Designing and Expression of Recombinant Chimeric Protein Containing CtxB and OmpW from *Vibrio Cholerae* and Evaluation of Its Immunogenicity

Atina Vakili[1], Seyed Latif Mousavi Gargari[1*], Shahram Nazarian[2], Jafar Amani[3]

[1]Department of Biology, Faculty of Basic Science, Shahed University, [2]Department of Biology, Faculty of Science, Imam Hussein University, [3]Applied Microbiology Research center, System biology and poisonings institute, Baqiyatallah University of Medical Sciences, Tehran, Iran

ABSTRACT

Background: Cholera disease caused by *Vibrio cholerae* remains a major cause of morbidity and mortality throughout the world. Various strategies with different proteins as immunogens have been tried for vaccine development, none of which have been sufficiently effective to preclude cholera. Chimeric proteins, with their ability to present multiple antigens at the same time, can play important roles in immunization. **Objective:** To evaluate the immunogenicity of a chimeric construct, comprised of OmpW and CtxB as immunogenic proteins of *Vibrio cholera,* in BALB/c mice. **Methods:** The construct was designed after bioinformatics assessments and then expressed in *E.coli*. Chimeric protein, OmpW, and CtxB were purified with Ni-NTA chromatography and confirmed by Western blotting. Mice were immunized with purified recombinant proteins. The antibody titers and specificity of the immune sera were then analyzed by ELISA and challenged on the pups of immunized mice with 1, 5 and 10 LD50. Mice ileal loop assay was also performed. **Results:** Significant differences were observed in antibody titers in immunized mice compared to the control groups. Infant mouse challenge was performed so as to compare the protective efficacies of the selected immunogen regimens. Of the Pups from dams immunized with chimeric protein which received 1 LD50, 75% survived. Pups belonging to PBS-immunized dams, experienced 100% mortality. The serum raised toward immunogenic construct, inhibited cholera toxin activity in ileal loop test up to 68%. **Conclusion:** Chimeric construct is able to induce the immune system and provide up to 75% inhibition of toxin activity against 1 LD50 of *Vibrio cholerae*.

Vakili A, et al. Iran J Immunol. 2018; 15(3):207-220.

Keywords: Bioinformatics, CtxB, Immunization, OmpW, Recombinant Protein, *Vibrio Cholerae*

---

*Corresponding author: Dr. Seyed Latif Mousavi Gargari, Department of Biology, Shahed University, Tehran, Iran, e-mail: slmousavi@shahed.ac.ir

INTRODUCTION

Cholera, a severe lethal secretory diarrheal disease, is caused by the *Vibrio cholerae*, a gram-negative, motile, curved-rod bacterium(1). Although there are more than 200 serogroups of *V. cholerae*, the epidemics of cholera are merely caused by strains of serogroup O1 and O139(2). These epidemics are most common in developing countries which suffer from unsafe drinking water, limited health care, and poor sanitary system; if untreated, fatality rate of cholera can reach as high as 70%(3). Despite the fact that the disease is easily curable, it is still deemed as a major health issue, with a rate of 3-5 million cases a year. Moreover, it is known as the second leading cause of death in children under the age of 5 years (4). One of the reasons for its sustainability is the absence of vaccines with long-term effects and protections(5). Bacterial action is made possible through its attachment to the small intestine and colonization. A large number of factors including lipopolysaccharide (LPS), OM porins (OMPs), TCP, accessory colonization factors (ACFs), the core oligosaccharide (core OS) lipid A and flagella are important in its colonization (6).After proliferation, the release of heat-sensitive exotoxin stimulates intestinal mucosa, disrupting sodium ion exchange in the intestinal mucosa cells, and releasing large amounts of water and electrolyte into the intestine which, in turn, reduces blood plasma levels and eventuates in death (6,7).

Cholera toxin (CT), a key factor in inducing such conditions, is encoded by the ctxAB gene located in bacteriophage CTXΦ (8). CtxA is the active unit and CtxB is a pentameric binding unit with no toxic properties, merely binding cholera toxin to GM1 ganglioside receptors(9).In addition to antitoxic immunity, antibacterial immunity has also an important role in preventing cholera, as it protects against both lipopolysaccharide (LPS) and outer membrane (OM) proteins (OMPs) of *V. cholerae,* through inhibiting the intestinal colonization of *Vibrio* (10).Outer membranes (OM_s) in Gram-negative bacteria work as barriers, and via their channels, they promote the uptake of necessary nutrients for the cells. These protein channels are monomeric or trimeric barrels with 12-22 anti-parallel β-strands. There is also a noticeable number of smaller, monomeric β-barrels which consist of 8 or 10 β-strands(11). One of these small monomeric β-barrels is OmpW, an 8-stranded β-barrel, 22 kDa protein, comprised of a narrow, long, hydrophobic channel acting as an ion channel. This hydrophobic channel causes distinction between OmpW and other OMP channels, which are usually hydrophilic(12). Various environmental conditions such as temperature, salinity, availability of food and oxygen influence the expression of OmpW in *V. cholerae*. As mentioned, this protein is effective in the attachment of pathogenic bacteria to the intestine and providing an immune response against them. A study on mutant *V. cholerae* that lacked OmpW showed a 10-fold reduction in the colonization of the bacteria in mice, compared to those containing OmpW (13). Attenuated or inactivated pathogens are traditionally used as vaccines. Nowadays, on the other hand, recombinant protein vaccines, chimeric ones in particular, offer several advantages for cloning, expressing and purification of genes from different etiologic agents to test as a vaccine. The ability of chimeric construct to present multiple antigens at the same time can play important roles in immunization(14). Bioinformatics approaches have recently been employed to identify protein subunits as vaccine candidates. Such methods ultimately offer more rapid advances towards preclinical studies with vaccines. Comparative structural and immunological analysis of antigens eventuates in a logical selection of a combination of immunogens for a multi subunit chimeric vaccine. Chimer proteins

contain many subunits and linker sequences with adjuvant properties, can increase the effect of recombinant proteins(15).Therefore, in this work, based on the immunogenic properties of these two proteins and the benefits of chimeric vaccines, a chimeric construct containing ompW and ctxB, was designed and expressed in *E.coli,* and its immunogenicity was evaluated against *V. cholerae* in mice.

