Cloning and Expression of N-terminal Region of *IpaD* from *Shigella dysenteriae* in *E. coli*

Mojtaba Saadati[1,2,*], Mohammad Heiat[2], Shahram Nazarian[1], Babak Barati[1], Hossain Honari[2], Mohammad Doroudian[1,2], Mahdi Hesaraki[1], Fatemeh Malaei[1], Marzieh Eghtedardoust[1], Mohammad Rahbar[3], Saeed Hossein zadeh[4], Mehrdad Hashemi[5]

[1]Department of Biology, Imam Houssein University, Tehran, Iran.
[2]Applied Biotechnology and Environmental Research Center, Baqiyatallah Medical Science University, Tehran, Iran.
[3]Microbiology group collaborating Center for Reference and Research on Rabies.
[4]Department of Food Microbiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[5]Department of genetics,Islamic Azad University.Tehran Medical Branch

*Corresponding Author: email address: Saadati_m@yahoo.com (Mojtaba Saadati)

ABSTRACT

Genus *Shigella* is one of the important members of the family enterobacteriacae. There are numerous antigens in *Shigella* carrying by a 220 kb plasmid. Among them, IpaD is the key virulence factor of *S. flexneri*. Apart from having effectors function that is essential for host cell invasion and intracellular survival, this protein also controls the secretion and translocation of other effector proteins into eukaryotic host cells. In the present study, we have cloned and expressed the *ipaD* in *E. coli*. The *ipaD* gene was amplified by PCR. Prokaryote expression vector pET-28a(+)- *ipaD* was constructed, and used to transform *E. coli* BL21DE3 plySs. The expression of recombinant protein induced by IPTG was examined by SDS-PAGE. Western blot were used to determine immunoreactivity of IpaD-His by a rabbit monoclonal antibodies against his-tag. SDS-PAGE demonstrated that the constructed prokaryotic expression efficiently produced IpaD at the 1 mmol/L of IPTG. IpaD protein was able to react with the rabbit monoclonal antibody against His-tag. IpaD is essential for *Shigella spp* invasion. N-terminal region is most significant functional fragment of IpaD. Purification of IpaD from the wild type of *Shigella* is difficult furthermore profound study on a specific domain on the N-terminal of IpaD by using the wild type of purified IpaD is not feasible.

Keywords: *Shigella ssp*; N-terminal region, *ipaD*; cloning; expression

INTRODUCTION

Enterobacteriacae, are large groups of the enteric, non-spore forming bacteria which considered as normal inhabitants of intestine in both animals and human. *E. coli*, salmonella, klebsiella, proteus and enterobacter are some important genus in this family. Some strains such as *E. coli* are considering as normal inhabitants while some are normal flora of gastro-intestinal tract (GIT). A few genuses in the family, enterobacteriaceae such as *Salmonella* and *Shigella* are pathogen for human and animals [1]. *Shigella dysentriae* is one of the major causes of dysentery during last century, with a different mortality rate [1, 2]. The prevention of such diseases is mainly depending on the level of sanitation. No effective vaccination program has been proposed, so far. Few antigens of shigella have expressed which were potentially immunogenic in human. A large number of these antigens are carrying by a 220 kDa plasmid [3, 4]. A number of operons are located on the plasmid in which the entry region is playing a major role in the antigenicity of the microorganism [5]. *ipaD* is one of the operon harboring *Ipa,a, b, c,* and *d* [6]. Each operon is playing a role in the invasion of shigella to the M-cells with the clone [7]. IpaD is a 37 kDa with a dumbbell shape structure which exactly localizes to the tip of the type III secretion system needle of Shigella spp [8]. IpaD is necessary to invade the microorganism to host cells, carrying two main regions; N- and C-terminals in which the latter is a hydrophobic domain and not available in the environment. IpaD is fused to the C-terminal domain of a protein called MxiH, the main subunit of T3SS, thus, the protein residues of MxiH is cleaving a ligand for the C-terminal portion of IpaD [9, 8]. IpaD is interacting to the surrounding environment by its globular N-terminal. The key factor in the invasion of shigella to host epithelial cells is depending on the function of IpaD [7, 8]. Bile salts are also necessary to induce the

