Iran Red Crescent Med J. 2017 April; 19(4):e43040. doi: 10.5812/ircmj.43040.

Published online 2017 February 27. **Research Article**

Expression and Purification of *gp40/15* Antigen of *Cryptosporidium parvum* Parasite in *Escherichia coli*: an Innovative Approach in Vaccine Production

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Received 2016 October 13; **Revised** 2016 November 30; **Accepted** 2017 January 16.

Abstract

Background: *Cryptosporidium* is a protozoan parasite that has medical and veterinary importance, and causes diarrhea and vomiting in a vast range of vertebrates. Some surface antigens, such as *gp40/15*, play important roles in adhesion and invasion of the parasite to host cells and consequently stimulate immune responses. Cloning and expression of the *gp40/15* gene to provide recombinant proteins of the parasite antigens is valuable.
Objectives: This study aimed at cloning and expression of the *gp40/15* gene in *Escherichia coli*.
Methods: In this experimental study, the *gp40/15* gene sequence was extracted from GenBank (No. AF155624) and cloned in the PET28a$^+$ plasmid. Colony polymerase chain reaction (PCR) and enzyme digestion methods by restricting enzymes, including BamHI and XhoI, were applied for verification. The recombinant plasmid was transferred to the *Escherichia coli* and the protein expression was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, and enzyme linked immunosorbent assay (ELISA) techniques using serum containing antibodies against *Cryptosporidium parvum*. The protein was further purified by column chromatography.
Results: The *gp40/15* gene was successfully cloned in the PET28a$^+$ plasmid. The colony PCR and enzymatic digestion methods showed a 921-bp fragment. Furthermore, expression of pEgp40/15 in *Escherichia coli* demonstrated a 43-kDa band. Antibody titrations in sera of test groups were significantly ($P < 0.0001$) higher than that of the control groups. Furthermore, antibody titration in test groups with four injections was significantly higher than that of the three injections ($P < 0.05$).
Conclusions: The *gp40/15* gene, which was cloned in the PET28a$^+$, was successfully expressed and produced in *E. coli*. Therefore, this protein can be used in future studies to develop recombinant vaccines and diagnostic kits.

Keywords: Protein Expression, *Cryptosporidium Parvum*, *Escherichia coli*, *gp40/15* Gene

1. Background

Cryptosporidiosis is a zoonotic disease created by a parasite from *apicomplexa* called *Cryptosporidium parvum*. This parasite has infected a wide variety of mammals, especially humans and is now considered a major public health problem, particularly among infants and immunocompromised individuals (1). There are no effective drugs for treatment of this disease (1). The parasite oocytes can enter the digestive tract through contaminated water and foods, and produce sporozoites that can invade and adhere to intestinal epithelial cells (2). Recent studies have focused to control parasite infection and identify antigens that are involved in stages of invasion and adhesion of parasite to host cells (3, 4). Studies have shown some important parasite antigens and proteins such as gp40/15, P23, gp40, gp15, and gp900 that have important roles in invasion and adhesion of the parasite to host cells (5). These proteins can induce production of some specific host immune responses against the infection, which can be detected by antibodies in serum of humans, who are infected with *Cryptosporidium parvum* (6). Accordingly, production of these immunogenic proteins as recombinant proteins and DNA vaccines provides a valuable strategy for the diagnosis of infection and immunize animals (3, 7-9). One of the first and important glycoprotein antigens that are considered as a surface radioactive glycoprotein is gp40/15. This antigen is similar to mucin, which is synthesized as the precursor protein and can be broken to two mature glycoproteins including gp40 and gp15 (10). Glycoprotein gp15 adheres to sporozoites' membrane by inositol phosphatidylinositol, while the glycoprotein gp40 is more soluble and has epitopes that are detected by the host cell receptors (10). The protein produced by these glycoproteins is able to stimulate