MATERIALS AND METHODS

Bioinformatics Construct Design. To design the recombinant construct, the sequences of genes, ompW and ctxB, were obtained from the NCBI. The presence of a signal peptide in the sequences was determined with Online Signal peptide prediction software(16). A suitable linker, including (EAAAK) amino acids, was selected to maintain the 3D structure of each protein separately within the chimer (17). The effect of the linker on the 3D protein structure of the chimeric protein was investigated by I-TASSER software(18). In order to recognize the errors in the generated models, coordinates were supplied by uploading 3D structures in PDB format into PROSA-web, which is frequently employed in protein structure validation. The structure was validated to see the quality of the resulting stereochemistry by Ramachandran plot in PROCHECK software (19). Structural features such as RNA stability were examined by RNA mfold program (20). The prediction of humoral immunity generated by recombinant protein was done by CBTOPE bioinformatics software for discontinuous epitopes and BCEpred for linear epitopes (21). The molecular weight of protein, the composition of its amino acids, the number of positive and negative amino acids, half-life and instability index were specified through the use of ProtParam program. In order to design the primers of the chimeric construct, a forward primer was designed and for the reverse primer, the stop codon of the ompW gene was omitted and the linker sequence and the sequence of restriction enzyme were then added to design the primers of the ompW segment. The primers of the ctxB segment were also designed and checked by Oligo Analyzer software. For OmpW recombinant protein, only a reverse primer was designed. The forward primer used for the amplification of OmpW was same as the one employed for the chimeric construct.

Table 1. The sequences of the primers used in this study.

Sequencename	Target gene	Primer sequence5′→3′	PCR product (bp)
Chimer	*ompW*	ompW forward:TTAAGGATCCCACCAAGAAGGTGAC ompW reverse with linker: TATAGAATTCTTTTGCCGCAGCTTCGAACTTATAACCACC	615
	ctxB	ctxB forward: TGCAGAATTCACACCTCAAAATATTACTG ctxB reverse: TGTCCTCGAGTTAATTTGCCATACTAATTG	312
ompW	*ompW*	ompW reverse : TCGTGAATTCTTAGAACTTATAACCACC	603

Cloning the Constructs. The ompW gene was amplified by ompW forward and ompW linker primers using *Vibrio cholera* cell suspension as template for PCR. PCR product was digested with Rapid Digest *BamHI* and *EcoRI* enzymes, ligated to the pET28a with T4 ligase and transformed into component *E.coli* B121 (DE3) cells. The cells were cultured on LB agar plate containing 70 µg/ml kanamycin. The colonies were analyzed with colony PCR and restriction digestion. To create the chimeric construct, the ctxB PCR product was digested with *EcoRI* and *XhoI* enzymes and following purification, it was ligated to the plasmid harboring ompW with the linker and transformed into component *E.coli* Bl21 (DE3)cells. ompW gene was amplified with forward and reverse primers containing the stop codon and ligated to pET28a plasmid with *BamHI* and *EcoRI* restriction sites.

Expression and Purification of the Recombinant Proteins. After confirming all the three clones, OmpW, CtxB, and chimer, the recombinant clones were inoculated into LB broth sublimated with70 µg/ml kanamycin and incubated at 37°C via shaking. At OD_{600} = 0.6-0.7, cells were induced with IPTG for 4-5 h with a final concentration of 1 mM. The cells were harvested and lysed and recombinant proteins in inclusion bodies were dissolved in phosphate buffer (100mM NaH_2PO_4, 10mM Tris-HCl,) containing 8M urea. Protein expressions were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)(22). Proteins were purified via nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen). The columns were equilibrated with binding buffer and 0.5-1 ml (approximately 400 µg) of proteins were loaded onto the columns. Each column was washed with 20, 40 and 100 mM concentration of imidazole to remove nonspecific binders; finally, the recombinant proteins were eluted with the help of 250 mM concentration of imidazole. All fractions were collected and analyzed on the SDS-PAGE and protein concentration was determined through Bradford assay.

Western Blotting. Recombinant proteins were evaluated by western blotting. Proteins were electrophoresed on SDS-PAGE and transformed onto the nitrocellulose membrane using transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% SDS and 20% methanol). The membrane was kept in the blocking buffer (PBST + 5% Skimmed Milk Powder) overnight and was then washed with PBST (137 mMNaCl, 2.7mMKCl, 4.3mM Na_2HPO_4 + 0.05% Tween). Mouse HRP-conjugated anti-His-tag IgG (1:10000 in PBST) was added to nitrocellulose membrane and incubated for 2h. The membrane was washed with PBST and DAB (3, 3´-diaminobenzidine) added as a chromogenic substrate. The reaction was stopped by distilled water (23).