invasive potential of the bacterium [10]. In fact, the reaction between bile salts especially deoxycolate with IpaD may recruitment and employment of other proteins to locate on cell membrane of the bacterium [8, 11]. The condition permits the bacterium to attach and invade the host cells [8, 12]. Laboratory techniques and statistical analysis of previous data are indicating that deoxycolate can directly attach to a specific region on the N-terminal portion of IpaD, between MixH and IpaD. Any interaction in the function of this region may limit the function of IpaD and thus block the attachment process and/or invasion of the bacterium to the target cells [8, 11]. Previous researches have shown that the antibody that detects the N-terminal of IpaD, can stop the ability of the microorganism in making pores in red blood cells (RBC) [8]. However, further researches have shown that anti- IpaD antibody may inhibit the entry of shigella into its host cells. Recent work on producing an effective vaccine against shigella is based on using IpaD and its functional derivatives was also successful [13]. Therefore, IpaD could be considered as potential candidate and thus, some more work on the antigenicity of IpaD are seems to be Recruitment. Cloning and expression of the *ipaD* and its derivatives are necessary to find more about the antigenicity of such compounds. In the present study, we have tried to clone and express the N-terminal portion of the IpaD.

MATERIALS AND METHODS

Enzymes, Vectors and Bacterial strains

Pfu DNA polymerase (2.5U/µl, Fermentas, Lithuania), Enzymes *Hind*III and *Eco*RI (Fermentas, Lithuania.), IPTG (Vivantis, Malesia), Vector pET-28a(+) (Novagen USA), Vector pGEM-T (Promega, USA), *S. dysenteria*, was prepared from Milad hospital and confirmed by biochemical and serological tests. the genomic DNA of *shigella dysenteria* was Extracted and used as the templet in PCR experiment. *E. coli* DH5α and *E. coli* BL21DE3 plysS were used for cloning and expression experiments respectively. Plasmid pGEM-T Vector and pET-28a(+) were used as cloning and expression vectors respectively.

Amplification of N-terminal section of ipaD gene

Genomic DNA was extracted by a routine CTAB NaCl method [14]. The DNA fragment coding for N-termial region of *ipaD* gene, with accession number NC_007607.1, was amplified by using 2 primers. These primers which were designed by oligo software were IpaDF (5´T CAT GAA TTC AGA ACA ACA AAT CAG 3) as a forward primer with an endonuclease site of *EcoR* I and IpaDR (5´T CTT AAG CTT TTA AGT ATA TGA ACT AAC G 3') as reverse primer with an endonuclease site of *Hind*III. Synthesis of those primers was performed by Cinaclon Bio-tech Company. Amplification was made in a total volume of 50 µl of reaction mixture containing 1µl of genomic DNA(0.5µg/µl) , 5 µl of 10× *Pfu* buffer with $MgSo_4$, 5 µl dNTP Mix(2mM each), 2 µl of each primer (10 pmol) and 0.25 µl of *Pfu* DNA polymerase (2.5 unit/µl). By adding double-distilled water, the reaction mixture reached the Final volume 50 µl. A total of 32 cycles was performed with the first denaturation at 95 ℃ for 5 min, then 30 cycles at 95 ℃ for 30 second, 57 ℃ for 30 second and 72 ℃ for 30 second, and the final extension at 72 ℃ for 5 min. The amplified products were analyzed by electrophoresis with a 1% agarose gel followed by ethidium bromide staining and UV-transilluminator visualization.