the host's immune responses and can be detected in sera of infected patients (11). The gp40 and gp15 glycoproteins can attack and connect to host cells surface, and thus can be considered as valuable targets for treatment and control of cryptosporidiosis (12). A few studies have focused on cloning and expression of *gp40/15* gene in eukaryotic and prokaryotic cells. Connor et al., transferred *gp40/15* gene to *Toxoplasma gondii* for protein expression. They showed that the recombinant protein gp40/15 expressed on the surface of *Toxoplasma tachyzoites* is identified by anti-gp15 and gp40 monoclonal antibodies (13, 14). They believed that these proteins play an important role in adhesion of the parasite to the intestinal wall of the host and create the first step of infection. Tilley et al. injected gp40/15 protein of the *cryptosporidium parvum* in mice. They positioned serum taken from mice adjacent to the parasite's sporozoites and observed that the serum had a high sensitivity to the parasite, and provided a good response. They used this protein for the detection of *Cryptosporidium parvum* parasite and believed that it can be used as a vaccine candidate for control and detection of parasites (15). In another study by Cevallos et al., they concluded that the *gp40/15* gene did not have an intron, and existed in a single copy in the genome of the parasite. This protein is the precursor of two proteins including Gp40 and gp15, which are expressed at intracellular stages of the parasite (10). Analysis of the amino acid sequence of the *gp40/15* gene, has demonstrated that these proteins have an N-terminal peptide signal area, a row of poly-serine amino, glycalization position, and hydrophobic region in the C-terminal, with a molecular weight of about 981 bp (10). In a study by Leave et al. (2002), they analyzed *gp40/15* gene sequence in children with Acquired Immunodeficiency Syndrome (AIDS), who were infected with cryptosporidium in South Africa. Their results showed that this gene has numerous polymorphic sites in different genotypes of the parasite. They also believed that the protein produced by this gene is an important factor in *Cryptosporidium parvum* and can be used to determine the subspecies of the parasite (16). To the best of our knowledge, there is no study to consider the *gp40/15* gene cloning in PET28a$^+$ plasmid and its expression in *E. coli*. This study aimed at considering the cloning and expression of the *Cryptosporidium parvum gp40/15* gene in *E. coli* to achieve recombinant protein of gp40/15 for further study. The results of this study can be used to produce recombinant vaccines and design cryptosporidiosis diagnostic kits.

2. Methods

In this experimental study, the complete sequence of *Cryptosporidium parvum gp40/15* gene was extracted from GenBank with code number AF155624 and a fragment with 921 bp was selected and cloned in the PET28a$^+$ plasmid. The GenScript software was used to optimize the gene. The PET28a$^+$ vector containing the *gp40/15* gene, pEgp40/15 recombinant plasmid, was transformed to *E. coli* cells BL21 (DE3) (stratagene) and then cultured in Lysogeny Broth (LB) agar containing kanamycin.

