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Cloning, Expression and Purification of HpaA Recombinant Protein of *Helicobacter pylori* as a Vaccine Candidate

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***Helicobacter pylori* is prevailing in gastric niches and chronic disease with this pathogen can cause increased risk of many diseases such as gastritis, duodenal ulcer, dysplasia, neoplasia, gastric B cell lymphoma. Emergence of antibiotic resistance makes to recommended different treatments, and prevention methods have offered against infection. Today, among these methods as a result of immunization both in prevention and treatment of the infection has been confirmed and great efforts for vaccine design have been made. This study aimed to evaluate the cloning, expression and purification of HpaA recombinant protein of *H. pylori* as a vaccine candidate. Bioinformatics designing for desired gene were performed by use of suitable softwares and the construct was ordered to shine company (China), after receiving construct, cloned in pET21b vector, and this process was confirmed by polymerase chain reaction (PCR), digestion and sequencing techniques. Then, it was successfully cloned in the *Escherichia coli* BL-21 as expression host and the protein expressed. Expression of protein was verified using SDS page and western blot, for purification of protein, the Ni-NTA column was used. Dialysis for removal of imidazole was applied. Finally, protein concentration was determined by Bicinchoninic Acid Protein assay Kit (Parstoos). In present study, HpaA recombinant protein with size 29 kDa was expressed and purified, successfully. In our study construct HpaA was synthesized, but complementary studies are necessary to assess the immunological characteristics of this construct as an appropriate and efficient vaccine candidate against *H. pylori*.**

Keywords: *Helicobacter pylori*, HpaA, vaccine candidate.

Helicobacter pylori is prevailing in gastric niches and chronic disease with this pathogen can cause increased risk of many diseases such as gastritis, duodenal ulcer, dysplasia, neoplasia and gastric B cell lymphoma[1]. *H. pylori* colonize the human gastric for years and even decades without any adverse impacts[2]. *H. pylori* is a member of class 1 carcinogens and there is a correlation between the incidence of gastric cancer and high prevalence of infection with it[3]. Gastric cancer is the second most common malignancy and is the fourth leading cause of death worldwide and is a major epidemiological problem in the 21st century[4]. It is estimated that in 2000 there were about 870,000 deaths due to the gastric

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cancer, which accounted for 12% of deaths[5]. The risk of gastric cancer associated with *H. pylori* infection in industrialized countries is 70% and in developing countries is estimated 80%[6]. *H. pylori* infection usually causes asymptomatic chronic gastritis and 10-15% of infected persons show ulcers or gastric cancer. The high prevalence of *H. pylori* infection in the world and its role in gastric cancer and other diseases, emergence of antibiotic resistance makes to recommended different treatments, and prevention methods have offered against infection. Today, among these methods as a result of immunization both in prevention and treatments of the infection has been confirmed and great efforts for vaccine design has been made[7]. Vaccinations can solve the problem of antibiotic resistance and contributing to the eradication of this pathogen[8]. This bacterium has several virulence factors which are useful for vaccine design which HpaA is one of those and is most important sialic acid adhesion[9]. HpaA protein is conserved, immunogenic, secreted or surface localized[10]. Also the role of HpaA in colonization has been confirmed[10]. Therefore, regarding the role of HpaA as a virulence factor in pathogenesis of *H. pylori* can apply it as a vaccine candidate. Therefore, this study aimed to evaluate the cloning, expression and purification of HpaA recombinant protein of *Helicobacter pylori* as a vaccine candidate.

MATERIALS AND METHODS

Construction of recombinant plasmid

Firstly, sequence of *hpaA* gene of *H. pylori* strain 26695 achieved from gene bank, whole genome of *hpaA* (sequence 666 bp) was selected, at the beginning and end of the selected fragments were placed restriction enzymes. Primers designed by Primer 3 and Gene runner softwares for *H. pylori* 26695 *hpaA*. Bioinformatics studies in silico with related softwares were performed. The primers sequences were (F: 5' - ACTGAAGCTTCCGG CGAAACAGCAGCATAAC -3' and R: 5' - GGTTGAGCTCGAATGCGAAAAAGTAGAGCG-3; which allowed amplifying *hpaA* fragment and protein modeling performed. Two enzymes cutting site; hindIII and XhoI (Fermentas, Lituania) inserted into the 5' and 3' ends of *hpaA* fragment. Then fusion fragment (after deletion of stop codons) was sent to shine company (China) for synthesis. The DNA construct was cloned into the multiple cloning sites (MCS) of PGH vector by mentioned company. After that the *E.coli*[1] strain Top10 was transformed. Transformed *E.coli* was selected on LB agar plate containing 100 mg/ml of ampicillin. For verification of transformed colonies, PCR, enzyme digestion and sequencing were used. PGH-G genes plasmids were extracted from *E.coli* (top10) by extraction plasmid kit (Genet Bio Inc., South Korea) and double digestion by using enzymes to get hpaA fragment based on manufactures 'direction. Double digested hpaA fragment was cloned into pET21b to create recombinant expression vector. The cloning process was confirmed by restriction endonuclease digestion, sequencing *(*Macrogen Company*)* and PCR of the insert using universal T7-promoter and T7-terminator primers (TAG Copenhage A/S Symbion, Denmark).