Cloning, subcloning and sequencing

The PCR products of N-terminus region of *ipaD* were trailed with dATP, ligated with pGEM-T vector and transformed into *E. coli* DH5α. Ampicillin selection and some control tests ensured the presence of the recombinant plasmid. In order to subcloning, Fragments of *Eco*RI and *Hind*III-digested *ipaD* were inserted into the *EcoRI/Hind*III site of expression vector pET-28a(+), through enzyme digestion and ligation reactions. Then the recombinant plasmid pET-28a(+)-*ipaD* was confirmed by PCR and restriction enzyme digestion. Recombinant pET-28a(+)-*ipaD* was amplified in competent *E. coli* DH5α and then extracted by Alkaline lysis method [14]. The resulting plasmid pET-28a(+)-*ipaD* was Transformed into competent final host *E. coli* BL21DE3 plysS and kanamycin resistance was used for selection [14]. A recombinant plasmid was prepared and identified by restriction enzymes. In order to sequence of inserted fragment, 20 µl of purified recombinant plasmid was sequenced by Koser Bio-tech Company.

Expression and identification of the fusion protein

The N-terminal *ipaD* expression system pET-28a(+)-*ipaD* BL21DE3 was cultured in LB medium at 37 ℃. After the OD of bacteria reached to 0.6, it induced by isopropylthio-β-D-galactoside (IPTG) at final concentration of 1 mmol/L. The precipitate and incubated for 5h. The bacteria were collected by centrifugation and the cell pellet was broken by B Buffer (NaH_2PO_4:13/8gr, Tris.HCl:1/2gr ,urea 480/5gr, add DDW to 1liter,adjust pH in 8). The molecular weight of IpaD fusion protein was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblot analysis

Immunoreactivity of IpaD fusion protein was determined by Western blot. For Western blotting, proteins were transferred to nitrocellulose membrane and incubated with the rabbit antiserum against Histidin and HRP-labeling sheep anti-rabbit IgG as the first and second antibodies, respectively.

RESULTS

Construction of recombinant pET-28a(+)-ipaD

The PCR product amplified from genomic DNA of shigella is shown in figure 1. The 344-bp expected fragment amplified by PCR contained a gene ipaD. The 344-bp PCR product was cloned into pGEM-T vector at first step. The recombinant pGEM-T-ipaD vector digested with EcoRI and HindIII enzymes and ipaD fragments ligated into the corresponding sites of pET-28a(+). The recombinant plasmids pET-28a(+)-ipaD were digested by EcoRI and HindIII and analyzed on agarose gel electrophoresis is shown in figure 2.

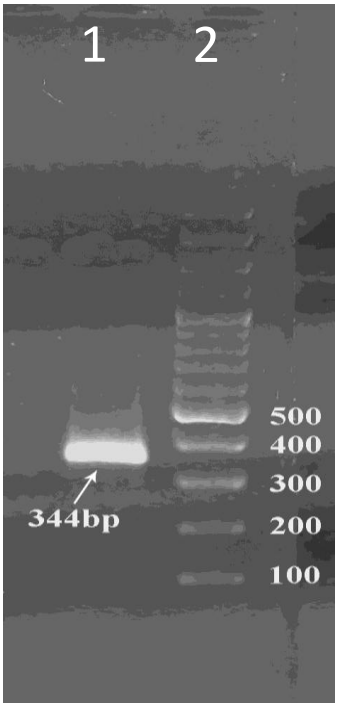

Figure 1. The target fragment of N-terminal of *ipaD* gene amplified from *Shigella dysenteriae*.
Lane 1: The target amplification of *ipaD* gene (*S. dysenteriae*)
Lane 2: 100 bp DNA size marker. (#SM0623 purchased from fermentas co.)