Selected clones were considered by Polymerase Chain Reaction (PCR) colony method in solid medium using forward (catcatcacagcagcggcc) and reverse (ggatctcagtggtggtggtgg) primers. The recombinant plasmid was cultured in liquid LB medium and was then extracted after amplification, and confirmed using BamHI and XhoI restriction enzymes (17). About 100 mL of the overnight-cultured clones were added to the 5-mL liquid LB medium. After the Optical Density (OD) reached 0.6 at wavelength of 600 nm (to obtain the bacterial growth rate), induction promoter (IPTG) at a concentration of 0.5 mM was added to the culture medium and was incubated at 37°C for 16 hours. Samples were electrophoresed on 12% gel along with a marker (SM0671) before and after induction with IPTG under denaturing conditions. To confirm the expressed recombinant proteins, the immunoblot technique with anti-His-tag antibody was used (17, 18). After expression, cellular extracts were transferred to a nitrocellulose paper using Western blotting (Bio-Rad; Mini Protein) and transferring buffer (192 mM glycine, 25 mM Tris, 0.1% SDS, 20% methanol, and pH: 8.3). Nitrocellulose paper was blocked using phosphate buffered saline (PBS) buffer (37 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 20% Tween and pH: 7.2) containing 5% dry milk for 16 hours at 4°C. After three washes with PBS buffer, the sample was placed adjacent to PBST buffer at room temperature for an hour and then diluted with conjugated anti-His-tag (Abcam) antibody with a dilution of 1.10000. Following three washes with PBS buffer, the substrate (50 mM Tris buffer; pH: 7.8, containing 6 mg DAB, 10 μL H_2O_2) was used for the detection. After substrate-conjugation interaction and the appearance of protein band on nitrocellulose paper, the reaction was stopped using H_2O. The protein was isolated under denaturing conditions using the Ni-NTA column and samples were electrophoresed on 12% gel. This recombinant protein was further considered and confirmed by the enzyme linked immunosorbent assay (ELISA) method using mice serum immunized with 20 μg recombinant gp40/15 proteins. The immunized serum and serum containing antibody against *Cryptosporidium parvum* were provided from the department of parasitology of Shahid Beheshti University of Medical Sciences. The concentration of the expressed protein was determined by the Bradford method, using bovine serum albumin (BSA; Cinnagen) as a standard (19, 20).

2.1. Statistical Analysis

The ELISA results of reaction between gp40/15 recombinant protein and antibody are presented as Mean ± Standard Deviation (SD). Diagrams are drawn using the Excel software. An independent sample t test was used to compare the mean of ELISA tests between the two groups, while one-way analysis of variance (ANOVA) was applied to compare results between several groups. Data were analyzed using SPSS, version 19 and a probability of less than 0.05 was considered as significant.

3. Results

3.1. The Presence of Synthetic Gene in Expressive Plasmid

After transforming of the recombinant plasmid containing the gene (pEgp40/15) to *E. coli*, direct colony PCR was applied for the accuracy of transformation. As shown in Figure 1, a 961-bp bound can be seen on electrophoresis gel. According to the applied primers in the transcription step, where 40 bp from both sides of the cloned gene were performed on PET28a^{+}. Therefore, 40 bp was added to the *gp40/15* gene with 921 bp, which was cloned on the PET28a^{+} vector and produced a band, 961 bp in length. This confirms the accuracy of pEgp40/15 recombinant plasmid transformation in the bacteria. After plasmid extraction, for confirming the presence of the fragment in the expressive pET28a^{+} vector, enzymatic digestion was used by BamHI and XhoI restriction enzymes.

As illustrated in Figure 2, the *gp40/15* gene is broken from both ends by BamHI and XhoI and the 921-bp *gp40/15* gene is separated from the vector, which suggests the existence of *gp40/15* gene in the expressive pET28a^{+} vector.

3.2. Expression and Purification of the gp40/15 Protein

The colony of bacteria containing pEgp40/15 was cultured in LB medium and then incubated at 37°C. When colonies achieved enough growth, 0.5 mM IPTG was added to induce expression and production of protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis was applied to evaluate the expression and quality of the protein (Figure 3).

The gp40/15 protein band was placed at around 43 kDa, whereas no band was detected in control samples. The recombinant protein was further purified by nickel column (Figure 4) and electrophoresed on the gel. As shown in Figure 4, the 43 kDa protein band was well-separated from the column by washing with a solution of imidazole 100. The protein concentration was measured by the Bradford method.

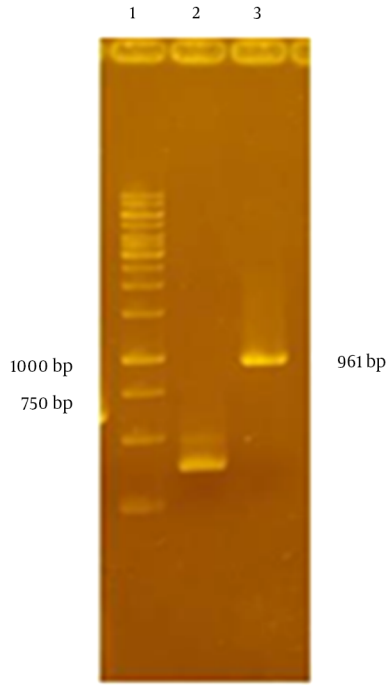


Figure 1. Nucleic Acid Marker (column 1); The Polymerase Chain Reaction (PCR) Product of *gp/15* Gene (Column 2); and PCR Product of *gp40/15* Gene (Column 3)