Immunization. 5-week old female BALB/c mice (20-25 gr) procured from the Razi Institute, Tehran, Iran, were selected and divided into five groups: control, chimer, ompW, ctxB and ctxB + ompW. The mice were housed under standard and ventilated situation in the animal care facility of Shahed University. All animal experiments were conducted in compliance with the Welfare Act and regulations related to experiments involving animals. The principles in the Guide for the Care and Use of Laboratory Animals were followed (24). The animal care rule was ethically certified by Shahed University. Except for the controls, all groups, received 4 subcutaneous injections of 20 µg of appropriated proteins in 100 µl of PBS mixed with 100 µl of alum adjuvant from Razi Institute on days 0, 15, 30 and 45. The control group received only PBS and adjuvant. Blood samples were collected on days 29, 44 and 59 from the eye corners of mice with a capillary; the sera separated from blood samples were stored at -20°C.

Determination of Serum IgG Titer. The Indirect Enzyme-linked Immunosorbent Assay (ELISA) was carried out to determine the IgG level in the mice sera. Five µg of

each recombinant protein in 100 µl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.5) were coated in ELISA plate wells and incubated overnight at 4°C. The wells were washed with 0.05% PBST and non-specific sites were blocked by blocking buffer (PBST + 5% Skimmed Milk Powder) for 1h at 37°C. Wells were washed with PBST and a serial dilution of antibody serum from 1:100 to 1:12800 in PBST was added to each well. The plate was kept for 2h at 37°C and then washed with PBST. Anti-mouse IgG HRP conjugate obtained from Abcam company (1/16000 dilution in PBST) was added to each well and incubated at 37°C for 1.5h. After washing, 100 µl of 3, 3´, 5, 5´-tetramethylbenzidine (TMB) substrate (0.4 g/liter) was added to each well; the reaction was stopped by H_2SO_4 3N and the plate was read at 450 nm(25).

Challenges. For LD50 Assay, 12 BALB/C mice infants (5 days old) of germfree dams were divided into three groups. Mice in each group were pipette fed with 10^6, 10^7, 10^8 CFU of *Vibrio cholera* in 20 µl normal saline. The bacterial dose, at which half of the infants died after 48 hours, considered as LD50 (26).To determine the immunity rate transformed from mother to the infants, all immunized mice were mated 15 days after the last injection. Infants under five days old were fed with 1, 5, 10 and 15 folds of LD50 of *Vibrio cholerae* and their mortality rate was monitored for 24 hours. The small intestine of all infants, either alive or dead, was removed and properly homogenized in PBS. Different dilutions were prepared from each homogenate and cultured in thiosulfate-citrate-bile salts-sucrose (TCBS) agar medium. The colony-forming units were further counted (1).

Ileal Loop Test. Two 6-week-old male mice were selected and fasted for 24 hours prior to the surgery.To prepare different combinations of serum and bacteria, 10^7 CFU of bacteria were mixed with the immunized mice sera from each group in ratios, 1:2 and 1:4 in a final volume of 50 µl and incubated for 45 min at 37°C. Animals were anesthetized with intraperitoneal injection of 20 µl ketamine and 10 µl xylazine in a total volume of 200 µl (3:2, 1 ml kg^{-1}). A small incision was created in the abdominal region of each mouse and the end of their ileum was knotted; three loops with lengths of 2 to 3 cm were created by knocking the nodes. Into the first node, 10^7 CFU of bacteria were injected as control and the two other loops were injected with 2 dilutions of serum and bacteria. The results were evaluated after 12 hours of recovery by reopening the abdomen, checking the loops, and obtaining the weight/length ratio. A ratio of at least 0.1 gr/cm indicates a positive reaction to diarrhea (27).

Statistical Analyses. The results of ELISA for antibody responses between immunized and non-immunized groups were analyzed using SPSS version 24 and One-Way ANOVA. Differences were considered statistically significant if $P<0.05$.

RESULTS

Bioinformatics Construct Design.

Gene's sequences of ompW and ctxB (Gene Bank Accession KJ722608, AB449339.1, respectively) were obtained; the signal peptide was omitted, and the total sequence of the chimeric construct was ultimately designed. Figure 1 shows the schematic diagram of the construct with each gene domain separated by the linker. The 3D modeled structure of protein was generated by I-TASSER software. The ribbon model of I-TASSER server predicted that the tertiary structure of the chimeric protein has two distinct domains, meaning the fusion protein could be efficiently separated

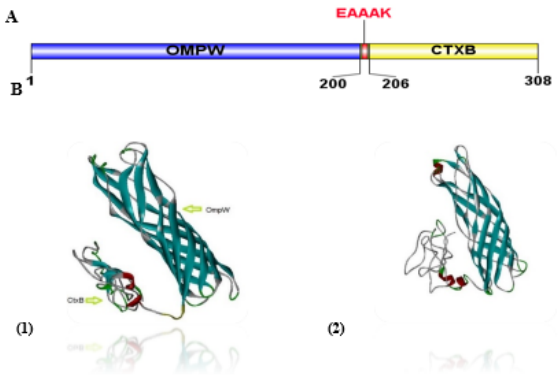

Figure 1. The chimeric construct structure. (A) Schematic diagram of chimeric construct containing OmpW, linker and CtxB. (B) 3D structure of chimeric protein predicted by I-TASSER. (1) With EAAAK linker. (2) Without linker.

through the inserted linkers. In the absence of linker, the protein structural collapse was well observed (Figure 1). The confidence score (C-score) for estimating the quality of the models predicted by I-TASSER was 1.34. C-score typically falls in the range of −5 to 2, where a C-score of higher values signifies a high model confidence. Ramachandran plot in RAMPAGE server estimates the potential for errors in tertiary structure prediction. The plot specified that 94.1% of the residues were in the favored and allowed area, while 5.9% belonged to the disallowed region (Figure 2). The quality of the predicted structure was evaluated by the PROSA software. The z-score of the input structure was within score ranges typically observed for native proteins of similar sizes (Figure 2).The predicted structure of the 5'mRNA initiation region of the chimeric gene is visible (supplementary Figure 1). The minimum energy for the structure was -280.30 kcal. Prediction of linear epitopes is possible by use of properties such as flexibility, hydrophobicity, polarity, and surface and bioinformatics algorithms.

