Vaccine Research
ISSN: 2423-4923
eISSN: 2383-2819

Design and Recombinant Expression of a multiepitope Vaccine Candidate Against Pathogenic Species of *Shigella*

Amir Namvar Vansofla, Abbas Hajizade*, Shahram Nazarian, Davoud Sadeghi, Mohammad Reza Akbari, Yousof Tarverdizadeh

Biology Research Center, Faculty of Basic Sciences, Imam Hossein University, Tehran, Iran

ARTICLE INFO

Research Article

VacRes, 2021
Vol. 8, No.1, 18- 22
Received: August 23, 2021
Accepted: September 17, 2021
Pasteur Institute of Iran

***Corresponding Author:**
Abbas Hajizade
Biology Research Center,
Faculty of Basic Sciences,
Imam Hossein University, Tehran, Iran
Email: abbashajizade@gmail.com
Tel/Fax: +989127239829

KEYWORDS: Shigella, Shigellosis, multi-epitope vaccine, Ipas, Omps, IcsA

ABSTRACT

Introduction: Shigellosis is a form of acute intestinal infection and one of the global health problems that occur in human by pathogenic species of *Shigella*. Producing a cost-effective and protective vaccine against the pathogenic strains of these bacteria will have a significant effect on the improvement of public health. The purpose of this research¬ was to design, express and purify a multiepitope protein as a candidate vaccine against *Shigella* pathogenic species. **Methods:** The multi-epitope protein-encoding Ipas, Omps and IcsA genes was designed based on previous bioinformatics assessments and synthesized in pET28a (+) expression vector. Since no detectable expression was observed, the gene was subcloned into pET32a (+). The pET32a (+) recombinant vector containing the desired gene was transferred into *Escherichia coli* BL21 (DE3) and the expression of the recombinant protein was induced using IPTG. The protein was purified using a nickel column. Finally, the Western blotting method was used to confirm the expression of the recombinant protein. **Results:** The sub-cloning of the gene was confirmed using PCR reaction. Gene expression analysis showed that the desired protein had a suitable expression. Western blotting analysis confirmed the expression of the recombinant protein. **Conclusion:** The expressed and purified multi-epitope recombinant protein, containing the main epitopes of the common antigens of pathogenic *Shigella* species could be achieved as the first step to design a multiepitope vaccine candidate against shigellosis.

Citation:

INTRODUCTION

Shigellosis is acute enteritis caused by pathogenic species of *Shigella* (S.) genus. The infection is accompanied by watery, and sometimes, bloody diarrhea. Four species of *Shigella* genus are able to develop the infection, namely *S. flexneri*, *S. sonnei*, *S. dysentery* and *S. boydii* [1]. Between 1966 and 1997, approximately 165 million cases of shigellosis have been reported annually worldwide with a mortality rate of ~0.67 %. More recent studies indicate that there are approximately 125 million shigellosis cases per year, of which ~98% of deaths in each case have decreased due to improvements in lifestyle and treatments [2-4]. Antibiotic therapy is a strategy of choice to combat the disease. However, due to the emergence of multi-drug-resistant of *Shigella* strains, this strategy has faced major challenges [5, 6].In this regard, vaccination is considered a good strategy to prevent the infection and can have a significant impact on the public health. Many attempts have been made to introduce an effective vaccine against the disease, some of which have been investigated in pre-clinical studies or clinical trials [7]. These vaccines can be mainly, but not merely, divided into two categories, namely, live-attenuated and subunit vaccines [8-11]. However, still there is no approved vaccine to prevent this disease.

Moreover, most of the candidate vaccines against *Shigella* are not able to protect against all pathogenic species of these bacteria. Since immunization against all four pathogenic species of *Shigella* is important, multi-epitope vaccine strategy can potentially help to achieve this goal. Multi-epitope vaccines (MEVs) are a class of recombinant vaccines that have the most potent epitopes of one or more major immunogens of one or more microbial pathogens. One of the advantages of these vaccines is the possibility to select and combine epitopes of the specific antigens of the species so that the resulting mixed protein is able to stimulate the immune system against all those antigens [12]. The purpose of this research was to subclone a gene fragment encoding a recombinant protein made of fragments of invasive plasmid antigens (Ipas), outer membrane proteins (Omps) and virulence plasmid factor VirG (IcsA) of

[DOI: 10.52547/vacres.8.1.18]

pathogenic *Shigella* Species, *including S. flexneri, S. sonnei, S.* dysentery and *S. boydii*, in pET32a (+) expression vector and its expression and purification.