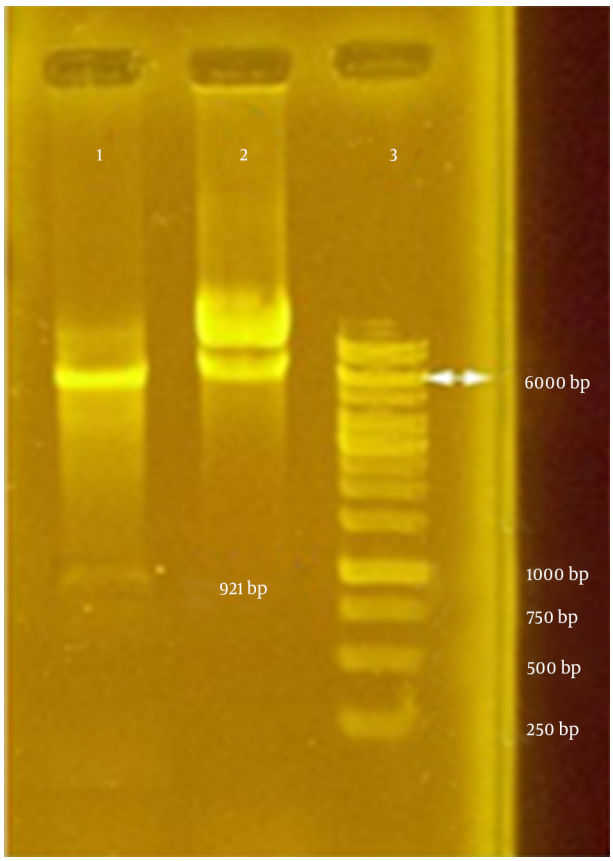
3.3. Western Blot Using Anti-His-Tag Monoclonal Antibody

Since the gp40/15 recombinant protein has His-tag sequence, its expression was confirmed by western blot using anti-His-tag antibody. The induced expression of gp40/15 recombinant protein with IPTG in bacteria interacted with anti-His-tag antibody and showed a band around 43 kDa (Figure 5; column 2). However, in the negative control (column 1), where bacterial cell containing the recombinant plasmid was used without induction step with IPTG, no band was detected.

3.4. Reaffirmation of the gp40/15 Recombinant Protein

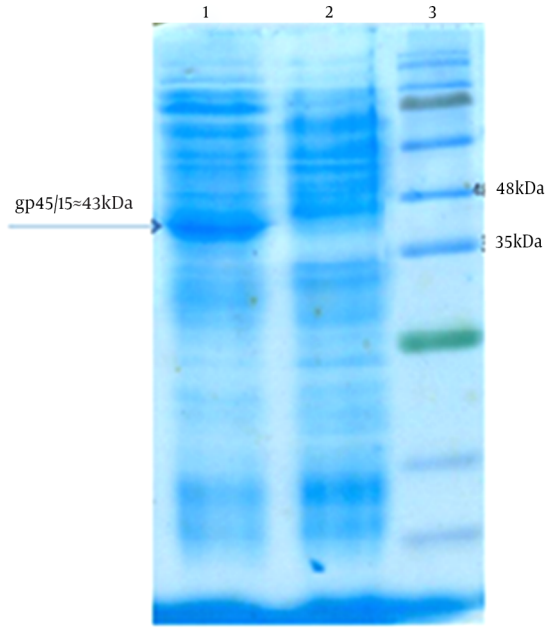
The results of the ELISA at a wavelength of 495 nm showed that the serum containing antibody against *Cryptosporidium parvum* and serum of mice immunized with the protein could detect the recombinant protein and react with it. This data reflect the accuracy of this protein and its reaffirmation by the serum containing antibody against this protein. As illustrated in Figures 6 and 7, the optical density caused by the reaction between the recombinant protein and antibody in serum was reduced at lower serum concentrations (1/12800) and was increased at higher serum concentrations (1/100). Antibody titrations in sera of test groups were significantly ($P < 0.0001$) higher than that of the control groups (Figure 6). Furthermore,

