprim (5 mg/l) antibiotics] (Oxoid). Bacterial cultures were kept under micro-aerophilic conditions for 24 h at 37 °C. Broth cultures of *H. pylori* in BHI were used to infect mice and challenge tests.

Mice infection and challenge

The immunized mice from groups I and II along with positive control group were orally gavaged with 10^9 CFU of live *H. pylori* suspensions at log phase (OD600 = 0.6) three times each every other day. The same volume of sterile BHI medium was used to gavage the negative control group. Mice had no access to food or water 6 h prior to or after inoculation.

Serological examination

Eight weeks after the last inoculation, blood was collected from all four groups of mice and sera were isolated. Establishment of *H. pylori* infection was assessed by ELISA as described under “Analysis of antibody response” except for the antigen which was *H. pylori* Hpa protein.

Histopathological examination

Eight weeks after oral administration of mice with *H. pylori*, the antral portion of stomach from mice population in each group were removed for histological examinations. Formalin-fixed tissues were processed routinely in paraffin and stained with hematoxylin and eosin (H&E) and were then studied by light microscopy. For evaluation of gastritis, the H&E-stained sections were examined based on the degree of infiltrating lymphocytes, plasma cells, and neutrophils.

Statistical analysis

Unless otherwise specifically stated, all the experiments were carried out in triplicate. The data obtained are an average of the experiments. Student paired *t* test was used to analyze the data.

**Results**

Expression and purification of the recombinant UreC and UreCc proteins

UreC gene with GeneBank accession number GU942733 was optimized based on *E. coli* codon bias. Highest blast score in NCBI data base for UreC gene was 1,370 belonging to *Helicobacter pylori* complete genome sequence. The closest score to non-*Helicobacter* result was 565 that belonged to Clostridium sp. The highest score (536) of UreC gene was also *Helicobacter pylori* complete genome sequence and the closest non-*Helicobacter* score of 208 was of *Pseudomonas fluorescens* Pf0-1. Amplification of UreC gene resulted in 1,710 bp and UreCc gene resulted in 610 bp amplicons. Digestion and sequencing of the cloned pET28a vectors also yielded the same size fragments confirming correct and successful cloning. IPTG induced UreC and UreCc recombinant proteins subjected to His-tagged affinity chromatography, the purified recombinant proteins analyzed by SDS-PAGE revealed a single band of approximately 61 kDa for UreC
and 31 kDa for UreCc proteins (Fig. 1). Western blotting of the recombinant proteins resulted in two bands of 61 kDa, 31 kDa for UreC and UreCc respectively (Fig. 2).

Immunization of mice with UreC and UreCc and analysis of antibody response

Sera from groups I and II of the immunized mice showed high level of antibody titer against UreC and UreCc antigens respectively (Fig. 3).

Serological analysis of immunized mice challenged with H. pylori

The immunized mice were challenged with live H. pylori in order to assess protective potential of UreC and UreCc antibodies against H. pylori infection. ELISA results of mice subjected to bacterial challenge are shown in Fig. 4. ELISA results of groups I & II immunized prior to the challenge did not show significant difference from negative control group ($P > 0.05$). Unimmunized group serving as positive control showed significantly high antibody response against Hpa antigen compared to the negative control and test groups ($P < 0.0001$). ELISA results from test groups I & II did not show significant difference compared to each other ($P > 0.05$).

Antral histopathology

The micro sections from antral portion of the stomach of the experimental mice groups viz, challenged immunized, negative and positive control, were histopathologically studied (Fig. 5). The infected (positive control) group showed high grade of gastritis. The gastritis in unimmunized infected mice was higher than that of immunized challenged mice (Fig. 5c). Infiltration of numerous neutrophils, plasma cells and mononuclear cells was observed in the stomach of the infected mice. The mucosa of vaccinated mice showed no significant signs of atrophy or inflammatory cell infiltration. A marked reduction of bacterial colonization was noticeable in this group.

Discussion

Emergence of antibiotic resistant H. pylori strains and high cost of antibiotic therapy are reasonable justifications for focusing research on alternative preventive measures. Although new treatments like antibody therapy are being introduced (Sumida et al. 2009; Malekshahi et al. 2011), most of the infections caused by this bacterium are without symptoms (Forman et al. 1993; Sykora et al. 2009). Development of vaccines that could inhibit H. pylori colonization could help to control the infection and avoid associated disease such as cancer. Vaccinations with whole cell or purified antigen have been suggested to prevent H. pylori infection (Goto et al. 1999; Summerton et al. 2010; Höffner et al. 2008; Zhang et al. 2010; Wu et al. 2008). Such goal seems to be difficult to achieve because researches have shown that despite strong immune response, infection continuous to persist in some cases. These could be due to the nature of antigens used in establishment of the immunity. Studies have been shown that antibodies produced against antigens that do not contribute to the bacterial colonization