Expression of recombinant proteins in E.coli BL21 strain

In the next step, pET21b/hpaA fragment transformed into the expression host (E.coli BL21 strain), successfully. To confirm proper transformation of selected colony, PCR, enzyme digestion and sequencing were applied. Transformed cells were cultured in 5 ml of LB (Luria bertani) broth containing 100 mg/ml ampicillin and shaken on shaker incubator at 37°C until OD reached 0.4-0.6 at 600 nm. Then, 2 ml of growing bacteria were harboring for inoculation of 500 ml of LB broth containing 100 mg/ml ampicillin and was shaken at 37°C until cell density in the OD 600 nm reached 0.4-0.6. The protein expression that was induced by IPTG (Isopropyl β-D-1-thiogalactopyranoside) in different concentrations which include 0.2, 0.5 and 1 mM, were submitted for shaking on the shaker incubator. In order to determine the best time and temperature for induction of cells, incubation was performed at 4, 18, 28 and 37°C for various times: 4, 8, 16 and 24 h. Subsequently, collected cells were sonicated 3 times with interval 2 minutes between cycles. Cells were pelleted using centrifugation at 14,000xg for 15 min at 4°C.

SDS-PAGE

To check the hpaA recombinant protein in the supernatant (periplasmic space) or pellet (inclusion bodies), SDS-PAGE was done.

Separation of protein carried out on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[11]. Pellet and supernatant of sonicated cells were suspended in sample buffer and heated at 95°C for 7 min; then, 10 µl of each sample was runned on SDS-PAGE gel. Coomassie Brilliant Blue R250 was used for staining of protein bands. Then, band size was assessed compared to protein marker (Thermo scientific).

Purification of recombinant protein from *E. coli* lysate

Ni-NTA resin affinity chro-matography column (QIAGEN Company) was applied to purification of recombinant protein. To resuspend cell pellet and supernatant, 10 ml of denaturing lysis buffer, potassium phosphate buffer, 150 mM NaCl were used. Then the sonication was performed and the lysates were loaded on the Ni-NTA column. For washing step, imidazole (30 mM) was used; subsequently recombinant protein was eluted with imidazole (500 mM). Finally, dialysis was performed by PBS buffer (pH= 7.4) at 4°C overnight to remove imidazole and other waste materials. The purity of recombinant protein was assessed by SDS-PAGE and Western blotting. Protein concentration was determined by Bicinchoninic Acid Protein assay Kit (Parstoos).

Western blot method

For western blot method[12], the protein which separated by SDS-PAGE gel was transferred to PVDF membrane (Amersham) and *immunobloting* was done by use of anti-poly histidine-peroxidase monoclonal antibody (Sigma-Aldrich). According to the standard protocol (manufacturer's direction), lastly, protein bands were shown by western blot Chemiluminescent kit (Parstoos). The antibody dilution in this experiment was 1/2000**.**

RESULTS

After receiving of *hpaA* construction in PGH vector and confirmation by mentioned methods in material & method section, has been successfully sub-cloned into the expression vector *pET21b*. The validity of sub-cloning process was proved by PCR, enzyme digestion and sequencing methods. Then sub-cloned *pET21b* (+) fragment was cloned into the *E.coli* strain BL21 as expression

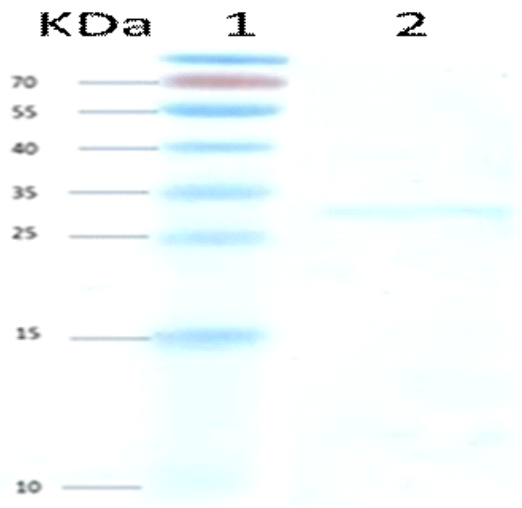

Fig. 1. SDS PAGE after purification of recombinant protein. Lane 1: protein marker and lane 2 shows the protein 29 KDa (HpaA)

