

Cloning of *Stx2* B Subunit Gene from Enterohemorrhagic *Escherichia coli* O157:H7 to the Expression Vector (pCDNA 3.1+) as DNA Vaccine Candidate

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Abstract: Shiga toxin (Stx) producing enterohemorrhagic *Escherichia coli* (EHEC) strains represent the major etiological agents of hemorrhagic colitis and hemolytic uremic syndrome (HUS). Production of *Stx* is the basis of *EHEC* pathogenesis. The aim of present study was cloning of *stx2* B subunit gene from enterohemorrhagic *Escherichia coli* serotype O157:H7 to the expression vector (pCDNA 3.1+) as DNA vaccine candidate. Bacterial DNA was extracted and *stx2* B subunit (*stxB*) gene was amplified from total genome of EHEC using *stx2* B specific primers. Then, DNA fragment of *stx2* B gene was cloned in pCDNA 3.1+ and this construct was transformed into *E. coli*. The results showed that *stx* gene was cloned in *E. coli* successfully. Therefore, it seems that the DNA construct that was produced in this study can be tested as DNA vaccine against Shiga toxin type 2 of enterohemorrhagic *Escherichia coli* in future researches.

Key words: *E. coli* • Stx Gene • Cloning.

INTRODUCTION

Strains of Shiga toxin (Stx)-producing *Escherichia coli*, or enterohemorrhagic *E. coli* (EHEC), are important food-borne pathogens for young, elderly and immunocompromised humans [1-4]. Infections with this bacterium are commonly associated with abdominal cramps and diarrhea which can be bloody, but more serious complications such as hemorrhagic colitis (HC), hemolytic-uremic syndrome and thrombocytopenic purpura and in some cases death can result [5]. A number of outbreaks and sporadic cases of EHEC infection caused by O157 as well as non-O157 strains have occurred in the US, Canada, Australia, UK, Germany and other European countries since the first reported outbreak in the US in 1982 [6].

A wide range of animal species are known to carry EHEC strains, but ruminants are the most important natural reservoir and excrete these bacteria with their feces [7]. Although the main infection routes are person-to-person transmission, as well as consumption of contaminated meat and milk, ingestion of contaminated vegetables or water and direct contact with animals or soil

have also been associated with EHEC associated outbreaks. It has been reported that human infections can result from ingestion of fewer than 100 viable EHEC cells [7]. Moreover, EHEC can persist and remain infectious for several weeks in slurries, farmyard manure and sewage sludge, as well as on pasture land [8-10]. The virulence of EHEC organisms is primarily associated with the production of one or two cytotoxins, Shiga-like toxin 1 (Stx 1) and Shiga-like toxin 2 (Stx 2), also known as verotoxins [5]. These cytotoxins target endothelial cells and are believed to mediate much of the tissue damage during HC and hemolytic uremic syndrome and can influence the duration of Stx-producing *E. coli* (STEC) shedding by ruminants [4, 5, 11]. Shiga toxins consist of a single A and a pentamer of B subunits [5]. The B subunit pentamer binds to globotriaosyl ceramide receptors on the cell membrane [5]. The B subunit of Stx1 and Stx2 binds to Gb3, which has been identified as the receptor for the Stx family. The A subunit of both toxins acts as an enzyme, RNA N-glycosidase, that catalyzes the release of an adenine at position 4324 in 28S rRNA of eukaryotic cells and inhibits protein synthesis [6]. Comparison of the nucleotide sequences of the A and B

subunits of Stx1 and Stx2 showed 57 to 60% homology, with 55 to 57% amino acid homology, respectively. Despite this degree of homology, Stx1 and Stx2 are reported to be immunologically distinct when reacted with polyclonal antiserum [7]. *stx* genes are carried by lysogenic bacteriophages and can be acquired by horizontal gene transfer [10,12,13]. The Stx converting phages are classified as lambdoid phages since a 8.1 kb *EcoRI* fragment of H-19B genome was shown to be homologous to a part of the lambda phage [14,15]. In addition, LamB, which is a receptor for lambda phage, was used as receptor by certain Stx phages [6,16].