MATERIALS AND METHODS

Enzymes, Chemicals, Vectors and Bacterial Strains

The genes encoding the multi-epitope protein fragments of invasive plasmid antigens (Ipas), outer membrane proteins (Omps) and virulence plasmid factor VirG (IcsA) of pathogenic *Shigella* Species, including *S. flexneri, S. sonnei, S. dysentery* and *S. boydii* as a fragment coding for a multi-epitope protein as described previously [23] was synthesized by Biomatik Company (Canada) in pET28a (+) expression vector. Briefly, the epitopes were selected so that they were common in four above-mentioned pathogenic *Shigella* species. Following the selection of the epitopes, they were joined together and the immunologic properties of the chimeric protein were evaluated through bioinformatics tools. Moreover, LT-IIc and ctxB mucosal adjuvants were added to the N- and C-terminal of the protein, respectively (Fig. 1).

pET32a (+) expression vector (Novagen) and bacterial strains of *Escherichia coli* DH5α and *E. coli* BL-21 (DE3) were obtained from Biology Research Center, Imam Hossein University. The Taq DNA Polymerase enzyme was purchased from SinaClone (Iran) and T4 DNA Ligase enzyme, along with the *Hin*dIII and *Eco*RI restriction enzymes were from ThermoFisher Scientific Company (USA). The materials required for PCR were obtained from SinaClone (Iran).

Fig. 1. Schematic diagram depicting the order of sequences on the multi-epitope protein. E1-E9: selected epitopes; LT-IIc and ctxB: mucosal adjuvants.

Amplification and Purification of the Desired Encoding Fragment

To amplify the desired gene, the PCR reaction was performed in a final volume of 25 µL (0.5 µM of each primer, 2.5 µl of PCR 10x buffer, 2.5 µl of MgCl2, 0.5 µM of each dNTP, 0.5 unit of Taq DNA Polymerase, 1 µl of template DNA and 17.5 µl of double-distilled water), using 5'-TAATACGACTCACTATAGGG -3' and 5'-GCTAGTTATTGCTCAGCGG-3' forward and reverse primers, respectively. After initial denaturing at 93 °C for 3 minutes, the chain-polymerase reaction was performed in 30 cycles. Each cycle consisted of three stages denaturing (30 seconds at the temperature of 92 °C), annealing (30 seconds at 60 °C) and elongation (1 min at 72 °C). PCR products were analyzed on a 1.5 % agarose gel. Then, the replicated fragments were cut off from the agarose gel and purified using a PCR product purification kit (Compony, Country [13].

Subcloning of the Chimeric Gene in the pET32a (+) Expression Vector

After the amplification of the gene, PCR products and pET32a (+) vector were digested by *Eco*RI and *Hin*dIII restriction enzymes and purified by DNA purification column [14] and salt precipitation, respectively. Digested PCR products and vector were ligated through the ligation reaction. The resulting recombinant construct¬ was transferred into *E. coli* DH5α cells that were chemically competent by thermal shock [15] and the bacterial transformation was confirmed by restriction digestion with *Eco*RI and *Hin*dIII restriction enzymes.

Expression of the Multi-Epitope Protein

To express the protein, the recombinant vector was extracted from the transformed *E. coli* DH5α by a plasmid extraction kit (GENALL, South Korea) and transferred into *E. coli* BL21 (DE3). The transformed colonies were confirmed by PCR. Expression of the recombinant protein was induced by the addition of IPTG with a final concentration of 1 mM. The expression of the recombinant protein was analyzed on a 12 % SDS-PAGE [16].

Denaturing purification of the recombinant protein using Ni-NTA chromatography column

Since the recombinant protein contained a histidine tag, it was purified using a nickel column (ShinGene, China). For this aim, the method described by Bagheri pour *et al.* was performed [17]. Shortly, the cell extract containing the recombinant protein was applied to the column which was pre-equilibrated by addition of the lysis buffer (100 mM NaH2PO4, 8 M urea, 10 mM Tris-HCl, pH=8). Then buffers C, D and E were added to the column, respectively (The composition of C, D and E buffers was as the B buffer unless the pH of these buffers were 6.3, 5.8 and 4.5, respectively). The flow-through of each washing step was collected for further analysis.