Figure 2. Enzymatic Digestion of pEgp40/15 Recombinant Plasmid



Well 1, enzymatic cutting of the vector by the BamHI and XhoI enzymes; well 2, pEgp40/15 recombinant plasmid without enzymatic cutting; well 3, nucleic acid marker.

Figure 3. Electrophoresis of gp40/15 Protein Expression Before Chromatography



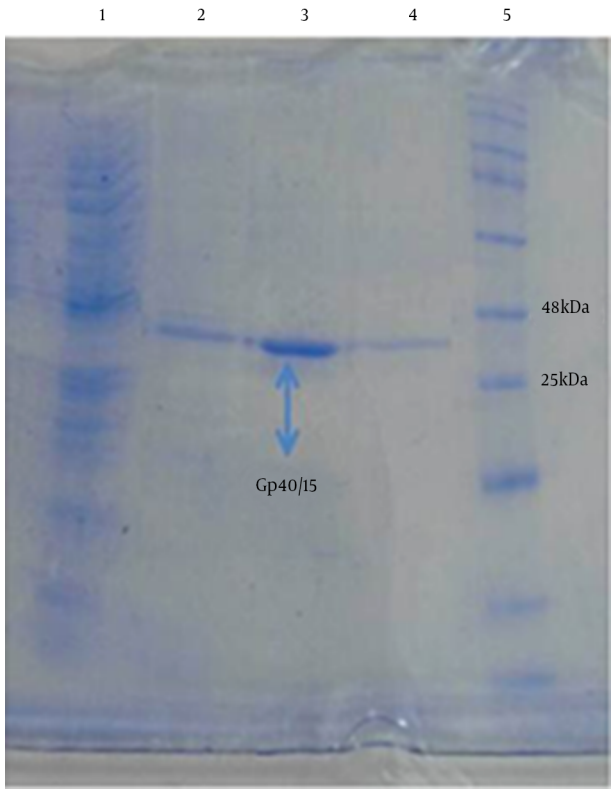
Column 1, protein band in the presence of IPTG; column 2, without IPTG; and column 3, protein marker.

antibody titration in test groups with four injections was significantly higher than that of the three injections ($P < 0.05$; Figure 7). Antibody titrations in serum of test groups (III or IV injections) were significantly ($P < 0.0001$) higher than that of control groups.