Stx1 is nearly identical to Stx, the principal extracellular cytotoxin of *Shigella dysenteriae* serotype 1 [17]. Usually, Stx2 is frequently associated with clinical manifestations of HUS [18]. Considering the pivotal role of Stx2 in the EHEC pathogenesis, it is desiderated to address its potential for the treatments of EHEC infections [19-21]. Large-scale production of the Stx2 B subunit (Stx2B) has not been efficient, probably due to the instability of the B multimers when synthesized without the A subunit [19]. An alternative approach could be to express the antigen in vivo, by developing a DNA vaccine [22]. The purpose of this study was cloning of *stx2* B subunit gene from enterohemorrhagic *Escherichia coli* serotype O157:H7 to the expression vector (pCDNA 3.1+) as DNA vaccine candidate.

MATERIALS AND METHODS

Bacterial Strains and Plasmids: Enterohemorrhagic *Escherichia coli* ATCC 3081 obtained from the Razi Institute (Karaj, Iran) was used for isolation of *stx2* B subunit gene. Also, pGEMT easy vector (Invitrogen, San Diego, CA) and pCDNA 3.1+ (Qiagen, Germany) together with *E. coli* strain Top10F' (Pasteur institute of Iran Tehran, Iran) were used for cloning and maintenance of DNA fragment and host strain.

DNA Extraction: Genomic DNA was extracted from EHEC ATCC 3081 using DNA extraction kit (Qiagen, Germany), according to manufacturer instructions and assayed on 1% agarose gel electrophoresis and measured at 260 nm optical density according to the method described by Sambrook and Russell [23].

Gene Amplification: Specific oligonucleotide primers for the *stx2* gene: F: 5'-AGCCCCATCCATGAAGAAGATGTTT-3' and R: 5'-TCAGTCATTATTAAGTG-3' (accession number:

DQ231595.1) were designed and used in the amplification reaction. The amplification was done using Thermal Cycler (Mastercycler Gradient, Eppendorf, Germany), in a final reaction volume of 25 µl. The PCR mixture consisted of 1 µg of DNA sample, 1 µM of each primer, 200 µM MgCl₂, 200 µM dNTPs, 2.5 µl of 10X PCR buffer and 1 U of *Taq* DNA polymerase (Fermentas, Germany) [23]. The following conditions were applied: initial denaturation at 95°C for 5 minutes, followed by 30 cycles; denaturation at 94°C for 1 min, annealing at 60°C for 1 min, elongation at 72° for 1 min. The program was followed by final elongation at 72°C for 5 minutes.

Evaluation of PCR Products: The amplification products were detected by 1.5% agarose gel electrophoresis [23]. The electrode buffer was TBE [Tris-base 10.8 g 89 mM, Boric acid 5.5 g 2 mM and EDTA 4 ml of 0.5 M EDTA (pH 8.0)]. Aliquots of 10 µl of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. After electrophoresis, the gel was stained with "ethidium bromide" and photographed under UVIdoc gel documentation systems (UK).

DNA Extraction from Gel: The products of PCR were extracted from the gel using DNA extraction gel kit (Bioneer Co., Korea) according to manufactures protocol.

Plasmid Construction: The amplified products was cloned in pGEM T easy vector (Invitrogen, San Diego, CA) and the construct was transformed into competent *E. coli* strain Top10F' in LB Media (CinnaGen, Iran). The presence of binding domain of *stx2* B subunit coding region was confirmed by restriction enzyme analysis [23].

Subcloning of the *Stx2* Gene and Construction of Expression Vector PCDNA 3.1+: The 286 bp fragment of *stx2* gene from recombinant plasmid (pGEM-*stx2*) and cloning vector (pCDNA3.1) were digested with *HindIII* and *XhoI* and then ligated to generate the recombinant plasmid (pCDNA3-*stx2*). After preparation, the plasmid was transformed under heat shock (42°C) and calcium chloride for 90s into *E. coli* TOP10F'. Extraction and purification of subcloned plasmids were done using SDS (1%), NaOH (0.02 N) and acetate sodium (3M) [24].

RESULTS

Gene Amplification: PCR amplified products for *stx 2* gene (286 bp length) are shown in Figure 1. The results showed that the bacterial cells included *stx2* gene.