Confirmation of the Recombinant Protein Expression by the Western Blot Analysis

In order to confirm the expression of the recombinant multi-epitope protein, Western blot analysis was performed as described by Sayadmanesh et al [18]. For this aim, at first, the protein samples were loaded on a 12 % SDS-PAGE. Then, the protein samples were transferred from the gel to PVDF paper [19]. Upon completion of the transfer process, PVDF paper was blocked with blocking buffer (5% w / w solution of skimmed milk in PBST) for an overnight at 4 °C. The paper was washed with PBST three times, and then, HRP-conjugated anti-His tag antibody (Sigma, Germany) was added to the paper and the paper was placed at 37 °C for 1 h. Finally, after washing the paper with PBST, the assay buffer (DAB, 50 mM Tris pH 7.8, H_2O_2) was poured onto the paper and the presence of the band was evaluated.

RESULTS

Amplification of the Multi-Epitope Gene by PCR and Subcloning it n pET-32a (+) Expression Vector

The desired gene was amplified by PCR and verified by 1% agarose gel. As shown in Fig. 2, the desired gene was amplified successfully and there was no non-specific band.

After amplification of the fragment, the PCR products were gel purified and inserted into pET-32a (+) expression

[DOI: 10.52547/vacres.8.1.18]

vector according to the procedure outlined above. Direct PCR of the grown colonies showed that the gene has been inserted into the vector (Fig. 3). pET universal primers were used for the amplification and PCR products have a size of about 2200 bp.

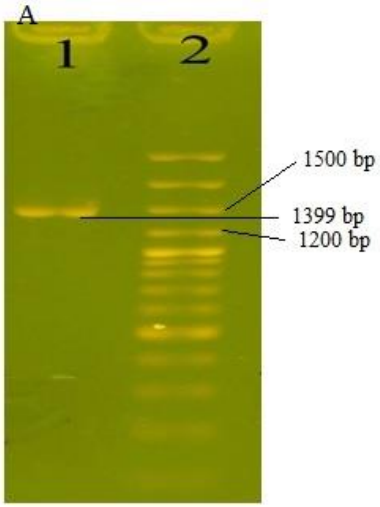

Fig. 2. The amplification of the desired gene by PCR for the subcloning. Lane 1: The amplified gene; Lane 2: DNA marker (100 bp Plus DNA Ladder).

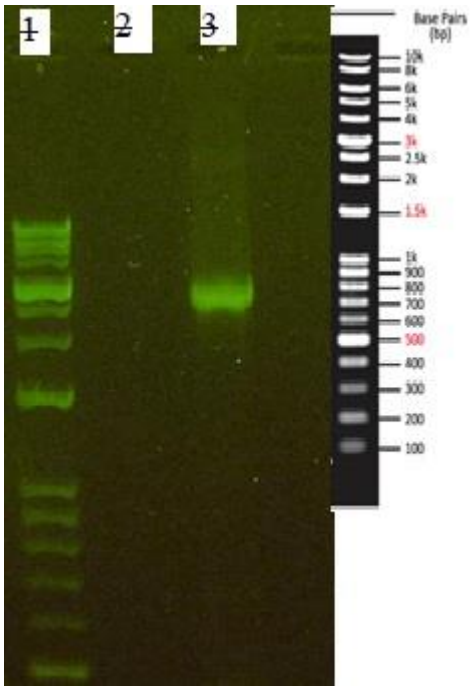

Fig. 3. The amplification of the desired gene using PCR for the confirmation of the subcloning process. Column 1: DNA marker (Excel Band 1kb Plus DNA Ladder); column 2: negative control; column 3: the amplified gene. Electrophoresis was allowed to last for 2 h, so the initial bands of the marker have exited from the gel. The last band in this picture is 500 bp.