Table 2.Results of linear epitopes prediction.

Sequence	Start position	Score	Sequence	Start position	score
YANIETTATYKAGADA	182	0.95	VFMIAGGYKFEAAAKT	208	0.78
GKREMAIITFKNGATF	255	0.92	SFEVLAATPFSHKIST	73	0.76
NGTGTNAGLSDLKLDD	141	0.92	TPQNITDLCAEYHNTQ	223	0.76
DLCAEYHNTQIHTLND	229	0.91	SQHIDSQKKAIERMKD	277	0.75
AIERMKDTLRIAYLTE	286	0.90	KLCVWNNKTPHAIAAI	306	0.74
DVEINPWVFMIAGGYK	201	0.90	YKFEAAAKTPQNITDL	215	0.74
SWGLAANVGFDYMLND	157	0.89	GELGSLGDIGETKHLP	91	0.71
GETKHLPPTFMVQYYF	100	0.89	QVEVPGSQHIDSQKKA	271	0.71
HKISTSGGELGSLGDI	84	0.88	AVLAALSSAPVFAHQE	9	0.70
YKAGADAKSTDVEINP	191	0.88	DTLRIAYLTEAKVEKL	292	0.70
YYFGEANSTFRPYVGA	113	0.86	GAGLNYTTFFDESFNG	127	0.69
YTESLAGKREMAIITF	249	0.83	GFDYMLNDSWFLNASV	165	0.67
AGLSDLKLDDSWGLAA	147	0.83	DKVLNTQSELAVNSNT	43	0.66
NDKIFSYTESLAGKRE	243	0.82	TEAKVEKLCVWNNKTP	300	0.66
SAPVFAHQEGDFIVRA	16	0.82	NSTFRPYVGAGLNYTT	119	0.64
IASVVPNDSSDKVLNT	33	0.81	LGLTLGYMFTDNISFE	60	0.59
SWFLNASVWYANIETT	173	0.80	HNTQIHTLNDKIFSYT	235	0.59

The molecular weight and PI of the chimer was 33978.99 Da and 5.17, respectively. The total number of amino acids with a negative charge (glutamic acid, aspartic acid) was 35, and the sum of amino acids with a positive charge (arginine, lysine) was 24. The chimer aliphatic index and instability was 77.44 and 33.10, respectively. The instability index expresses the sustainability of the recombinant protein. Proteins with an index less than 40 are considered as stable. Primer sequences were designed after analysis with Oligo Analyzer software. *BamH1, EcoRI & XhoI* restriction enzymes were applied (Table 1).

Expression and Purification of Recombinant Proteins.

The expression of each clone was analyzed on SDS-PAGE and the proteins with 15.4 kDa for CtxB, 24.8 kDa for OmpW and 36.8 kDa for chimeric construct appeared on the gel (supplementary Figure 2). Recombinant proteins were purified by Ni-NTA affinity chromatography using different concentrations of imidazole and verified with western blotting (Figure 3).

Table 3.Results of discontinues epitopes prediction.

Amino acid	position	score	Amino acid	position	score	Amino acid	position	Score
GSH	1-3	4	SDLKLDDSWG	131-140	4	TFKN	244-247	4
SVV	16-18	4	A	142	4	G	248	5
P	19	5	VG	145,146	4	AT	249,250	6
N	20	4	D	148	4	FQV	251-253	5
DS	21,22	4	W	155	4	E	254	6
L	33	4	AS	159,160	4	VPG	255-257	8
S	70	4	VW	161,162	5	S	258	7
LPP	86-88	4	YANI	163-166	4	QH	259,260	8
A	99	4	A	174	5	I	261	7
STFRP	101-105	4	GA	175,176	4	D	262	8
VG	107,108	4	D	177	5	S	263	6
F	116	4	AKS	178-180	4	Q	264	5
DES	118-120	4	D	182	4	KLCV	287-290	4
FN	121,122	4	PQNITDLCAEY	205-215	4			
TNAGL	125-130	4	H	221	4			

Determination of Serum IgGTiter.

Blood samples were collected and IgG antibody titers were evaluated. Indirect-ELISA confirmed the most significant ($P<0.05$) antibody titer after each booster dose in mice test group administrated with chimeric protein, CtxB, and OmpW mixture compared

with the control group (Figure 4,5). The effect of booster immunization number was analyzed via repeated measurements. Results indicated that the increase in antibody titer following each booster dose administration was significant (P<0.05).

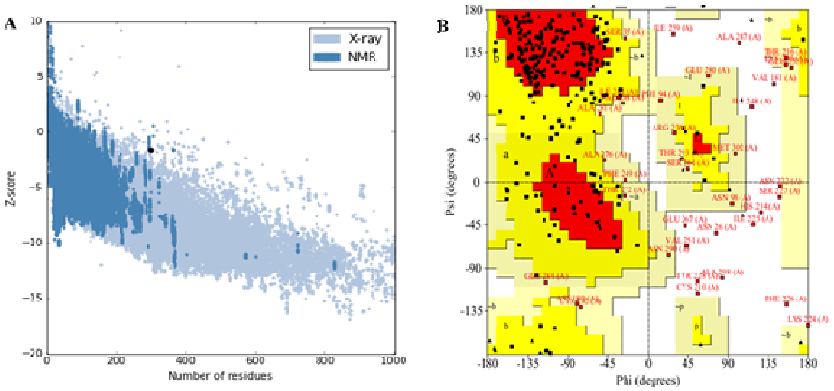

Figure 2. PROSA-web z-score chimeric protein plot.(A) The plot shows results with a z-score≤10. The chimeric z-score is highlighted as a large dots. The value is in the range of native conformations. Validation of protein structure using Ramachandran plot. **(B)** The Ramachandran plot revealed that 84.8% of amino acid residues from modeled structure were incorporated in the favored regions (A, B, and L) of the plot. 9.6% of the residues were in allowed regions (a, b, l, and p) of the plot.