![Fig. 1 a SDS-PAGE result of recombinant UreC purification by Ni-NTA affinity chromatography. Lane M molecular weight marker; Lane F column outcome after loading cell lysate; Lane W column outcome after washing with Qiagen C and D buffers; Lanes E1, E2 column outcome after elution with Qiagen E buffer. b SDS-PAGE results for purification of UreCc recombinant protein using Ni-NTA affinity chromatography. Lane M molecular weight marker; Lanes I & F column outcome after loading induced cell lysate, Lane W column outcome from washed with Qiagen C and D buffers, Lanes E1, E2 column outcome after washing with Qiagen elution buffer](image)
do not eliminate the bacteria (Sutton et al. 2001; Garhart et al. 2002). Many \textit{H. pylori} antigens have been introduced as vaccine candidates while urease showed more promising results (Londono-Arcila et al. 2002; Jungblut et al. 2000; Kabir 2007). Anti UreC antibodies of this enzyme have always been present in the sera of all patients infected with the \textit{H. pylori} (Kabir 2007). In this study epitopic regions responsible for protective response produced against UreC subunit were identified by sequence and structural analysis of several bacterial urease enzymes. The data showed that C-terminal region of UreC subunit could serve as an antigen, and antibodies produced against this region could be protective against \textit{H. pylori} infection. Both UreC and UreC C-terminal fragment gene sequences underwent blast against NCBI data base indicated their specificity in \textit{Helicobacter} bacteria. C-terminal fragment of UreC subunit was compared to total UreC protein and used to vaccinate mouse models. We estimated immunogenicity by measuring IgG antibodies. The mice immunized with Urec and UreCc showed negligible gastritis and challenge resulted in reduced bacterial colonization followed by mild inflammation (Fig. 5). The findings are in support of Garhart et al. (2002) who reported that prophylactic immunization does not prevent colonization but enables immunized mice to reduce or clear the bacteria. Other researchers have suggested that cell-mediated immunity or IgA could be more important against \textit{H. pylori} infection (Crabtree 1996; Aebischer et al. 2010; Wilson and Crabtree 2007). Goto et al. (1999) showed that causing a severe inflammation in mice models could lead to protection and several researchers showed that immunized mice with different \textit{H. pylori} antigens will develop some level of inflammation after challenge with live bacterium. This post immunization gastritis might be related to protection against infection with

\begin{figure}
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\includegraphics[width=\textwidth]{image1.jpg}
\caption{Western blot result of UreC and UreCc recombinant protein after 6 h induction with IPTG. Anti His-Tag antibody was used for targeting the recombinant proteins. 61 kDa band is related to UreC recombinant protein and 31 kDa band is associated to UreCc recombinant protein.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image2.jpg}
\caption{Immunogenicity of UreC and UreCc recombinant proteins. Both proteins brought about high antibody response. Immunogenicity of UreCc is equal to that of the UreC protein.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image3.jpg}
\caption{ELISA results of mice challenged with live bacterium. Only positive control group of mice showed high antibody response against Hpa antigen. Mice from groups 1 and 2 received same amount of bacterium as positive control group did not show antibody response against Hpa antigen. Negative control group only received sterile BHI medium.}
\end{figure}
H. pylori infections (Mohammadi et al. 1996). Until now it has not yet been clarified as to whether antibodies produced against Urease inhibit bacterial colonization. However, inhibition of urease enzyme will reduce bacterial survival in acidic environment. Establishment of same level of protection obtained in UreCc compared to UreC suggested that C-terminal fragment could be used instead of total UreC protein. Both recombinant proteins resulted in high immune response and immunoprotection obtained was almost same in both proteins \((P > 0.05)\). Having a smaller antigen will reduce chance of allergy and immune response in host. The antibodies produced against such antigens will however lessen the chance of cross-reacting with other bacteria including normal flora. The findings showed that UreCc could be used as a vaccine candidate for prevention of H. pylori infections.

**Conclusion**

Immunization with UreC and UreCc proteins from H. pylori can protect infection with H. pylori. Efficiently specific antibodies produced against UreC and UreCc could be processed further to assess feasibility of their application as protective measures against H. pylori infections.

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**Conflict of interest** We declare no conflict of interest that could inappropriately influence our work.

**Ethical consideration** All animal experiments were carried out in accordance with Shahed University guidelines.

**References**


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Fig. 5 Gastric histology in C57BL/6j mice post-challenge with H. pylori. a Infected unimmunized mice showing severe inflammatory infiltration in the mucosa and submucosal regions. b Colonization of H. pylori in mucosa. (HE stain, ×100) c A histologically normal gastric mucosa of mice prophylactically vaccinated with recombinant protein. d Gastric histolograph of negative control mouse. (H&E stain, ×400)
treating Helicobacter pylori strains from several regions of the world.
J Clin Microbiol 48(3):690