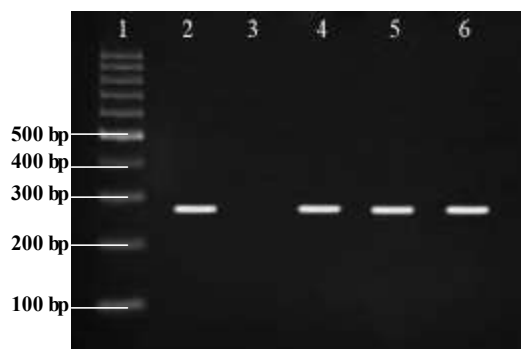


Fig. 1: Analysis of PCR amplified *stx 2* gene products by agarose gel electrophoresis. Lane 1: 100 bps DNA marker, lanes 2 and 3: positive and negative controls, respectively, and lanes 4-6: *stx* amplified fragments.

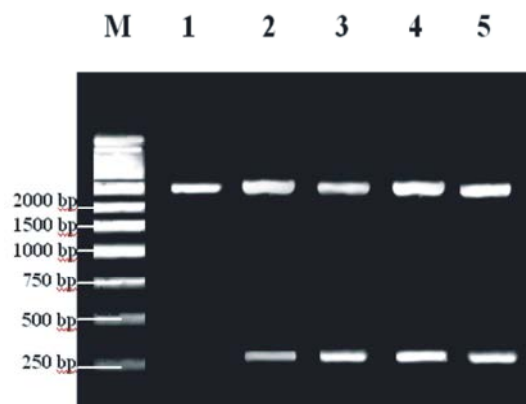


Fig. 2: Analysis of digested pCDNA-*stx* plasmid by *Hind*III and *Xho*I. Lane 1: uncut plasmid, lanes 2 - 5: fragments of *stx* and pCDNA, and lane M: 1 kbp DNA marker.

Subcloning of the *Stx2* Gene: *Stx2* gene fragment which has restriction point of *Hind*III and *Xho*I was inserted in polyclonal site (PCS) in pCDNA 3. 1+ plasmid. TOP10F' competent cells were used for transformation and culturing in LB media containing AMP. In addition some colonies as a positive control were obtained. Using alkaline procedure, recombinant plasmids of pCDNA-*stx* from transformed cells were isolated and then restriction enzymes (*Hind*III and *Xho*I) were applied for further clarification and the fragments with length of 286 bp and 5428 bp were ran on agarose gel electrophoresis (Figure 2).

DISCUSSION

Shiga toxin is one of the exothermic factors produced by *Shigella* and *E. coli*, which initially was identified in *S.*

dysenteriae (biotype 1). In addition to its destructive effect on gastrointestinal (especially intestine), it can be transferred to central nervous system (CNS) via blood stream and lead to destructive effect on this system [17].

Shiga toxin producing *E. coli* (STEC) is capable to causing life-threatening illness such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Although there are many different O:H serotypes associated with production of Shiga toxins and illness, *E. coli* O157:H7 serotype is the pathogen implicated in a large number of food-borne outbreaks of diseases [19]. This serotype alone is estimated to be responsible for over 73,000 illnesses annually in the United States, in which HUS occurs in about 4% of the reported cases [23].

The incidence of EHEC infections varies by age group, with the highest incidence occurring in children aged less than 15 years old (0.7 cases per 100,000 in the United States). Up to 85% of the cases are food-borne and up to 20% of EHEC infections progress to the severe sequel HUS, the leading cause of acute renal failure in children. Furthermore, up to 40% of patients with HUS develop long-term renal dysfunction and about 3-5% of patients even die during the acute phase of the disease [10].

Our results showed that *stx* gene was cloned in *E. coli* successfully and these findings showed DNA construct that can be used for DNA vaccine against Shiga toxin type 2 enterohemorrhagic *E. coli* in future researches. Many studies were performed about cloning of virulence genes of *E. coli* in bacterial cells. The research of Huang, *et al.* [25] for cloning and expression of the genes specifying Shiga-like toxin production in *E. coli* H19 showed that this gene was successfully cloned to pUC vector and almost is the same to this study. The study of Acheson *et al.* [26] for expression and purification of Shiga-like toxin 2 B subunits showed that *stx 2* B subunit was cloned to the pET9 and is the same as this study but the plasmid and restriction enzyme of that study differed from our study. The research of Cha *et al.* [27], for cloning and expression of *stx 2* gene of *E. coli* O157:H7 to pUC118 and pUC119 vector showed that cloning of this gene was successful and is the same to the results of present research [27]. The study of Capozzo *et al.* [28] for development of DNA vaccines against *E. coli* producing Hemolytic-Uremic Syndrome in a murine model showed that 282bp of *stx 2* B subunit was cloned to the pCDNA 3+ and is the same to our study. Another study of Bentancor *et al.* [29] for cloning of the encoding enterohemorrhagic *Escherichia coli* Shiga-like toxin 2 A and B subunits to pGEM T easy vector showed that these genes were cloned to that vector and their findings are the

