IJM Iranian Journal of Microbiology
Volume 6 Number 2 (April 2014) 68-73

Recombinant toxin-coregulated pilus A (TcpA) as a candidate subunit cholera vaccine

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Received: December 2012, **Accepted:** November 2013.

ABSTRACT

Background and Objectives: The toxin co-regulated pilus A (TcpA) has been described as a critical pathogenicity factor of *Vibrio cholerae*. TcpA is a candidate for making subunit vaccine against cholera. The aim of this study was to produce a candidate vaccine by expressing recombinant TcpA in *E. coli*.
Materials and Methods: In this study, the toxin co-regulated pilus A gene from EL-Tor, *V. cholerae* subspecies, was amplified by PCR and sub-cloned into prokaryotic expression vector pGEX4T1. *E. coli* BL21 (DE3) was transformed with pGEX4T1- TcpA and gene expression was induced by IPTG and purified by GST resin. The integrity of the product was confirmed by Western blot analysis using a standard rabbit anti-*V. cholerae* antibody. Sera reactivity of infected individuals was further analyzed against the recombinant TcpA protein.
Results: The concentration of purified recombinant protein was calculated to be 8 mg/L of initial culture. The integrity of product was confirmed by Western blot analysis using a standard rabbit anti *V. cholerae* antibody. Sera reactivity of infected individual was further analyzed against the recombinant TcpA protein. The obtained data indicated that recombinant TcpA protein from *V. cholerae* was recognized by patient serum and animal sera.
Conclusion: These results show that the recombinant TcpA is antigenic and could be used in a carrier host as an oral vaccine against cholera.

Keywords: *Escherichia coli*, Toxin co-regulated pilus, Vaccine, Recombinant, *Vibrio cholerae*

INTRODUCTION

Cholera is an aggressive diarrheal disease caused by the Gram-negative bacterium *Vibrio cholerae* (1). This disease remains a major cause of morbidity and mortality throughout the world. Currently, two types of vaccines are accepted for use in humans: (i) a killed-whole-cell formulation and (ii) a genetically engineered, live-attenuated *V. cholerae* vaccine (e.g., CVD 103-HgR, O1, classical biotype, Inaba serotype)(3). Several doses of the killed-whole-cell vaccine gave 50% protection in field trials. However, the main target population, young children, were even less well protected by this vaccine (less than 25%) (4). Clinical trials in North America demonstrate a single dose of the live-attenuated vaccine in adults gave > 90% protection against the virulent homologous strain and 65 to 80% protection against virulent El Tor biotype, Inaba serotype strains (5-7). In a large-scale field trial in endemic area of Indonesia, administration of the CVD 103-HgR vac-

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cine did not show protection (8). New oral vaccine strain CVD 111, which is a live-attenuated El Tor biotype, Ogawa serotype strain, provided 80% protection in adult volunteers (9). This vaccine is being evaluated collectively with CVD 103-HgR to establish if the mixture can give more protection against both biotypes in a single dose(10). Two problems of live vaccine strains, in spite of their advantages, exist:1) the live-attenuated strains cause side effects such as abdominal pain, moderate diarrhea, and low fever, 2) reversion to virulence, because these live strains are attenuated by deleting the *ctx* genes that are carried on a bacteriophage, vaccine strains can obtain *ctx*-carrying phage in endemic area (11). An alternative approach to cholera vaccine design is the development of a subunit vaccine. Much research has focused on *Vibrio cholerae* colonization factors such as Toxin co-regulated pili (TCP), for development subunit vaccines. TCP is a thin filamentous pilus forming distinctive clusters on the surface of *V. cholerae* when cultured under high toxin-expressing conditions. TcpA a critical role in colonization in the infant mouse cholera model and following in human volunteers (1).This antigen, TcpA, could be a candidate for research and development of subunit vaccine.

Isolation and expression of *V. cholerae* TcpA gene in *E. coli* as a host was presented in this paper. We also showed that recombinant TcpA protein was recognized by infected human serum and animal sera using western blot analysis.

MATERIALS AND METHODS

Bacterial strains and Vectors. *V. cholerae (*a gift from the Pasteur Institute of Iran) was grown on TCBS (Thiosulfate-citrate-bile salts-sucrose agar) for 24 hours. For recombinant protein production, a prokaryotic expression vector pGEX4T1 was used. The recombinant pGEX4T1 (pGEX4T1-TcpA) is transformed into *E. coli*, BL21 (DE3) (*f –ompt hsdB, rB- mB-, dcm gal, DE3*) as host strain. The required antibiotics were added to LB media according to the reference recommendation (12). We received standard rabbit anti *V. cholerae* sera from Tarbiat Moddares University (Department of Microbiology, Tehran, Iran) and acute phase patient serum (Immunology Department, Arak University of Medical Sciences Arak, Iran). All chemicals were obtained from Merck Co. (Germany).