The level of anti-OmpW antibodies increased in mice vaccinated with proteins containing OmpW (OmpW, OmpW+CtxB groups). Moreover, elevated levels of anti-CtxB subunit antibodies appeared in mice immunized with recombinant proteins containing CtxB subunit (P<0.05).

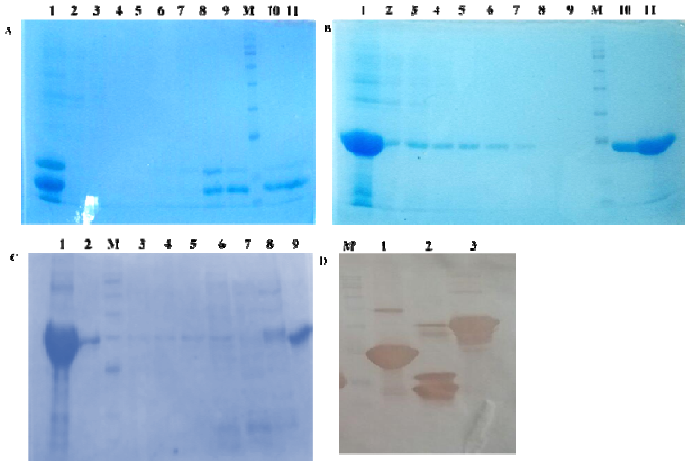

Figure 3. SDS-PAGE analysis of proteins purification.(A) CtxB, lane 1 clear cell lyset, lanes 3-5: washing the columns with 20 mM imidazole, 6-7: with 60 mM imidazole, 8-9: with 100 mM imidazole, 10-11: CtxB eluted with 250mM imidazole. **(B)** OmpW, lane 1 clear cell lyset, lanes 3-5: washing the columns with imidazole 20 mM, 6-9: with imidazole 40 mM, 10-11: purified OmpW with imidazole 250mM.**(C)** Chimer, lane 1 clear cell lyset, lanes 3-8: washing the columns with imidazole 20 mM, 9: purified Chimer with imidazole 250mM.**(D)** Western Blotting analysis of the 3 proteins, lane 1: OmpW, lane 2: CtxB, lane 3: chimeric protein. M: Molecular weight marker.

There were no significant differences (P>0.05) in the ELISA results of IgG generated against OmpW + CtxB or OmpW alone when OmpW protein was used as an antigen. Similar results were obtained when CtxB was coated as an antigen on ELISA wells (supplementary Figure 3).

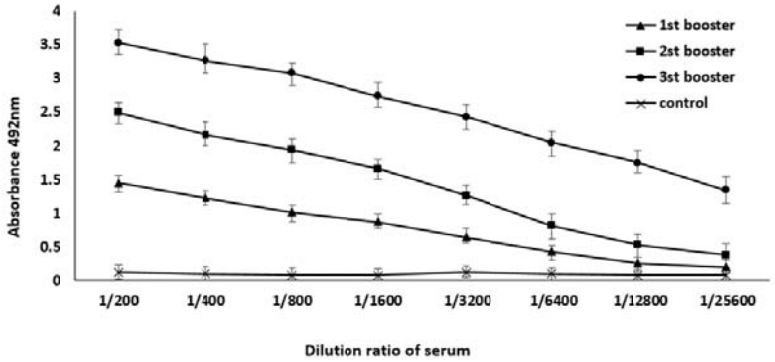

Figure 4. Immune response type analysis of mice immunized with chimeric protein. Serum specific IgG titers from mice immunized. The detection was performed by ELISA. Purified chimeric protein was used as antigen.

Challenges.

The LD50 dose was 10^7 CFU bacteria, considered as the base for the animal challenge. After mating, the childbirth was monitored, where only animals receiving the chimer and OmpW+CtxB antigens and the control group had childbirth. The challenge was performed on mice pups, on the basis of the protocol mentioned in the methodology. All control pups died within 24 hours. In the chimeric pups, all infants receiving 5LD50 died (uncountable CFU, like the control group), whereas out of the four pups which received 1LD50 of bacteria, three stayed alive (average of 1400×10^3 CFU on TCBS agar medium). Surprisingly, of the three pups of OmpW+CtxB group receiving 5LD50, one stayed alive (640×10^3 CFU on TCBS agar medium), CtxB and OmpW groups were eliminated, as no pups were obtained after mating. Results showed a 75% inhibition in mortality among the infants who received 1LD50. Concerning ileal loop test, the result showed a 68% inhibition of toxin activity in the mouse treated with the chimer serum and 40% inhibition of toxin activity in the mouse treated with CtxB+OmpW serum. Control group obtained a weight/length ratio of 0.1 gr/cm, indicating a positive reaction to diarrhea (Figure 6).

DISCUSSION

Vibrio cholerae, a gram-negative, motile bacteria, and the causative agent of a severe diarrheal disease, is still a major health issue taking the lives of myriads throughout the world (1). Following after oral entry, the bacteria quickly transmits itself to the small intestine, the first colonization site, and induces its pathogenic factors, such as cholera toxin.