4. Discussion

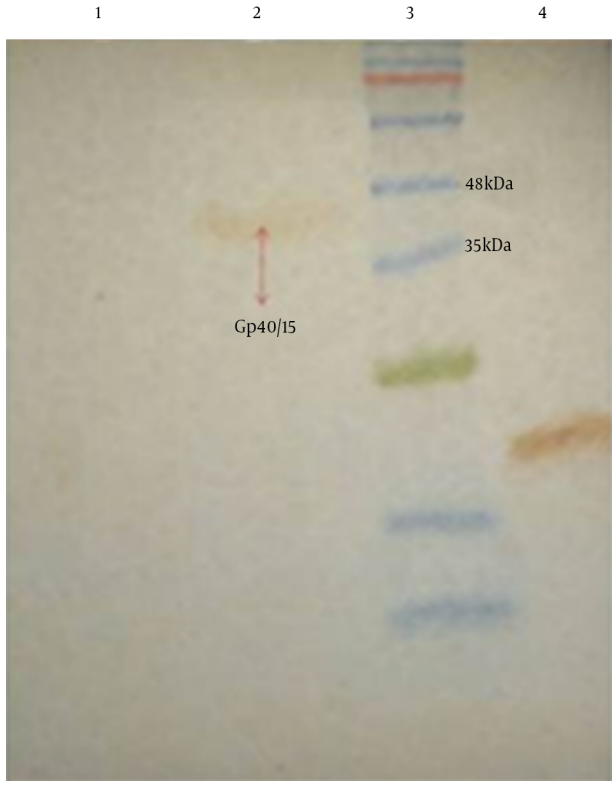
Cryptosporidiosis is an infectious disease that is caused by a protozoan parasite called *Cryptosporidium parvum* (21). This parasite is a potential life-threatening factor in patients with suppressed immune system, and can cause diarrhea (22, 23). Currently, no specific, full, and effective treatment for cryptosporidiosis has been discovered (24). Surface and apical region antigens of the parasite such as cp23, gp900, gp15, gp40/15, gp40, and CSL are involved in the process of adhesion and invasion of *Cryptosporidium* sporozoite to target cells. This is a vital step in inducing infection by this parasite (25, 26). Several studies have shown that parasite antigens and immunogenic parasite recombinant proteins can be identified by antibodies of human serum and a number of other infected animals. These antigens and proteins are considered as promising candidates for vaccine development and diagnosis of infection (25). A number of them are mucin or pseudo-mucin glycoproteins, which play an important role in the production of host immune response against infection. The best mucin-like glycoprotein is the gp40/15 antigen, which is expressed as a precursor and can be broken to gp40 and gp15 proteins. These proteins are immunogenic and use of antibodies against them can control the infection and prevent *Cryptosporidium parvum* contamination. These proteins can be detected within the serum of infected patients (10). A few studies have considered the cloning and expression of *cryptosporidium* antigens, especially gp40/15 antigen. To our knowledge, this is the first study in Iran and the world, which investigated the expression of *gp40/15* gene embedded in the pET28a+ plasmid. In the present study, we selected the *gp40/15* gene due to its critical role in the process of parasite virulence. In this study, a fragment of 921 bp in length was selected from the *gp40/15* gene, and cloned in the $pET28a^{+}$ plasmid. After cloning, they were transformed to

Figure 4. Purification of gp40/15 Recombinant Protein by Nickel Chromatography Column



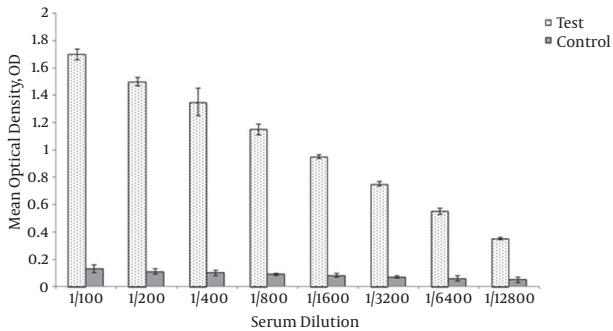
Column 1, flow leachate; column 2, the leachate washed by 40 mM imidazole (E40); column 3, the leachate washed by 100 mM imidazole (E100) (the protein with molecular weight of about 43 kDa was isolated from the column); column 4, the leachate washed by 250 mM imidazole (E250); and column 5, protein marker.

Figure 5. Results From Western Blot



Column 1, negative control (bacteria cell containing recombinant plasmid without induction with IPTG); column 2, expressed gp40/15 recombinant protein in bacteria induced by IPTG; column 3, protein marker; column 4, control protein to consider and ensure the function of materials and the antibody used in the western blot.