DNA extraction and PCR method. *V. cholerae* subsp. EL-Tor chromosomal DNA for using in PCR was extracted by standard CTAB/NACL method (13). Briefly, *V. cholerae* was grown on TCBS for 24 hours. One colony cultured in 1.5 ml nutrient broth for 24 hours. After resuspending the pellet of bacterial culture in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8), the bacterial cell was lysed by SDS and proteinase K, the chromosomal DNA was extracted by CTAB/NACL solution (10% CTAB and 0.7 M NACL).The cell debris and proteins were removed by two times phenol/chloroform/isoamylalcohole (25:24:1) mixture. DNA precipitated by isopropanol and washed in ethanol (70%). The TcpA gene (accession number U09807) was amplified using a forward primer (5' TGG ATC CAT GCA ATT ATT AAA ACA 3') and a reverse primer (5' ACT CGA GTT AAC TGT TAC CAA AAG 3'), that were manufactured by MWG company (Germany). PCR was carried out in 50 µl volumes containing 1 µl each deoxynucleotide (dATP, dTTP, dGTP, and dCTP), 2.5 µl each primer (10pM), and 1 U of *Expand DNA* polymerase (Fermentas). Buffer was added as described in the manufacturer's instructions. Reaction mixes placed in an automated thermal cycler (eppendorf). Amplification conditions for primers were 5 min of Primary denaturation at 95˚C, 25 cycles (30 s of Secondary denaturation at 95˚C, 1 min of annealing at 61˚C, and extension at 72˚C for 1.5 min) and final extension at 72˚C for 5 min. After amplification, the reaction mix was assayed on an agarose gel in the presence of 0.5 µg of ethidium bromide per ml. The tcpA gene fragment obtained from PCR was purified by High Pure PCR Product Purification Kit (Roche) (12).

Cloning. The PCR product of TcpA gene was digested by *Bam* HI (Fermentas) and *XhoI* (Fermentas) and cloned into expression vector pGEX4T-1.The ligated vector (pGEX4T-1- TcpA) was transformed into *E. coli* DH5α and *E. coli* BL21 (DE3) competent cells and then they were grown at 37˚C. Colonies were isolated, and their plasmid DNA was extracted by miniprep plasmid extraction (14). The extracted plasmid was assayed on 1% agarose gel.

DNA Sequencing. The constructed vector (pGEX4T-1-tcpA) was sequenced by MWG Company (Germany) to confirm that the desired product had been obtained. Sequence data has been blast to

GenBank, accession number U09807.

Expression and purification of TcpA. *E. coli* BL21 was transformed with pGEX4T1- TcpA and grown in 2 ml LB broth supplemented with Ampicillin (100 µgml-1) at 37°C with agitation. A colony which contained recombinant plasmid was cultured on shaking incubator for overnight at 37°C in 2 ml LB medium containing 100 µg ml-1 ampicillin. The next day, 500 µl of culture was removed and inoculated in 50 ml LB broth (per liter contains: 10 g yeast extract (Difco), 20 g Bactotryptone broth (Difco), 0.2% (mass/vol.) glucose, 10 g NaCl, 1 g KCl, 0.5 g $MgCl_2$, 0.5 g $CaCl_2$, 100 mg ampicillin), incubated at 37°C with at 200 rpm shaking with vigorous agitation until the optical density reached to an absorbance of 0.5 to 0.8 at 600 nm. Expression of the TcpA protein was then induced by the addition of IPTG to a final concentration of 1 mM and incubation was continued for further 4 h. The expressed protein was purified using GST-sepharose column according to manufacture instruction (Pharmacia). The purified protein was dialyzed twice against phosphate buffered saline (NaCl 140 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO 1.8 mM, pH 7.2) at 4°C overnight. The quality and quantity of purified recombinant hylA protein was analyzed by SDS-PAGE (15%) and Bradford methods, respectively (15).

In order to analyze the cross-reaction between fused segment (near 20 kDa) of recombinant TcpA protein with patient serum, an *E. coli* BL21 (DE3) containing pGEX4T1 a vector was induced by IPTG.

Mice Immunization. 100 µl of emulsion containing bacteria and complete freund´s Adjuvant (50 µg *vibrio cholerae* + 50 µg complete Freund´s Adjuvant) (Sigma, St. Louis Ma) at three weeks injected to five mice. In 21st and 28th days incomplete freund´s Adjuvant (50 µg vibrio cholerae + 50 µg incomplete Freund´s Adjuvant) was injected. 10 days after the last injection.

Immunoblot analysis of recombinant tcpA. For Western blot analyses, 0.5 µg of purified recombinant TcpA protein was used per well. As a negative control, the bacterial lysate from induced *E. coli* BL21 (DE3) contain pGEX4T1 vector was analyzed by Western blot.