Expression and Purification of the Recombinant Protein

Following the induction of protein expression with 1 mM IPTG, cell lysis was performed using a lysis buffer ((Na_2HPO_4 50 mM, NaCl 300 mM, Urea 8 M, pH 8) and the result was evaluated on a 12% SDS-PAGE. The resulted recombinant protein, which is composed of the desired protein fused to thioredoxin reductase (24 kDa), had an expected molecular weight of 67 kDa that can be seen in the Fig. 4.

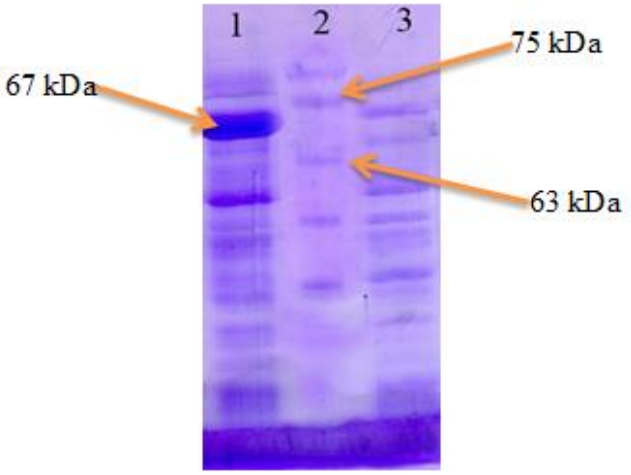

Fig. 4. Evaluation of the expression of the multi-epitope protein using a 12% SDS-PAGE. Column 1: IPTG-Induced Sample; Column 2: Protein marker (SinaClone, Iran); Column 3: Non-induced sample.

Nickel column was exploited to purify the recombinant protein. As shown in Fig. 5, the protein has been purified following the wash with the buffer containing 40 mM imidazole with a concentration of 600 µg / ml though some of the protein has been dissociated from the column after the addition of the buffer containing 100 mM imidazole.

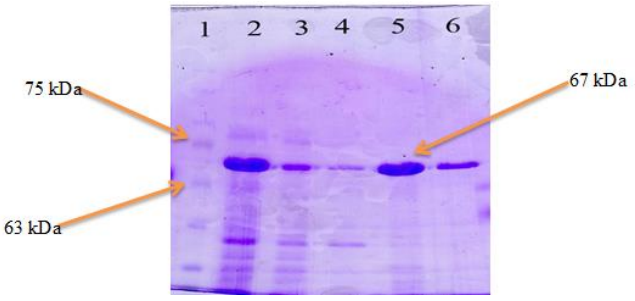

Fig. 5. Purification of the recombinant protein with nickel column. Column 1: Protein marker (10-170KDa, SinaClone, Iran); Column 2: Cellular extract of the recombinant protein before applying to the column; Columns 3, 4, 5 and 6: Flowthrough of the column following the addition of the cellular extract, 20 mM immidazole, 40 mM immidazole and 100 mM immidazole, respectively.

Western blot analysis with anti-His tag antibody

To confirm the expression of the recombinant protein which has a histidine tag, the Western blot method was used using an anti-His tag antibody. As shown in Fig. 6, Western blotting confirms that the expressed protein is the desired multi-epitope protein.

[DOI: 10.52547/vacres.8.1.18]

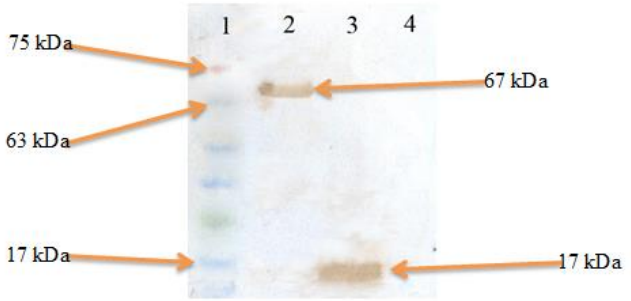

Fig. 6. Result of the Western blotting. Column 1: Protein marker (10-170KDa, SinaClone, Iran); Column 2: The recombinant protein; Column 3: CTXB recombinant protein containing a His-tag as the positive control; Column 4: BSA as a negative control.

