Immunogenicity of a New Recombinant IpaC from *Shigella dysenteriae* Type I in Guinea Pig as a Vaccine Candidate

Fatemeh Malaei[1], Mahdi Hesaraki[2], Mojtaba Saadati[1*], Ali Mohammad Ahdi[3], Mohammad Sadraeian[4], Hussein Honari[1], Shahram Nazarian[1].

[1]Biology Research Center, Faculty of Science, Imam Hussein University, [2]Department of Stem Cell and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, [3]Department of Genetics, Faculty of Science, Shahrekord University, Shahrekord, [4]Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

ABSTRACT

Background: Recombinant vaccine technology is one of the most developed means in controlling infectious diseases. However, an effective vaccine against *Shigella* is still missing. **Objective:** To evaluate recombinant IpaC protein of *Shigella* as a vaccine candidate. **Methods:** In this study we cloned IpaC gene into an expression vector in prokaryotic system. The protein expression was evaluated by SDS-PAGE and Western-Blotting analysis. The recombinant protein was purified using Ni–NTA affinity chromatography. Guinea pigs were immunized with the recombinant protein and the level of immunogenicity was examined by ELISA and Western blotting of IpaC. Challenge test was done through the intraoculary injection of *Shigella dysenteriae* (6×108 CFU/eye) and after 48 hours was scored for keratoconjunctivitis. **Results:** The results showed a remarkable level of immunogenicity in terms of antibody response and protection against keratoconjunctivitis in tested animals. The recombinant IpaC protein provided a protective system against *Shigella dysenteriae* type I during the challenge test. **Conclusion:** The results showed the potential of using recombinant IpaC in preparation of vaccine in perspective studies.

Malaei F, et al. Iran J Immunol. 2013; 10(2):110-17

Keywords: Expression, IpaC Gene, Recombinant Vaccine, *Shigella dysenteriae*

*Corresponding author: Dr. Mojtaba Saadati, Biology Research Center, Faculty of Science, Imam Hussein University Tehran Iran, Tel: (+) 98 21 77104934, Fax: (+) 98 21 77104934, e-mail: saadati_m@yahoo.com

INTRODUCTION

It is estimated that 600,000 children less than 5 years of age living in developing countries die by shigellosis every year (1). Despite the fact that *Shigella* was discovered over a century ago, shigellosis remains as an important public health challenge, especially in developing countries. The infectious dosage, which can be as low as 10 cells, enables the disease to spread effectively through contaminated food or water, and also person to person contact (2). Approximately 99% of total new incidence is spread out in developing countries, where poor hygiene and limited access to refined drinking water promote the spread of enteric diseases. Malnutrition and the lack of appropriate medical intervention contribute to the high mortality rate, especially for young children. Mechanism of pathogenesis in shigellosis is based on type III secretion system (TTSS). This procedure forms a pore in the host cell membranes and delivers translocator and effector proteins through the pore into the target cells (3).

The genes involved in the cell entry of organisms are located on a 30-kb region in sd197 plasmid. This region contains the *ipa*, *mxi*, and *spa* operons, as well as the *virB* gene, which encodes a transcriptional activator. Four Ipa proteins, IpaA to IpaD, which are encoded by the *ipa* operon, are secreted through a type III secretion apparatus which is encoded by the *mxi* and *spa* operons. Although *ipa*, *mxi*, and *spa* operons are expressed by bacteria growing in vitro, only a small proportion of Ipa proteins is actually secreted by the wild-type strain under these growth conditions. In contrast, the secretion of presynthesized Ipa proteins, *Shigella* invasion plasmid antigen, is activated upon contact of bacteria with epithelial cells (4).

IpaC is necessary for the invasion of bacteria into epithelial cells. It triggers signals that causes actin polymerization. It is also shown that IpaC can assemble actin filaments in vitro which are associated with its Salmonella homolog SipC behavior (5). The putative extracellular IpaB-IpaC complex has been proposed to mediate pathogen entry possibly through binding of $\alpha_5\beta1$ integrin receptors (6). IpaB and IpaC separately bind to the chaperone IpgC in the bacterial cytoplasm and assemble upon secretion, thereby forming the pore inserted into host cell membranes for translocons (5). IpaB and IpaC contain hydrophobic segments and remain associated with the membrane of lysed erythrocytes, suggesting that these two proteins are components of the *S. flexneri* translocator. In addition, some effector functions have been proposed for IpaB and IpaC (7). The IpaB and IpaC have some roles in eventual escape from phagolysosomes. The Ipa proteins induce uptake of latex beads and noninvasive *Shigellae*, in addition to traditional vaccine antigens (8). The N terminus of IpaC harbors sequences for TTSS export and interaction with IpaB and recognition by IpgC. The central hydrophobic region is involved in IpaB binding, IpaC penetration of phospholipids membranes, and possibly protein stabilization. The C terminus of IpaC possesses essential oligomerization and effector domains (9,10).