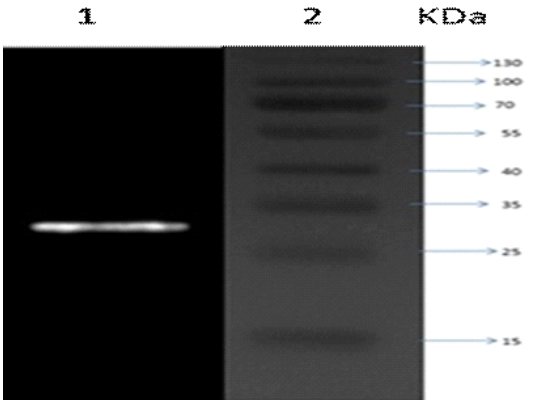

Fig. 2. Western blot for verification of recombinant protein expression. Lane 1: protein 29 KDa (HpaA) and lane 2: protein marker

host and this step was verified by above mentioned techniques. Expression host (*E.coli* strain BL21) containing plasmid encoding the recombinant protein was cultured overnight in different temperatures and was induced by IPTG until the recombinant protein expressed. The optimal temperature and IPTG concentration were 37 °C and 0.2 mM, respectively. Production of recombinant protein was successfully completed and the result was confirmed by means of the SDS page. Purification of protein was carried out successfully by loading of supernatant on Ni-NTA

column, for removal of imidazole the dialysis was performed, and then SDS page and western blot carried out (Fig. 1 & 2).

DISCUSSION

During many years, extensive efforts have been made to develop new vaccines against pathogenic *H. pylori*, but after passing 25 years of research and obtaining appropriate protective immunity in animal models, there is currently no a vaccine licensed for use in humans, yet, apparently, the existing vaccine candidates cannot to affect bacterial colonization and thus find a vaccine that affects disease caused by this bacterium even in the absence of appropriate safety, would be valuable[8]. The use of whole-cell vaccines in humans could be inhibited by bacterial components that probably cause undesirable responses[13]. Therefore, the focus is on vaccines containing recombinant protein antigens[8]. As well as, the immunity against pathogens would be more active using a combination of diverse antigens which involved in different phases of the colonization and infection pathogenesis and immunization pathways[14, 15]. A vaccine candidate antigens would be immunogenic, surface exposed and conserved among different species of *H. pylori*[16]. Currently, bioinformatics genome analysis tools are useful in selecting the desired correct antigen or epitope directly from the genomes of pathogens for design a vaccine[17]. Hence, selection of peptides derived from conserved regions of the protein is extremely important[18]. The T-cell responses are activated by cellular peptides are attached to the surface of antigen-presenting cells[17]. All pET vectors have many benefits for expression of recombinant proteins; all of these are available in three reading frames. And all of them using IPTG as the inducer, also, owing to protease deficiency in BL21 (DE3) is suitable for expression of recombinant proteins[19]. The expression of proteins in *E. coli* have some advantages such as; It is a easiest, quickest and cheapest method[20]. The *hpaA* gene is located in *H. pylori* genome and remarkably sequences of nucleotide and amino acid are conservative. Moreover, in all patients affected by *H. pylori*, antibody against HpaA nearly would be found in their serum, thus, will be an ideal candidate for vaccine[21]. HpaA is necessary for bacterial colonization in gastric mucus of mice and mutants lacking this factor have problem in gastric colonization[10]. Previous different studies have used of HpaA as a vaccine candidate. Of innovations of this study is that the PET 21 has been used. Johanna Nystrom et al for study protective effects of HpaA, immunized the mice intragastrically and intraperitoneally, the protection was strongly caused by specific mucosal immune responses (both CD4+ T cell production and IgA responses)[22]. Moreover, a study has shown that the immunogenicity role of HpaA in combination with OMP22 as fusion (Omp22–HpaA)[23]. Since HpaA is a small protein with size (~29 kDa)[24], in this study whole genome of HpaA was selected. Unlike our study, Peerayeh SN and colleagues[25] used from prokaryotic expression vector pET28a and like present study, E. *coli*, BL21 (DE3) was applied as expression host. They did ligation in at 20°C overnight, and technique (double cut) was used to clone *hpaA* gene, the protein purification was done by Ni-NTA agarose resin affinity chromatography (Qiagen, USA). As well as in study conducted by Neda Soleimani et al, the *hpaA* gene was inserted into pET28a (+) as cloning and expression vectors respectively. E. *coli*, BL21 (DE3) was applied as expression host and the restriction enzymes; *Nde*I and *Xho*I were used in their study, kanamycin was added as antibiotic to the LB medium and for purifica-tion fast protein liquid chromatography (FPLC) method was used[26]. However, in this study, HpaA recombinant protein synthesized, successfully, but the protective effects of this recombinant protein as a vaccine candidate should be evaluated.

CONCLUSION

In our study HpaA construct was synthesized, but complementary studies are necessary to assess the immunological characteristics of this construct as an appropriate and efficient vaccine candidate against *H. pylori*.

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