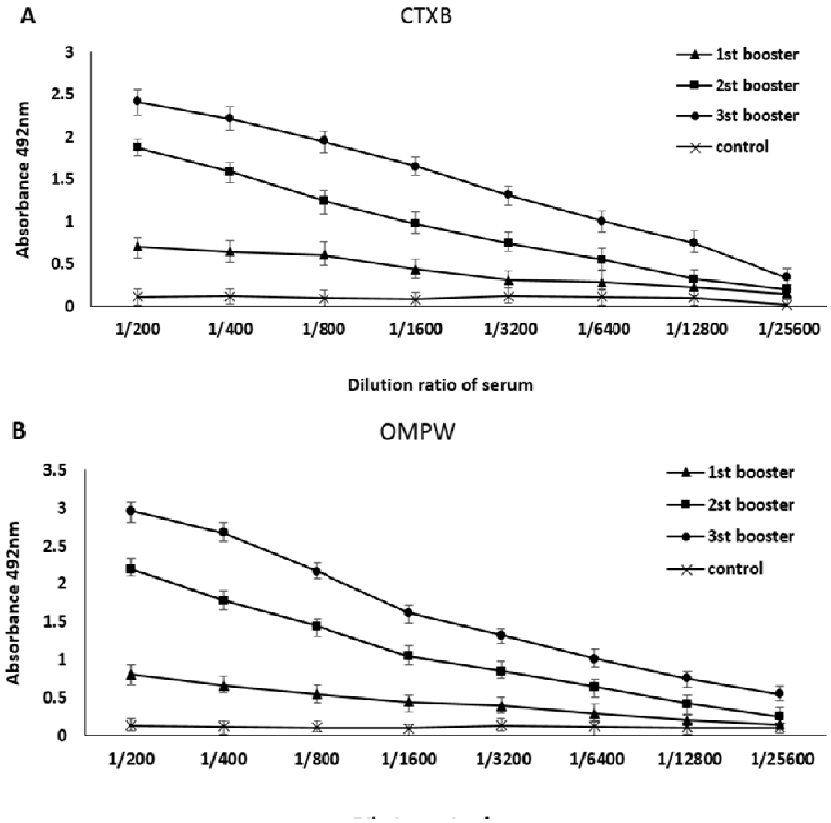

Figure 5. IgG response to CTXB and OMPW mixed proteins in sera of immunized mice. A) Analysis of the CTXB-specific IgG titers. **B)** Analysis of the OMPW-specific IgG titers.

Severe watery diarrhea, caused by cholera toxin, leads to serious dehydration in the body, which, if not timely treated, eventuates in shock and death. Rehydration with oral or intravenous serums containing glucose, sodium chloride, potassium chloride and trisodium citrate, and, in some cases, antibiotics is a general treatment of cholera disease(2). In recent years, antibiotics like tetracycline or quinolone have been extensively utilized to reduce the symptoms of cholera; however, the advent of strains showing antibiotic resistance has curbed antibiotic treatment (3).

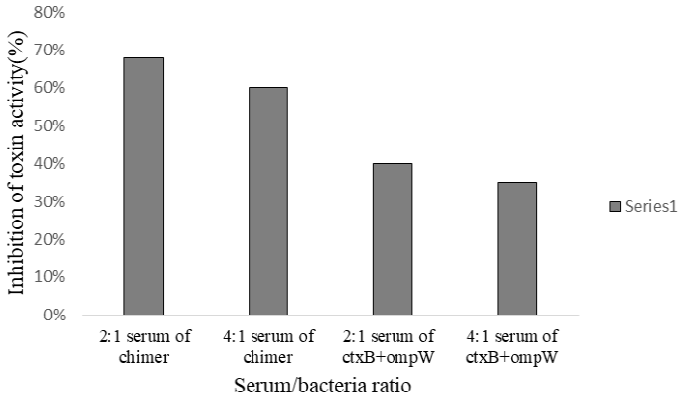

Figure 6. *In vivo* inhibitory effect of chimer and CtxB+OmpW sera on cholera toxin.

Therefore, finding a proper treatment with long-term effects is indispensable. In this regard, vaccination is one of the most efficient ways of decreasing the outbreak of cholera. The objective in the present research was to find out an appropriate vaccine candidate with broad immune response against the pathogenic agents of *V.cholorea*, especially those conducing to its colonization and toxin inoculation into the host cells. Our DNA fragment consisted of two putative antigens, CtxB and OmpW with a proper linker (EAAAK) in between.CtxB is a non-toxic part of CT which is responsible for attaching the toxin to the epithelial cells. This subunit is used in many vaccines as a mucosal adjuvant and is able to provide an immune response (4). Honari *et al.* expressed CtxB with StxB from *Shigella* in the presence of a non-Forine Linker in *E. coli*. In addition to benefiting from the natural adjuvant properties of the CtxB, an appropriate immune response was developed against both *Vibrio* and *Shigella* (5). Recombinant CtxB as an immunogen, was able to act as an effective immunogen for the induction of humoral immunity against cholera toxin, and the antibodies raised toward toxin could preclude bacterial binding to the GM1 receptor (7). OmpW is highly conserved in *Vibrio cholerae* strains, playing very important roles in bacterial food harvesting, disabling the host defense system and the interaction with host cells during bacterial adhesion, hence the fact that it is regarded as a vaccine candidate(32). Karunasagar *et al.* evaluated the immunogenicity of recombinant ompW and aha1 from *Aeromonashydrophila* in a particular type of fish; both proteins were immunogen and provided protection in fish, yet OmpW was nominated as a better vaccine candidate (33).Yu *et al.* expressed a chimer containing ompW and four other genes from the omps of *Vibrio parahaemolyticus* in *E. coli.* When used as an immunogen in fish, this chimer was able to protect 80–90% of vaccinated groups, whereas all 10 control fish died challenged with 10^8cfu ml^{-1} of *V. parahaemolyticus* (34). Cai *et al.* developed a DNA vaccine containing the ompW gene from *Vibrio alginolyticus*in pcDNA plasmid; they injected the vaccine into *Lutjanus erythropterus* fish and measured the rate of expression in fish organs using RT-PCR. The results from ELISA showed the apparent production of antibodies in fish serum, and a viability of 92.53% was observed in vaccinated fish, indicating the effective protection of the vaccine against the infection of *Vibrio alginolyticus*(35). Nezafat *et al.* carried out a bioinformatics analysis on a chimeric construct containing epitopes of several different proteins, one of which was OmpW (36). In our study, all proteins of OmpW and CtxB were bioinformatically analyzed, and a chimeric protein was constructed and its immunogenicity evaluated.