same to our research. According to the results of the present study DNA construct that was produced can be used for DNA vaccine against Shiga toxin type 2 enterohemorrhagic *E. coli* in future researches.

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REFERENCES

- Kaper, J.B., J.P. Nataro and H.L. Mobley, 2004. Pathogenic *Escherichia coli*. Nature Reviews Microbiol., 2: 123-140.
- Agin, T.S., Ch. Zhu, L.A. Johnson, T.E. Thate, Z.H. Yang and E.C. Boedeker, 2005. Protection against hemorrhagic colitis in an animal model by oral immunization with Isogeneic Rabbit Enteropathogenic *Escherichia coli* attenuated by truncating intimin. Infection and Immunity, 73: 6608-6619.
- Rangel, J., P. Sparling, C. Crowe, P. Griffin and D.L. Swerdlow, 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. Emerging Infectious Dis., 11: 603-609.
- Hoffman, M.A., Ch. Menge, T.A. Casey, W. Laegreid, B.T. Bosworth and E.A. Dean-Nystrom, 2006. Bovine Immune Response to Shiga-Toxigenic *Escherichia coli* O157:H7. Clinical and Vaccine Immunol., 13(12): 1322-1327.
- Jinnemana, K.C., S.D. Weagant, J.M. Johnson, S.H.L. Abbott, W.E. Hill, B.J. Tenge, N.L. Dang, R. Ramsden and C.J. Omiecinski, 2000. A large insertion in the Shiga-like toxin 2 gene (stx) of an *Escherichia coli* O157:H7 clinical isolate. International J. Food Microbiol., 57: 115-124.
- Sato, T., T. Shimizu, M. Watarai, M. Kobayashi, S.H. Kano, T. Hamabata, Y. Takeda, S.H. Yamasaki, 2003. Distinctiveness of the genomic sequence of Shiga toxin 2-converting phage isolated from *Escherichia coli* O157:H7 Okayama strain as compared to other Shiga toxin 2-converting phages. Gene, 309: 35-48.
- Caprioli, A., S. Morabito, H. Brugere and E. Oswald, 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. Veterinary Research, 36: 289-311.
- Duffy, G. 2003. Verocytotoxic *Escherichia coli* in animal faeces, manures and slurries. J. Applied Microbiol., 94: 94-103.
- Hepburn, N.F., M. MacRae and I.D. Ogden, 2002. Survival of *Escherichia coli* O157 in abattoir waste products. Letters in Applied Microbiol., 35: 233-236.
- Loukiadis, E., M. Ke'roue'dan, L. Beutin, E. Oswald and H. Bruge're, 2006. Characterization of Shiga toxin gene (stx)-positive and intimin gene (eae)-positive *Escherichia coli* isolates from wastewater of slaughterhouses in France. Applied and Environmental Microbiol., 72(5): 3245-3251.
- Proulx, F., E.G. Seidman and D. Karpman, 2001. Pathogenesis of Shiga toxin-associated hemolytic uremic syndrome. Pediatric Res., 50: 163-171.
- Muniesa, M. and J. Jofre, 2004. Abundance in sewage of bacteriophages infecting *Escherichia coli* O157:H7. Methods in Molecular Biol., 268: 79-88.
- Muniesa, M. and J. Jofre, 2000. Occurrence of phages infecting *Escherichia coli* O157:H7 carrying the Stx 2 gene in sewage from different countries. FEMS Microbiology Lett., 183: 197-200.
- Huang, A., S. DeGrandis, J. Friesen, M. Karmali, M. Petric, R. Congi, J.L. Brunton, 1986. Cloning and expression of the genes specifying Shiga-like toxin production in *Escherichia coli* H19. Journal of Bacteriol., 166: 375-379.
- Huang, A., J. Friesen and J.L. Brunton, 1987. Characterization of a bacteriophage that carries the genes for production of Shiga-like toxin 1 in *Escherichia coli*. J. Bacteriol., 169: 4308-4312.
- Sato, T., M. Kobayashi, T. Shimizu, S. Yamasaki, T. Tobe, C. Sasakawa, Y. Takeda, 1998. Identification and characterization of a newly isolated Shiga toxin 2-converting phage from Shiga toxin producing *Escherichia coli*. Infection and Immunity, 66: 4100-4107.
- Cornick, N.A., S.L. Booher, T.A. Casey and H.W. Moon, 2000. Persistent colonization of sheep by *Escherichia coli* O157:H7 and other *E. coli* pathotypes. Applied and Environmental Microbiol., 66: 4926-4934.
- Sheoran, A.S., S. Chapman-Bonofiglio, B.R. Harvey, J. Mukherjee, G. Georgiou and A. Donohue-Rolfe, 2005. Human antibody against Shiga toxin 2 administered to piglets after the onset of diarrhea due to *Escherichia coli* O157:H7 prevents fatal systemic complications. Infection and Immunity, 73: 4607-4613.