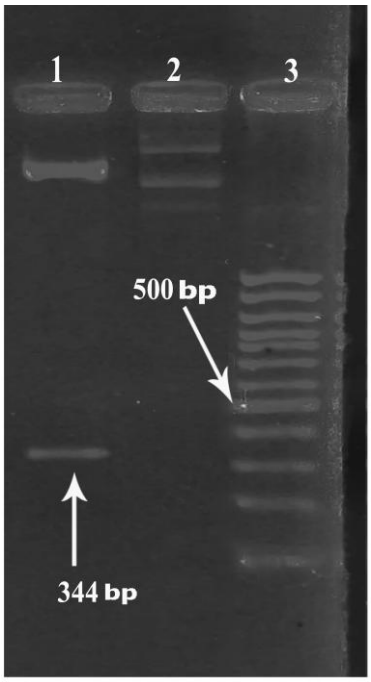

Figure 2. Agarose gel electrophoresis analysis of recombinant pET-28a(+)-*ipaD*
Lane1: Double digests of recombinant pET-28a(+)-*ipaD* with *Eco*RI and *Hind*III
Lane2: Recombinant pET-28a(+)-*ipaD* without digestion
Lane3: 100bp DNA size marker (#SM0623 purchased from fermentas co.)

Expression of recombinant fusion protein

The recombinant pET-28a(+)-ipaD was transformed into E. coli BL21DE3 strains and the fusion protein was expressed. The 1 mmol/L of IPTG was able to efficiently induce expression of IpaD fusion protein with a predicted molecular mass of 16.7 kDa is shown in figure 3. A culture of recombinant E. coli BL21DE3 plysS that wasn't induced by IPTG used as a negative control.

Western blot analysis using a specific

antibody against Histidine tag.

Expression of the N-terminal region of IpaD was investigated in E. coli. For this reason, an antibody against the Histidine tag was employed. The expression of the recombinant protein was then confirmed by appearing a proper band control is shown in figure 4.

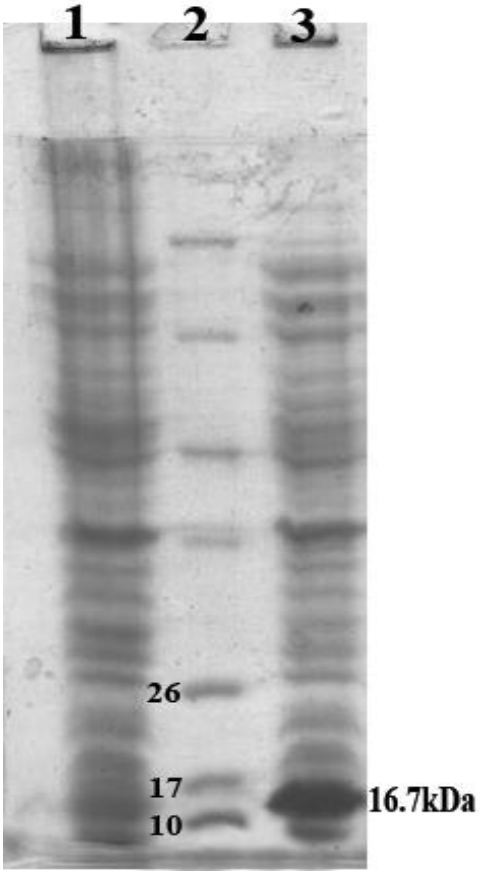

Figure 3. Expression of IpaD protein in pET-28a(+)-*ipaD*-BL21DE3 plysS
Lane 1: Negative control (Non-induced recombinant bacterial cells)
Lane 2: Protein size marker (cat No:#sm0671 purchased from fermentas co.)
Lane 3: 16.7 kDa protein resulted from crud extraction of bacterial cells.