In this research, we used EAAAK linker to separate two recombinant proteins. The linker prevented the structural interference of each protein, and both fragments in our immunogenic structure could retain their independent folding. The salt bridge with the glutamic acid and lysine in the linker created sustainable Helix structure for preventing domain interference (37). The same linker sequence was successfully used in other studies, resulting in separate presentations of their structures(15,38).The c-score and the z-score of the input structure were within the range of scores typically found for native proteins of similar sizes. The RNA stability was examined and the minimum energy for the structure was -280.30 kcal. Strong B cell and T cell immune responses are really important in vaccine effects. Predicting B cell linear epitopes is possible by use of properties such as flexibility, hydrophobicity, polarity, and surface properties and bioinformatics algorithms. The results of the prediction of the linear and discontinuous epitopes of the B cell showed that both were scattered throughout the chimer sequence

and belonged to both proteins that make up the structure. Despite the advantages of recombinant vaccines over the traditional ones, most have a low immunogenicity, if employed alone. Therefore, it is necessary to use an adjuvant beside the recombinant protein to generate a long-term protective and immune response (39). The success of recombinant vaccines, such as hepatitis B vaccine, was due to the aluminum salt used as adjuvant (40). We further used aluminum salt as an adjuvant, with results comparable to the previous findings.The chimeric construct expressed in *E.coli* could significantly induce the immune response and antibody titer increased in the immunized animals, compared to the control group.

Antibodies produced in mothers can be transmitted to infants through the transfer of IgG antibody in the mother's blood by placenta as passive immunity, thereby resulting in immunogenicity in the pups. Pups of immunized mothers resisted against 1LD50 of *Vibrio cholera* when administered orally.*V.cholerae* binds to the intestinal epithelial cells leading to subsequent toxin production and infection. Immunogens, which produce antibodies against bacterial attachment and colonization, are considered as relevant vaccine candidates. To examine this feature of our construct, the ileal loop test was carried out, showing that the loops treated with serum obtained from mice immunized with chimer or OmpW+CtxB prevented 68% and 40% of bacterial toxicity in the ileal loops, respectively. In conclusion, chimeric construct is able to induce the immune system and inhibit pathogens of *Vibrio cholerae*.

ACKNOWLEDGEMENTS

The authors thanks to Mr. Ehsan Derakhshanifar for his help in conducting the animal challenges. This work was founded by the Deputy Research, Shahed University, Tehran, Iran (grant no.4/494).

REFERENCES

1. Schild S, Nelson EJ, Bishop AL, Camilli A. Characterization of Vibrio cholerae outer membrane vesicles as a candidate vaccine for cholera. Infect Immun. 2009; 77:472-84.
2. Adriani R, Gargari SLM, Nazarian S, Sarvary S, Noroozi N. Immunogenicity of Vibrio cholerae outer membrane vesicles secreted at various environmental conditions. Vaccine. 2018; 36:322-30.
3. Chawley P, Samal HB, Prava J, Suar M, Mahapatra RK. Comparative genomics study for identification of drug and vaccine targets in Vibrio cholerae: MurA ligase as a case study. Genomics. 2014; 103:83-93.
4. Nelson EJ, Harris JB, Morris Jr JG, Calderwood SB, Camilli A. Cholera transmission: the host, pathogen and bacteriophage dynamic. Nat Rev Microbiol. 2009; 7:693-702.
5. Farkas P, Korcova J, Kronek J, Bystricky S. Preparation of synthetic polyoxazoline based carrier and Vibrio cholerae O-specific polysaccharide conjugate vaccine. Eur J Med Chem. 2010; 45:795-9.
6. Schild S, Nelson EJ, Camilli A. Immunization with Vibrio cholerae outer membrane vesicles induces protective immunity in mice. Infect Immun. 2008; 76:4554-63.
7. Nazarian S, Arefpour M, Bagheripour M. Production and Purification of Polyclonal Antibody against Cholera Toxin. Journal of Babol University of Medical Sciences. 2015; 17:7-14.
8. Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science. 1996; 272:1910.