According to previous studies on Shigella vaccine based on LPS and the IpaB and IpaC invasive proteins are important in pathogenesis and immunogenicity (11,12,13,14). However, due to lack of effective vaccine against Shigella strains and increasing frequency of antibiotics resistance, there is an urgent need for finding candidate molecules to be used in vaccination against Shigella. Therefore, the main goals of this study were cloning, expression, purification and functional analysis of *IpaC* produced in prokaryotic system.

MATERIALS AND METHODS

Construction of Recombinant Pasmid *IpaC*-pET-28a (+). Forward primer with *EcoR*I restriction site 5'-GAAGTA GAATTC ATG GAA ATT CAA AAC ACA A-3' and reverse One with *Hind*III restriction site 5'-GAAAAT AAG CTT TTA AGC TCG AAT GTT ACC AG-3' were designed and qualified by Gene runner software (V.3.o5). Reference sequence number for primer designing was NC_007107 in NCBI gene bank.
Total DNA was extracted from 5 ml LB (Luria Bertani) media using CTAB/NaCl method (15). PCR was performed in 25µl total volume include 1u *Pfu* DNA polymerase (Fermentas, USA), 0.2 mM dNTPs, 2 mM $MgSo_4$ and 0.8 pmol of both forward and reverse primers in final concentration. PCR program performed as initial denaturation in 94°C for 5 min followed by 30 cycles: 40 s at 94°C, 30 s at 57°C, and 1 min at 74°C and a final extension for 5 min in 74°C. PCR product was evaluated 1% agarose gel electrophoresis and detected by ethidium bromide, and visualized under a UV transilluminator (15).
PCR product was purified using gel extraction kit (Fermentas #K069). Purified PCR product and pET-28a(+) vector were double digested by using of 1µ *EcoR*I and *Hind*III in total volume digestion reaction with 2x Tango buffer (Fermentase) concentration. Digested fragment and vector were ligated using T4 ligase. *E.coli* DH5α competent cells were transformed by pET-28a/*IpaC*. After one round recombinant DH5α culture and plasmid extraction using plasmid extraction kit (Fermentas #0503). Recombinant vector containing *IpaC* gene was transformed into *E.coli* BL21DE3 pLysS competent cells.
Colonies were screened by three tests; digestion of a miniprep plasmid DNA with *EcoR*I and *Hind*III, colony PCR and sequencing of purified plasmid using T7 terminator primer ABI pyrosequences by 3130 xlgenetic analyzer-20253-001 from human genetic laboratory (15).
Expression and Purification of Recombinant IpaC. *E.coli* BL21DE3 harboring recombinant plasmid pET-28a/*IpaC* was grown in 5 ml LB containing kanamycin antibiotic 37°C at 150 rpm for overnight. For optimization of protein expression, various times, temperatures and IPTG concentrations were tested and the best condition was optimized. Bacterial culture (in OD of 0.6 at 600 nm) was induced with 1 mM isopropylthio-β-galactoside (IPTG) at 37°C for 4 h. Harvested cells were lysed in 5x sample buffer [100mmol Tris HCl pH 8, 20% glycerol, 4% sodium dodecyl sulfate (SDS), 2% beta mercapto- ethanol, 0.2% bromo phenol blue] and analyzed with 12% SDS polyacrylamide gel with protein marker (SM 0671) as described by Sambrook (15). The gel was stained with Coomassie brilliant blue R-250. Non-induced cell culture was analyzed as negative control. For high level purification of IpaC, the supernatant also was mixed by Ni-NTA column (QIAGEN). Purified proteins were dialyzed against pH 7.2, Tris-Hcl to remove urea and protein refolding (16).
Western Blotting Analysis. Western blotting was performed using monoclonal Rabbit anti His-tag antibody or immunized guinea pigs sera. Protein samples were under electrophoresis using SDS-PAGE and then transfer to nitrilocellulose membrane. Nitrilocellulose membrane impute blocking buffer at 4°C for overnight. Briefly, membrane was incubated by primary antibody (anti-His tag) and detected by HRP-conjugated mouse anti-rabbit antibody or HRP-conjugated goat anti guinea pigs IgG as secondary antibody, subsequently was detected by DAB (3,3- Diaminobenzidine) as the chromogenic reaction substrate (17).