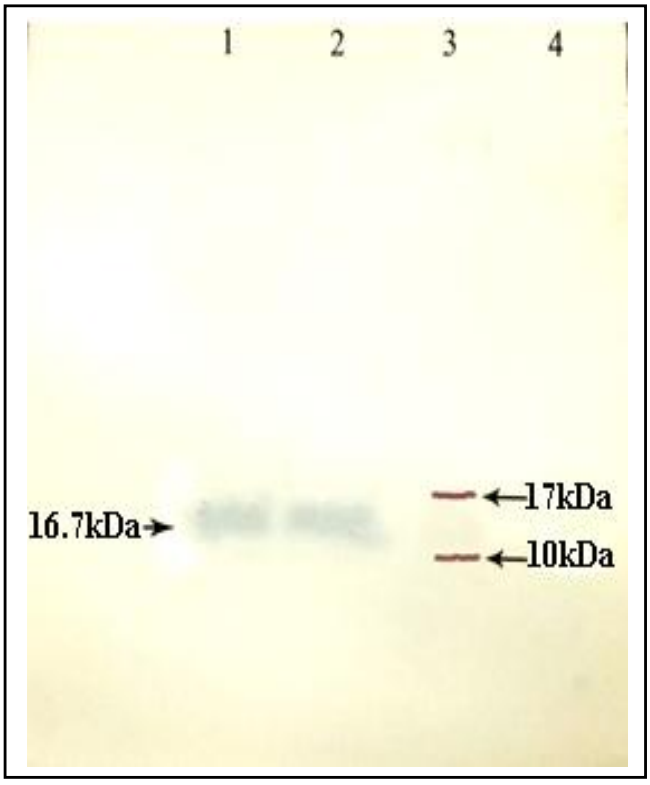

Figure 4. Western blotting analysis of expressed pET-28a-*ipaD* products.
Lane 1: Induced recombinant bacterial cells
Lane 2: Induced recombinant purified protein by nickel column
Lane 3: Protein size marker (cat No:#SM0671 purchased from fermentas co.)
Lane 4: Negative control (Non-induced recombinant bacterial cells)

DISCUSSION

The main agent of shigellosis is S. dysentriae, which is a gram negative pathogenic bacterium characterized by its invasion to the epithelial cells of large intestine. Attachment of the microorganism to the host cells may induce an explosive secretion of proteins via the type three secretion system (TTSS) of the bacterium. *Shigella* is transmitted by the fecal-oral route, typically via contaminated water, and is spread efficiently due to an unusually low 50% infective dose [7]. After ingestion, acid-tolerant *S.* dysenteriae passes through the stomach to the colonic mucosa, by transcytosis through M cells and encounters resident macrophages [15]. There, the shigellae enter macrophages and efficiently induce apoptosis, leading to the eventual release of the pathogen on the basal side of the colonic epithelium [16]. The *Shigellae* then invade these epithelial cells by inducing major cytoskeletal rearrangements [17]. The *Shigella* invasive genes have located on a 31-kb region of its large virulence plasmid which includes the genes for the type III secretion system (TTSS) [18]. The first group of proteins in which are excreted are; IpaD, IpaB and IpaC. The IpaD is essential component to invade the host cells. Antibodies are able to recognize the surface

epitopes of IpaD. In the present study, the antigenic properties of IpaD was investigated by calculative and laboratory techniques. The overall surface available epitopes of the IpaD are surrounded within the first 180 amino-acids composition of the protein, whereas, the carboxyl region in the surface of *Shigella* is not available. Even though, hydrophilic areas are present in the carboxyl side, recent studies on the serum of animals have shown that the production of antibodies against the C-terminal is considerably lower than the N-terminal region [19]. Purification of IpaD from the wild type of *Shigella* has previously explained. However, the technique is difficult, firstly because the microorganism produces the IpaD protein in low level. The second difficulty in the purification of IpaD is referring to find it among the plenty of proteins which need expensive and sophisticated biochemical techniques [20]. However, a profound study on a specific domain on the N-terminal of IpaD by using the wild type of purified IpaD is not feasible; furthermore, the risks of working with the wild type of *Shigella* and also high costs experiments are making them to be impractical.

The microorganism produces the IpaD protein in low level and purification of IpaD among the plenty of proteins is very difficult. one of the suitable method for producing of high level of protein is utilizing of recombinant method. Using of this method also endow the ability of studying on a specific region of antigen like IpaD. Although purification of IpaD from wild type bacterium is difficult but it can proposed as a vaccine candidate for shigellosis.

REFERENCES

1. Key B, Clemens J, Kotloff KL.Generic protocol to estimate the burden of Shigella diarrhoea and dysenteric mortality. Geneva: WHO document WHO V&B 1999; 99: 26.