9. Mousavi SL, Nazarian S, Amani J, Karimi RA. Rapid screening of toxigenic vibrio cholerae O1 strains from south Iran by PCR-ELISA. Iran Biomed J. 2008; 12:15-21.
10. Sengupta DK, Sengupta TK, Ghose AC. Major outer membrane proteins of Vibrio cholerae and their role in induction of protective immunity through inhibition of intestinal colonization. Infect Immun. 1992; 60:4848-55.
11. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. MicrobiolMolBiol Rev. 2003; 67:593-656.
12. Touw DS, Patel DR, Van Den Berg B. The crystal structure of OprG from Pseudomonas aeruginosa, a potential channel for transport of hydrophobic molecules across the outer membrane. PLoS One. 2010; 5:e15016.
13. Nandi B, Nandy RK, Sarkar A, Ghose AC. Structural features, properties and regulation of the outer-membrane protein W (OmpW) of Vibrio cholerae. Microbiology. 2005; 151:2975-86.
14. Salamaszynska-Guz A, Klimuszko D. Strategies of constructing recombinant vaccines. Pol J Vet Sci. 2009; 12:133-9.
15. Alerasol M, Gargari SLM, Nazarian S, Bagheri S. Immunogenicity of a fusion protein comprising coli surface antigen 3 and labile B subunit of enterotoxigenic Escherichia coli. Iran Biomed J. 2014; 18:212-218.
16. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011; 8:785-6.
17. Chen X, Zaro JL, Shen W-C. Fusion protein linkers: property, design and functionality. Adv Drug Deliv Rev. 2013; 65:1357-69.
18. Roy A, Kucukural A, Zhang Y. I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc. 2010; 5:725-38.
19. Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res. 2007; 35(Web Server issue):W407-10.
20. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 2003; 31:3406-15.
21. EL□Manzalawy Y, Dobbs D, Honavar V. Predicting linear B cell epitopes using string kernels. J MolRecognit. 2008; 21:243-55.
22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. nature. 1970; 227:680-5.
23. Bollag DM, Rozycki MD, Edelstein SJ. Protein methods: Wiley-Liss New York; 1996.
24. Council NR. Guide for the care and use of laboratory animals: National Academies Press; 2010.
25. Crowther JR. ELISA: theory and practice: Springer Science & Business Media; 1995.
26. Mohammad Pour Ghazi F, Gargari SLM. Development of lipopolysaccharide□mimicking peptides and their immunoprotectivity against Vibrio choleraeserogroup O1. J Pept Sci. 2016; 22:682-688.
27. Sawasvirojwong S, Srimanote P, Chatsudthipong V, Muanprasat C. An adult mouse model of Vibrio cholerae-induced diarrhea for studying pathogenesis and potential therapy of cholera. PLoSNegl Trop Dis. 2013; 7:e2293.
28. Sawasvirojwong S, Srimanote P, Chatsudthipong V, Muanprasat C. An adult mouse model of Vibrio cholerae-induced diarrhea for studying pathogenesis and potential therapy of cholera. PLoSNegl Trop Dis. 2013; 7:e2293.
29. Garg P, Sinha S, Chakraborty R, Bhattacharya S, Nair GB, Ramamurthy T, et al. Emergence of fluoroquinolone-resistant strains of Vibrio cholerae O1 biotype El Tor among hospitalized patients with cholera in Calcutta, India. Antimicrob Agents Chemother. 2001; 45:1605-6.
30. Odumosu O, Nicholas D, Yano H, Langridge W. AB toxins: a paradigm switch from deadly to desirable. Toxins. 2010; 2:1612-45.
31. Madanchi H, Honari H, Sadraeian M, Hesaraki M. Fusion of CtxB with StxB, Cloning and Expression of in Esherichia coli: A challenge for Improvement of Immune Response againstStxB. Iranian Journal of Pharmaceutical Sciences. 2011; 7:185-90.
32. Maiti B, Raghunath P, Karunasagar I. Cloning and expression of an outer membrane protein OmpW of Aeromonashydrophila and study of its distribution in Aeromonas spp. J ApplMicrobiol. 2009; 107:1157-67.
33. Maiti B, Shetty M, Shekar M, Karunasagar I, Karunasagar I. Evaluation of two outer membrane proteins, Aha1 and OmpW of Aeromonashydrophila as vaccine candidate for common carp. Vet ImmunolImmunopathol. 2012; 149:298-301.

34. Mao Z, Yu L, You Z, Wei Y, Liu Y. Cloning, expression and immunogenicty analysis of five outer membrane proteins of Vibrio parahaemolyticus zj2003. Fish Shellfish Immunol. 2007; 23:567-75.
35. Cai S-H, Lu Y-S, Jian J-C, Wang B, Huang Y-C, Tang J-F, et al. Protection against Vibrio alginolyticus in crimson snapper Lutjanuserythropterus immunized with a DNA vaccine containing the ompW gene. Dis Aquat Organ. 2013; 106:39-47.
36. Nezafat N, Karimi Z, Eslami M, Mohkam M, Zandian S, Ghasemi Y. Designing an efficient multi-epitope peptide vaccine against Vibrio cholerae via combined immunoinformatics and protein interaction based approaches. ComputBiol Chem. 2016;62:82-95.
37. Arai R, Ueda H, Kitayama A, Kamiya N, Nagamune T. Design of the linkers which effectively separate domains of a bifunctional fusion protein. Protein Eng. 2001; 14:529-32.
38. Amani J, Salmanian AH, Rafati S, Mousavi SL. Immunogenic properties of chimeric protein from espA, eae and tir genes of Escherichia coli O157: H7. Vaccine. 2010; 28:6923-9.
39. Perez O, Batista-Duharte A, González E, Zayas C, Balboa J, Cuello M, et al. Human prophylactic vaccine adjuvants and their determinant role in new vaccine formulations. Braz J Med Biol Res. 2012; 45:681-92.
40. Perrie Y, Mohammed AR, Kirby DJ, McNeil SE, Bramwell VW. Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. Int J Pharm. 2008; 364:272-80.