The gel was blotted on to Poly vinylidine difluoride (PVDF Membrane, Roche Diagnostic) membrane using transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine and %20 methanol at 90 volts for 1.5 hours at 4°C. The blotted membrane was blocked with 2.5% (w/v) BSA in TBST buffer (0.5M NaCl, 0.02 M Tris pH 8.5, 0.05% Tween 20) for 1 hour at room temperature. Membranes were incubated for 2 hours at room temperature with rabbit, mice sera and patient serum, diluted 1:100 and 1:50, respectively. Normal sera from rabbit, mice and human were used as controls. After reactions with the primary antibody, the blots were washed three times with TBST and incubated with peroxidase conjugated goat anti-rabbit IgG , anti mouse IgG and antihuman Ig (G, A, M) at a 1:2500 dilution in TBST. The blots were then washed three times with TBST and reactions were developed by diamino Banzidine (DAB) Solution (Roche Diagnostic).

RESULS

PCR was used successfully to amplify a gene fragment encoding the sequence of tcp A. PCR product has about 598bp on 1% agarose gel electrophoresis. The sequencing of the PCR product fragment revealed complete homology at the nucleotide level to *tcpA* gene in NCBI.

Expression and purification. The recombinant protein from *E. coli* BL21 (DE3) harboring pGEX4T-1 containing the coding sequences of tcp A gene was detected in cell lysates and appeared at about 45 kDa on SDS-PAGE gel (Fig.1). The recombinant Tcp A protein was collected and further purified by using GST-sepharose. Purified recombinant Tcp A protein appeared at about 45 kDa (Fig. 1).

The concentration of recombinant protein was assayed and calculated to 8 mg purified protein per liter of the initial culture.

Western blotting of fusion protein. Serum from patient recovered, immunized rabbit and mouse were used for blotting (Fig. 2). The result shows that TcpA protein can be detected. This suggested that the recombinant Tcp A protein was successfully produced by *E. coli* harboring pGEX4T1 with tcpA gene and in mouse immune system could be induced (16).

There was no reaction between the expressed pGEX4T1 in *E. coli* BL21 (DE3) and patient serum (data not shown).

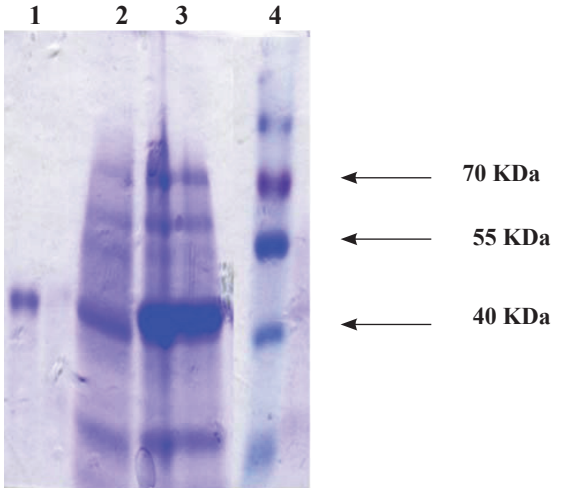

Fig. 1. Comassie blue-stained SDS-PAGE of recombinant protein, Lane 1: Extract proteins after Glutathione sepharose 4B affinity chromatography, lane 2: non-induced cells of pGEX4T1-TcpA without using IPTG, lane 3: induced cells of pGEX4T1-TcpA with using IPTG at a long time 4 hours, lane 4: protein marker (Fermentas).

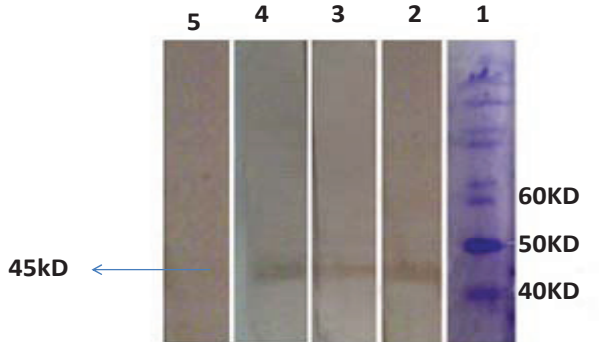

Fig. 2. Western blot analysis of recombinant TcpA: lane 1: protein marker (Fermentas) , lane 2: serum of mice immunized by whole kill cell of *E. coli* harboring constructed vector pGEX4T1-TcpA, lane 3: serum of patient recovered from cholera, lane 4: serum of rabbit immunized with purified recombinant TcpA protein, lane 5: serum of negative control.