2. 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. W.H.O 1999; 77:651–666

3. Yang F, Yang J, Zhang X, Chen L, Jiang Y, Yan Y, et al. Genome dynamics and diversity of Shigella species, the etiologic agents of bacillary dysentery. Nucleic Acids Res 2005; 33:6445–6458.

4. Buchrieser C, Glaser P, Rusniok C, Nedjari H, D'Hauteville H, Kunst F, et al. The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of Shigella flexneri. J. Mol. Microbiol 2000; 38:760–771

5. Sansonetti PJ, Kopecko DJ, Formal SB. Involvement of a plasmid in the invasive ability of Shigella flexneri. J. Infect Immun 1982; 35:852–860.

6. Venkatesan MM, Buysse JM, Kopecko DJ. Characterization of invasion plasmid antigen genes (ipaBCD) from Shigella flexneri. Proc. Natl. Acad. Sci. USA 1988; 85:9317-9321.

7. Hayward RD, Cain RJ, McGhie EJ, Phillips N, Garner MJ, Koronakis V.Cholesterol binding by the bacterial type III translocon is essential for virulence effector delivery into mammalian cells. J. Mol. Microbiol 2005; 56: 590–603.

8. Espina M, Olive AJ, Kenjale R, Moore DS, Ausar SF, Kaminski RW, et al. IpaD localizes to the tip of the type III secretion system needle of Shigella flexner. J. Infect. Immun 2006; 74: 4391–4400.

9. Lingling Z, Wang Y, Olive AJ, Smith ND, Picking WD, De Guzman RN, et al. Identification of the MxiH Needle Protein Residues Responsible for Anchoring Invasion Plasmid Antigen D to the Type III Secretion Needle Tip. J. Biol. Chem 2007; 44:32144–32141.

10. Pope LM, Reed KE, Payne SM. Increased protein secretion and adherence to HeLa cells by Shigella spp. following growth in the presence of bile salts. J. Infect. Immun 1995; 63: 3642–3648.

11. Stensrud KF, Adam PR, La Mar CD, Olive AJ, Lushington GH, Sudharsan R, et al. Deoxycholate Interacts with IpaD of Shigella flexneri inInducing the Recruitment of IpaB to the Type III Secretion Apparatus Needle Tip. J. Biol Chem 2008; 238:18646-18654.

12. Skoudy A, Mounier J, Aruffo A, Ohayon H, Gounon P, Sansonetti P, et al. CD44 binds to the Shigella IpaB protein and participates in bacterial invasion of epithelial cells. J. Cell. Microbiol 2000; 2: 19–33.

13. Allaoui A, Sansonetti Ph, Sani M, Botteaux A, Parsot C, Boekema EJ. Shigella Ipad protein and its use as a vaccine against Shigella infection. International Application patent 2008; PCT/IB2007/004192

14. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.

15. Schroeder GN, and Hilbi H. Molecular Pathogenesis of Shigella spp.: Controlling Host Cell Signaling, Invasion, and Death by Type III Secretion. Clinical Microbiology Reviews 2008; Jan. 2008: 134–156.
16. Zychlinsky A, Prevost MC, Sansonetti PJ. Shigella flexneri induces apoptosis in infected macrophages. J. Nature 1992; 358: 167-169.
17. Sansonetti PJ, Ryter A, Clerc P, Maurelli AT, Mounier J. Multiplication of Shigella flexneri within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. J. Infect. Immun 1986; 51: 461-469.
18. Cossart P, Sansonetti PJ. Bacterial invasion: the paradigms of enteroinvasive pathogens. J. Science 2004; 304: 242-248.
19. Turbyfill KR, Jennifer AM, Corey PM, Edwin VO. Identification of Epitope and Surface-Exposed Domains of Shigella flexneri Invasion Plasmid Antigen D (IpaD). J. Infection and Immunity 1998; 66: 1999–2006.
20. Parsot C, Sansonetti PJ. Invasion and the pathogenesis of Shigella infections. J. Microbiol. Immunol 1996; 209: 25-42.