DISCUSSION

Recombinant DNA technology has influenced almost all areas of biology and health, including pharmaceutical and vaccine industries. It has been possible to design, manufacture and produce the pharmaceutical proteins and vaccine candidates that have not already existed [20]. On the other hand, the development and advancement of bioinformatics has made it possible to evaluate the efficiency of newly-designed drugs and vaccines before experimental tests [21, 22]. In this study, a multi-epitope protein composed of major immunogens of pathogenic *Shigella* species whose efficiency as a candidate vaccine had been confirmed by bioinformatics tools [23], was synthesized in pET28a (+), subcloned in pET32a (+), expressed in *E. coli* BL21(DE3), confirmed by Western blot analysis and purified by a nickel column.

Due to the lack of an approved vaccine against pathogenic *Shigella* species, which infect millions of people in the developing countries, an effective vaccine is an urgent need. In this study, we chose the most immunogenic surface proteins of these bacteria, which were highly conserved in pathogenic strains to enhance the possibility of protection of the immunized animals against all pathogenic species of *Shigella* in future steps of evaluation of this vaccine candidate. Since the oral route is the preferred route for the vaccine delivery against enteric pathogens, LT-IIc and ctxB, two mucosal adjuvants, were also added to the chimeric antigen for the improvement of its immunogenicity. After the bioinformatics and immunoinformatics evaluations of the proposed multi-epitope vaccine [23], here, we used the exact protein sequence from that study, containing selected epitopes and the mucosal adjuvants, synthesized in pET28a (+) expression vector. The recombinant protein was successfully produced in *E. coli* BL21 (DE3) cells and was purified.

There are several studies that have exploited different antigenic proteins for immunization against *Shigella*. The use of one or more antigens from our list (of which we selected the final epitopes) has resulted in proper protectivity against *Shigella*. Heine *et al.* have used two components of the *Shigella* type III secretion system, IpaB and IpaD, to evaluate their immunogenicity in mice. They reached 40% and 90% protection levels following the oral and intranasal routes, respectively (24). Honari *et al.* have evaluated the immunogenicity of a fusion form of IpaD (STXB-IpaD) in guinea pigs via intraperitoneal route, and the animals were able to tolerate $28LD_{50}$ of the live bacteria (25). The combination of IpaB and IpaC with purified *S. flexneri* 2a LPS and intranasal administration to Aotus nancymaae, a non-human primate challenge model, has resulted in a proper immunity in this animal model (26).

There are different heterologous protein expression systems, including bacteria, yeasts, insect cell lines, mammalian cell lines and plants. Among them bacterial expression systems are frequently used for the expression of bacterial proteins or those proteins that do not require post-translational modifications, such as glycosylation. Bacteria have a simple and well-understood genetics, short generation times, low food requirements, etc. Indeed it is easy to manipulate these organisms and their scale-up id so easy. Since the desired protein had been derived from bacteria, *Shigella* species, in this study we used *E. coli* expression system for the production of the chimeric protein.

pET expression vectors were used to increase the expression of the desired gene. In these vectors, the transcription of the gene is intended to control via T7 promoter, so the use of DE3 *E. coli* strains, in which the T7 RNA polymerase gene is incorporated into the bacterial genome and under the control of the lacUV5 promoter, can greatly increase the expression of the desired gene [24]. Here at first, the synthetic gene was cloned into pET28a (+). However, the gene was not expressed in this vector and the application of different conditions did not improve the expression (results are not provided). For this reason, the gene was subcloned in pET32a (+) expression vector. The multiple ¬cloning site of this vector is located downstream of the thioredoxin protein-encoding gene and the protein will be expressed as a fusion protein which has a Trx tag. This 109 amino acid tag solubilizes the recombinant proteins expressed in *E. coli* host [25]. However, in our study the fusion protein was expressed as insoluble inclusion bodies. Indeed, genes located downstream of trx are efficiently expressed. This is due to the high efficacy of the transcription initial of the trx gene, which results in high transcription and as a result high expression of the gene [24]. Here, following the sub-cloning of the desired gene in the pET32a (+) vector¬, a high expression of the desired protein was observed (600 µg / ml). The desired recombinant protein expressed with the antibody against the histidine sequence expressed in the recombinant protein is confirmed.