DISCUSSION

Colonization of the human small intestine by *Vibrio cholerae* is an important step in pathogenesis that needs the type IV toxin-coregulated pilus (Tcp). Three function of Tcp have been described: it serves as the CTXphi receptor, secretes the colonization factor TcpF, and functions in microcolony formation by mediating bacterium-bacterium interactions. TcpA protein is assembled into toxin-coregulated pilus (Tcp), a type IVb pilus. The toxin co-regulated pilus (TCP) that is required for colonization of *V. cholerae* is a fiber of assembled TcpA. The TcpA predecessors for the TCP (a type IVb pilus) are processed at the cytoplasmic side of the IM by TcpJ (Type Four Prepilin Peptidase, TFPP) (22). Mature TcpA subunits assemble into TCP via the TCP biogenesis appendages (21). Thus, it is a strong candidate for a cholera subunit vaccine (17).

Recently, it was shown that immunization with natural TcpA induces protective immunity against *V. cholerae* O1 challenge in mice (18). It was reported earlier that larger size of the fusion partner and position of the fusion, i.e., at NH2-or COOH terminal, affects the pentamerizing ability or induces conformational changes that lead to reduced GM1 binding efficiency of CTB or no binding at all (19). Also, it has been found that linking the peptide to NH2-terminal end of CTB may reduce its GM1 binding efficiency as N-terminus lies near to GM1 binding pocket of CTB (20). The specific resides around the disulfide loop between a 120 and 186, and the region immediately adjacent on the carboxyl-terminal side were recognized by the antibodies (21).

In this study, we have cloned and expressed TcpA gene that encodes toxin-coregulated pilus under the control of Trp promoter. Present data shows that recombinant TcpA can be detected as an antigen by serum in patient, immunized mouse and rabbit. Therefore, recombinant TcpA protein has same epitopes with natural form of this antigen. Recombinant TcpA also seemed to be a promising antigen for the vaccination of cholera

The results obtained here demonstrate the Glutathione s transfers has weight about 20 kDa , was added to N-terminal of the recombinant TcpA protein.

In order to investigate the effect of additional amino acids in plasmid (GST), pGEX4T1 vector in *E. coli* BL21 (DE3) was also induced by IPTG and further analyzed by Western blotting with mice and human sera. The results showed that there was no interfere related to fused amino acids.

In Previous studies, volunteers were immunized by Fimbrial antigens cholera were examined for immune responses to natural TcpA (23). These studies have shown that there are low rates of seroconversion. Here, by using the Western blot analysis, we showed that the *Vibrio cholerae* purified recombinant TcpA doesn>t dose not have any cross reactivity with normal human sera which was vaccinated against pathogenic bacteria such as *Mycobacterium tuberculosis*. Other groups demonstrated that

Fimbrial antigens of *Vibrio cholerae* acts as an immunogenic and induces partial immunity in animal models (23), but there are no reports concerning the antigenicity of the recombinant TcpA in human. Our data showed that recombinant TcpA protein could be detected as an antigen by serum from acute phase of cholera in infected human. Additionally, other studies demonstrated that this protein can elicit immunity in animals (3, 5, 22), but in human models it needs more investigation. We suggest that TcpA in combination with other molecular subunits of *Vibrio cholerae* would provide superior protection to *Vibrio cholerae* infection. As observed in malaria and leprosy models, solid protective immunity requires immunization with several proteins rather than a single moiety (23, 24).

So, recombinant TCP (TcpA) is produced in *E. coli* (DE3) Bl21 is more safe in comparable with natural protein was purified. These results in this work may provide conception on the recombinant TCP (TcpA) is immunogenic in future study.

In the recent availability of recombinant produced and purified El Tor TcpA (26) has provided a more suitable reagent for assessing these immune responses. Earlier studies various epitopes have been described within the TcpA protein, of which epitopes P4 and P6 have been shown to provide protective immunity against the challenge (27). Peptides P4 and P6 were selected as fusion partner and linked to C-terminal end of CTB through a hinge linker. Fusion of GST with the TcpA and expression of chimeric proteins in bacterial cells may result into production of vaccine, which could be more efficient because of anti-colonization immunity.

In summary, we constructed a recombinant *E. coli* BL21 (DE3) harboring pGEX4T1-TcpA strain for producing recombinant Toxin co-regulated pili (TcpA). Oral administration of this strain in mouse could induce serum antibodies; therefore this construct can be used in oral host such as *Lactococcus lactis* or attenuated *S. typhimurium*. Protection of this recombinant protein in mice is under investigation.

Our results indicated sera from infected mouse, rabbit and patient recovered with *V. cholerae* O1 serotype Inaba could be detected by recombinant TcpA protein, this indicated antigenic dominants of natural protein present in fusion protein, and if this protein use it can exposure to immune system (28, 29).

ACKNOLEDGEMENT

The authors would like to thank Deputy of Research and Technology of Arak University of Medical Sciences, Iran, for financial and equipments support.

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