19. Marcato, P., G. Mulvey, R.J. Read, K. Vander Helm, P.N. Nation and G.D. Armstrong, 2001. Immunoprophylactic potential of cloned Shiga toxin 2 B subunit. *J. Infectious Dis.*, 183: 435-443.
20. Mukherjee, J., K. Chios, D. Fishwild, D. Hudson, S.O. Donnell and S.M. Rich, 2002. Human Stx2-specific monoclonal antibodies prevent systemic complications of *Escherichia coli* O157:H7 infection. *Infection and Immunity*, 70: 612-619.
21. Maa, Y., X. Maoa, J. Li, H. Li, Y. Jiang, S. Gua, Y. Hao, Z. Gang, G.K. Yangb and Q. Zoua, 2008. Engineering an anti-Stx2 antibody to control severe infections of EHEC O157:H7. *Immunology Lett.*, 121: 110-115.
22. Abe, T. and H. Takaku, 2000. Protection against influenza by genetically engineered vaccines. *Nippon Rinsho*, 58: 2313-2320.
23. Sambrook, J. and D.W. Russell, 2001. Molecular cloning: A laboratory manual. 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
24. Clemson, M. and W.J. Kelly, 2003. Optimizing alkaline lysis for DNA plasmid recovery. *Biotechnology and Applied Biochemistry*, 37(3): 235-244.
25. Huang, A., S. De Grandis, J. Friesen, M. Karmali, M. Petric, R. Congi and J.L. Brunton, 1986. Cloning and expression of the genes specifying Shiga-like toxin production in *Escherichia coli* H19. *J. Bacteriol.*, 166(2): 375-379.
26. Acheson, D.W., S.A. De Breucker, M. Jacewicz, L.L. Lincicome, A. Donohue-Rolfe, A.V. Kane and G.T. Keusch, 1995. Expression and purification of Shiga-like toxin II B subunits. *Infection and Immunity*, 63(1): 301-308.
27. Cha, H.J., S.K. Bae, H.Y. Lee, H. Sato, M. Seiki, C. Park and K.W. Kim, 1996. Antiinvasive activity of urosolic acid correlates with the reduced expression of matrix metalloproteinase-9 (MMP-9) in HT1080 human fibrosarcoma cells. *Cancer Res.*, 56: 2281-2284.
28. Capozzo, A.V.E., V.P. Creydt, G. Dran, G. Fernández, S. Gómez, L.V. Bentancor, C. Rubel, C. Ibarra, M. Isturiz and M.S. Palermo, 2003. Development of DNA Vaccines against Hemolytic-Uremic Syndrome in a Murine Model. *Infection and Immunity*, 71(7): 3971-3978.
29. Bentancor, L.V., M. Bilen, R.J.F. Brando, M.V. Ramos, L.C.S. Ferreira, P.D. Ghiringhelli and M.S. Palermo, 2009. A DNA Vaccine Encoding the Enterohemorrhagic *Escherichia coli* Shiga-like toxin 2 A2 and B subunits confers protective immunity to Shiga toxin challenge in the Murine model. *Clinical and Vaccine Immunol.*, 16(5): 712-718.