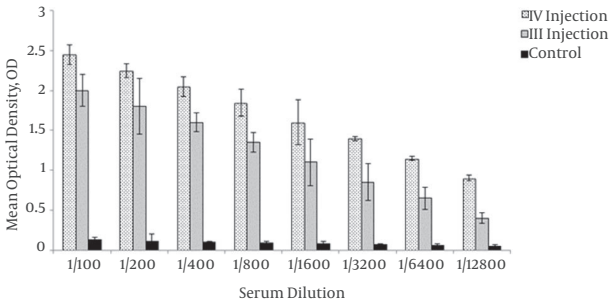
Figure 6. Results of the Reaction Between gp40/15 Recombinant Protein and the Serum Containing Antibody Against *Cryptosporidium parvum* by the Enzyme Linked Immunosorbent Assay Method in Control and Test Groups



Antibody titrations in sera of test groups were significantly ($P < 0.0001$) higher than those in control groups.

the *E. coli* BL21 for protein expression. Results of SDS-PAGE and Western blot showed that the protein's molecular weight was 43 kDa. The only study that evaluated the expression of these genes in prokaryotic cells was conducted by Cevallos et al. (10). They expressed the *gp40/15* gene in prokaryotic cells, and after gene sequencing they showed that it was 981 bp in length. They used the PET-32 LIC/Xa vector for gene cloning and transferred it to E. coli AD494. They discovered that its molecular weight was about 49 kDa (10). Due to the glycosylation process, the molecular weight of the gp40/15 protein generated in the parasite was approximately 55 to 60 kDa, yet, the molecular weight of the gp15 and gp40 glycoproteins was around 15 and 45 kDa, respectively. Due to the lack of glycosylation in prokaryotic cells and small number of selected nucleotides as well as type of vector, the molecular weight of the expressed protein ranged from 40 to 49 kDa (10). Strong et al. believed that gp40/15 has approximately 330 amino acids with a molecular weight of 34 kDa (27). They showed that the main scaffold of the antigen is formed by

Figure 7. Results of the Reaction Between gp40/15 Recombinant Protein and Serum of Mice Immunized with the Protein by Enzyme Linked Immunosorbent Assay Method



Antibody titration in test groups with four injections was significantly ($P < 0.05$) higher than that in test groups with three injections. Antibody titrations in sera of test groups (III or IV injections) were significantly ($P < 0.0001$) higher than that of the control groups.

gp15 and gp40 proteins, with a molecular weight of 11 and 20 kDa, respectively. However, these molecular weights are different from the molecular weight of expressed proteins in the parasite due to the large amount of mucin, serine and threonine, as well as glycosylation at antigenic sites that increase the molecular weight of the protein in the life cycle of intracellular stages (27).

The difference in molecular weight of the expressed protein between our study and Cevallos et al. can be related to the type of vector, number of selected bases, and kind of bacteria. Nevertheless, these proteins are somewhat similar. The ELISA results showed that the protein could be identified by serum containing antibodies against *Cryptosporidium parvum* and serum of mice immunized with the protein. It seems that the serum could identify the antigenic epitopes of recombinant protein gp40/15 and reaction with it; this may indicate the immunogenicity of the protein. This result is also consistent with the results of other researchers based on immune responses created by antigen gp40/15 or gp40 and gp15 glycoproteins (3, 11, 13). It should be noted that the evaluation of immunogenicity of these proteins will be examined in future studies.

This study showed that the *gp40/15* gene cloned was successfully in the PET28a^{+} vector and the related protein was expressed in E. coli. Thus, this recombinant protein can be used as a potential tool for production of diagnostic kits and vaccines to detect infection and immunize animals and humans. Additionally, it is an important step in detecting and combating parasites for infection control.

Acknowledgments

We appreciate all professors and researchers from cellular, molecular, and biochemistry departments of the University of Imam Hussein (PBUH); Dr. Farid Tahvildari and Dr. Seyed Jawad Seyed Tabaei, members of the faculty of the Department of Parasitology, Faculty of Medicine at Shahid Beheshti University, who helped with conducting the research. This Article was extracted from a student thesis.

Footnote

Conflict of Interest: There was no conflict of interest.

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