In conclusion, the designed recombinant protein harboring well-conserved epitopes of potent immunogens from *Shigella*, as well as two mucosal adjuvants could successfully be expressed in a prokaryotic expression system. Based on previous *in silico* evaluations, this candidate vaccine can potentially provide an appropriate oral recombinant vaccine upon further *in vivo* immunogenicity assessments

[DOI: 10.52547/vacres.8.1.18]

ACKNOWLEDGEMENT

This work has been carried out in the Biology Research Center, Imam Hossein University, Tehran, Iran. Authors thank all staff and students of the center

CONFLICT OF INTEREST

The author declares that she has no conflict of interest.

REFERENCES

1. Kotloff KL, Riddle MS, Platts-Mills JA, Pavlinac P, Zaidi AK. Shigellosis. Lancet. 2018 Feb 24;391(10122):801-12. doi: https://doi.org/10.1016/S0140-6736(17)33296-8
2. Bardhan P, Faruque A, Naheed A, Sack DA. Decreasing shigellosis-related deaths without Shigella spp.–specific interventions, Asia. EMERG INFECT DIS . 2010;16(11):1718. doi: https://doi.org/10.3201/eid1611.090934.
3. Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow DL, Sansonetti PJ, et al. Global burden of Shigella infections: implications for vaccine development and implementation of control strategies. Bull World Health Organ. 1999. doi: https://doi.org/10.1086/386326. Epub 2004 Apr 28.
4. Von Seidlein L, Kim DR, Ali M, Lee H, Wang X, Thiem VD, et al. A multicentre study of Shigella diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. PLoS Med. 2006;3(9):e353. doi: https://doi.org/10.1371/journal.pmed.0030353.
5. Mani S, Wierzba T, Walker RI. Status of vaccine research and development for Shigella. Vaccine. 2016;34(26):2887-94. doi: https://doi.org/10.1016/j.vaccine.2016.02.075.
6. Avakh Majalan P, Hajizade A, Nazarian S, Pourmand MR, Amiri Siyavoshani K. Investigating the prevalence of Shigella species and their antibiotic resistance pattern in children with acute diarrhea referred to selected hospitals in Tehran, Iran. Appl. Biotechnol. Rep. 2018;5(2):70-4. doi: https://doi.org/10.29252/JABR.05.02.06.
7. Sansonetti PJ. War and peace at mucosal surfaces. Nat Rev Immunol. 2004;4(12):953. doi: https://doi.org/10.1038/nri1499.
8. Livio S, Strockbine NA, Panchalingam S, Tennant SM, Barry EM, Marohn ME, et al. Shigella isolates from the global enteric multicenter study inform vaccine development. Clin Infect Dis. 2014;59(7):933-41. doi: https://doi.org/10.1093/cid/ciu468.
9. Camacho AI, Irache JM, Gamazo C. Recent progress towards development of a Shigella vaccine. Expert Rev Vaccines. 2013;12(1):43-55. doi: https://doi.org/10.1586/erv.12.135.
10. Martinez-Becerra FJ, Kissmann JM, Diaz-McNair J, Choudhari SP, Quick AM, Mellado-Sanchez G, et al. Broadly protective Shigella vaccine based on type III secretion apparatus proteins. Infect Immun. 2012;80(3):1222-31. doi: https://doi.org/10.1586/erv.12.135.
11. Pore D, Chakrabarti MK. Outer membrane protein A (OmpA) from Shigella flexneri 2a: a promising subunit vaccine candidate. Vaccine. 2013;31(36):3644-50. doi: https://doi.org/10.1016/j.vaccine.2013.05.100
12. Suhrbier A. Multi-epitope DNA vaccines. Immunol. Cell Biol. 1997;75(4):402-8. doi: https://doi.org/10.1038/icb.1997.63.
13. Joshi M, Deshpande J. Polymerase chain reaction: methods, principles and application. Int J Biomed Res. 2010;2(1):81-97. doi: https://doi.org/10.7439/ijbr.v2i1.83.
14. LaMontagne M, Michel Jr FC, Holden P, Reddy C. Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. J Microbiol Methods. 2002;49(3):255-64. oi: https://doi.org/10.1016/S0167-7012(01)00377-3.
15. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press. 1989. Cold Spring Harbor, USA.
16. Hajizade A, Salmanian AH, Amani J, Ebrahimi F, Arpanaei A. EspA-loaded mesoporous silica nanoparticles can efficiently protect animal model against enterohaemorrhagic E. coli O157: H7. Artif Cells Nanomed Biotechnol. 2018;46(sup3):S1067-S75. doi: https://doi.org/10.1080/21691401.2018.152967617.
17. Bagheripour M, Ebrahimi F, Hajizadeh A, Nazarian S. Immunogenicity Effect of Chitosan Nanoparticles Containing Botulinum Neurotoxin E binding Domain Recombinant Protein in Mice. J Mazandaran Univ Med Sci. 2015;25(124):37-47. doi: https://doi.org/10.22038/ijbms.2019.25333.6258.
18. Sayadmanesh A, Ebrahimi F, Hajizade A, Rostamian M, Keshavarz H. Expression and purification of neurotoxin-associated protein HA-33/A from Clostridium botulinum and evaluation of its antigenicity. Iran Biomed J. 2013;17(4):165. doi: https://doi.org/10.6091/ibj.1216.2013.
19. Darrow RA, Darrow RM, Organisciak DT. Biochemical characterization of cell specific enzymes in light-exposed rat retinas: oxidative loss of all-trans retinol dehydrogenase activity. Curr Eye Res. 1997;16(2):144-51. doi: https://doi.org/10.1076/ceyr.16.2.144.5092.
20. Patten PA, Howard RJ, Stemmer WP. Applications of DNA shuffling to pharmaceuticals and vaccines. Curr Opin Biotechnol. 1997;8(6):724-33. doi: https://doi.org/10.1016/S0958-1669(97)80127-9.
21. Bernstein FC, Koetzle TF, Williams GJ, Meyer Jr EF, Brice MD, Rodgers JR, et al. The Protein Data Bank: A computer-based archival file for macromolecular structures. EUR J BIOCHEM. 1977;80(2):319-24. doi: https://doi.org/10.1016/S0022-2836(77)80200-3.
22. Hajizade A, Ebrahimi F, Amani J, Arpanaei A, Salmanian AH. Design and in silico analysis of pentavalent chimeric antigen against three enteropathogenic bacteria: enterotoxigenic E. coli, enterohemorragic E. coli and Shigella. Biosc. Biotech. Res. Comm. 2016;9(2):229-43. doi: https://doi.org/10.21786/bbrc/9.1/9.
23. Nosrati M, Hajizade A, Nazarian S, Amani J, Vansofla AN, Tarverdizadeh Y. Designing a multi-epitope vaccine for cross-protection against Shigella spp: An immunoinformatics and structural vaccinology study. Mol Immunol. 2019;116:106-16. doi: https://doi.org/10.1016/j.molimm.2019.09.018.
24. Heine SJ, Diaz-McNair J, Martinez-Becerra FJ, Choudhari SP, Clements JD, Picking WL, Pasetti MF. Evaluation of immunogenicity and protective efficacy of orally delivered Shigella type III secretion system proteins IpaB and IpaD. Vaccine. 2013 Jun 19;31(28):2919-29. https://doi.org/10.1016/j.vaccine.2013.04.045
25. Honari H, Amlashi I, Minaee ME, Safaee S. Immunogenicity in guinea pigs by IpaD-STxB recombinant protein. J A M . 2013 Jul 10;16(4):83-93.
26. Walker RI, Wierzba TF, Mani S, Bourgeois AL. Vaccines against Shigella and enterotoxigenic Escherichia coli: a summary of the 2016 VASE Conference. Vaccine. 2017 Dec 14;35(49):6775-82. https://doi.org/10.1016/j.vaccine.2017.09.045
26. Walker RI, Wierzba TF, Mani S, Bourgeois AL. Vaccines against Shigella and enterotoxigenic Escherichia coli: a summary of the 2016 VASE Conference. Vaccine. 2017 Dec 14;35(49):6775-82. https://doi.org/10.1016/j.vaccine.2017.09.045
27. Mahmoudi S, Abtahi H, Bahador A, Mosayebi G, Salmanian AH, Teymuri M. Optimizing of nutrients for high level expression of recombinant streptokinase using pET32a expression system. Mædica. 2012;7(3):241.
28. Gopal GJ, Kumar A. Strategies for the production of recombinant protein in Escherichia coli. The protein journal. 2013;32(6):419-25. https://doi.org/10.1007/s10930-013-9502-5.

[DOI: 10.52547/vacres.8.1.18]