Immunogenicity and Protective Efficacy of IpaC in Guinea Pigs. In order to evaluate immunogenicity of the recombinant IpaC, 10 male guinea pigs (Hartley albino strain) were injected with 30µg of recombinant IpaC in 250 µl of phosphate-buffered saline (PBS) and 250 µl Freund's complete adjuvant (Razi, Ins) as the test group. Three booster does were given at 14 day intervals, using Freund's incomplete adjuvant. Also, we consider 10 other guinea pigs as control group. Blood samples from guinea pigs were collected before each booster injection and 14 days after the last injection to determine the antibody titration by ELISA assay. Sera obtained after centrifugation of blood at 1000 rpm for 10 minute. Two weeks after the final immunization (recombinant IpaC), all guinea pigs (control and test) were challenged intracellulary with *S. dysenteriae* Type 1(6×10^8 CFU/eye). Following challenge with the virulent strain, animals were inspected daily (48 h) for the development of keratoconjunctivitis.

Indirect ELISA. ELISA method utilized to measure serum antibody levels. Briefly, Serial dilutions of sera (1:400- 1:6400) and HRP-conjugated goat anti guinea pigs IgG (DAKO, Co) were used against coated recombinant IpaC. For detection, the substrate (OPD + H_2O_2) was added, the reaction mixture was incubated for 10 min and then reaction was stopped by the addition of 0.5 M H_2SO_4. The OD of the reaction mixture was measured at 490.

RESULTS

PCR Amplification of *IpaC* Gene and Restriction Map. 1092 bp PCR product was detected using 1% agarose gel electrophoresis followed by Et Br staining. To confirmation of PCR product fidelity, we analyzed a restriction map on it. According to restriction map in NEB cutter, *IpaC* gene produced two DNA segments of 299 and 800 bp after *Sac*I digestion (Figure 1).

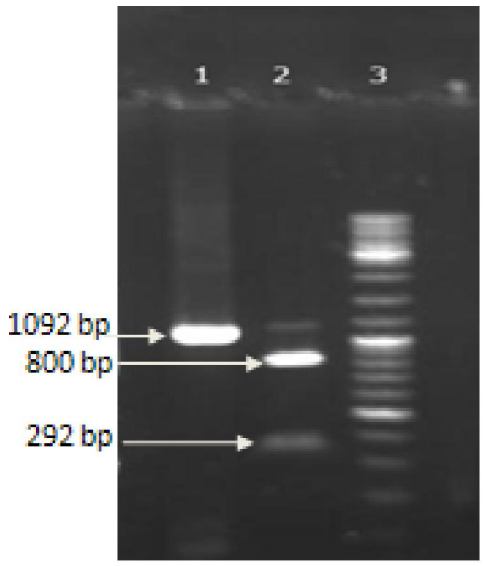

Figure 1. Analysis of PCR product on 1% agarose gel electrophoresis. Lane 1, PCR product of *IpaC* gene. Lane 2, PCR product was digested with *Sac*I. Lane 3, 10 kb DNA size marker.

Construction of Recombinant Plasmid and Restriction Enzyme Confirmation. The produced construct, pET28a(+)-E7-STxB, was confirmed by 3 methods; First, by restriction enzyme analysis, then by PCR reaction of extracted vectors and finally by

sequencing of the inserted fragment. Digested pET28a/*IpaC* with *Eco*RI and *Hind*III confirmed insertion in expression plasmid. The sequence get in this step was submitted to gene bank (GenBank accession JQ657702).

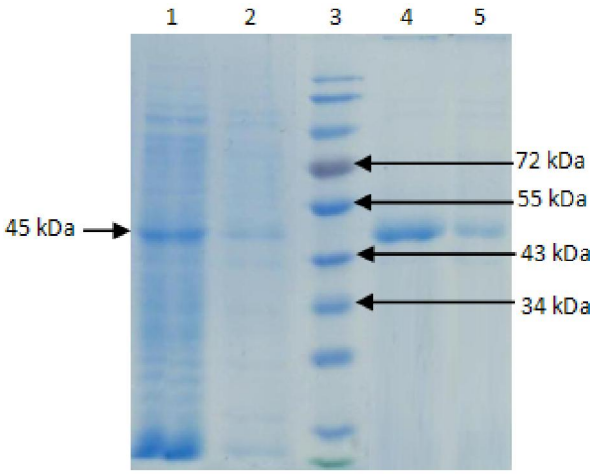

Figure 2. SDS-PAGE analysis of purified IpaC recombinant protein from soluble fraction by using of Ni-NTA column. Lane 1,2,3,4,5. Induce cells (1), exited sample of buffer C(2), protein marker SM0671(3), exited sample of buffer E (4), exited sample of buffer MES(5).

Expression and Purification of *IpaC* Gene in *Escherichia coli*. Recombinant protein was purified by using of Ni-NTA column affinity chromatography after induction recombinant bacteria harboring *IpaC*- pET28a (+) by 1 mM IPTG for 4 h cell lysis and protein precipitation was carried out. SDS-PAGE analysis confirmed a 45-kDa molecular weight size for recombinant protein as we expected. SDS-PAGE analysis was followed by Western-Blot analysis, using anti His-tag monoclonal antibodies. Western-Blot assay confirmed fidelity of expressed recombinant protein (Figure 2).

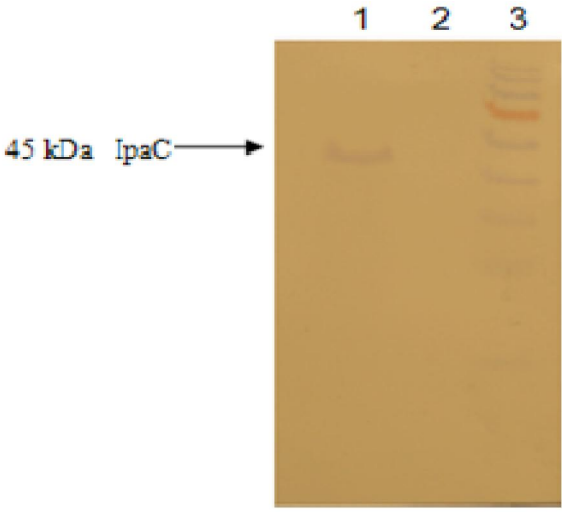

Figure 3. Western blot of IpaC. Lane 1, supernatant from *E. coli* clone with insert. Lane 2, from *E. coli* clone without insert. Lane 3, Protein Molecular Weight Marker.

Immunogenicity and Assay of Recombinant Fusion Protein. Purified collected recombinant IpaC was applied for immunologic tests by ELISA analysis showed positive results for IgG (Figure 3) in sera from 10 guinea pigs immunized by recombinant IpaC. In contrast, serum from the control group showed negative results. Also data indicated that the production of antibody was increased after last injection lower than 1/1600 dilution (Figure 4).

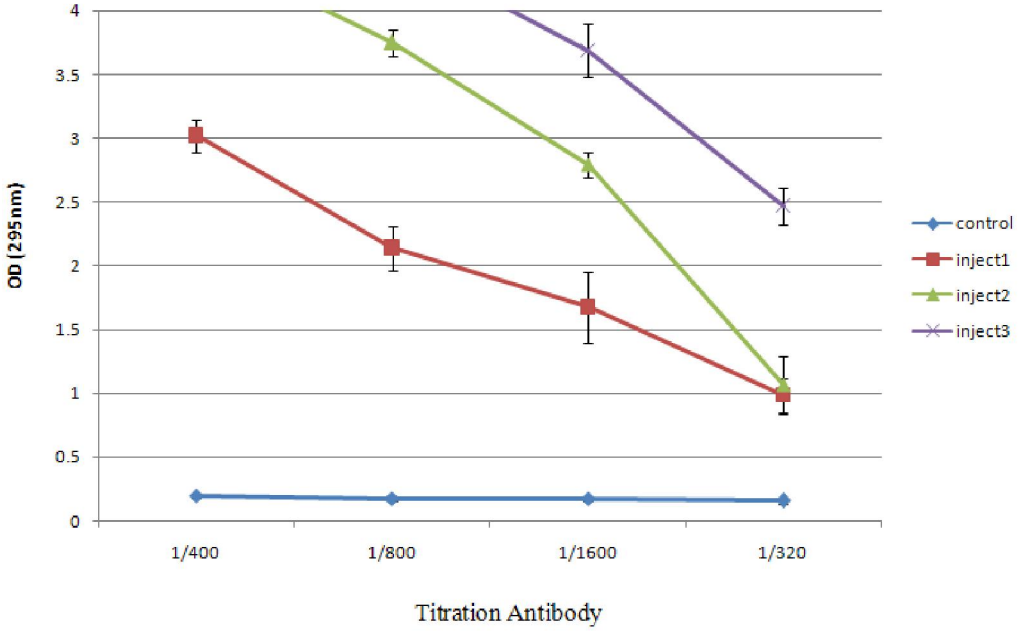

Figure 4. Investigation antibody titration in the serum of guinea pigs against IpaC. Titers over 4 OD could not be measured.

Western blot analysis demonstrated correct interaction between protein purified (recombinant IpaC) and produced antibody (Figure 5).

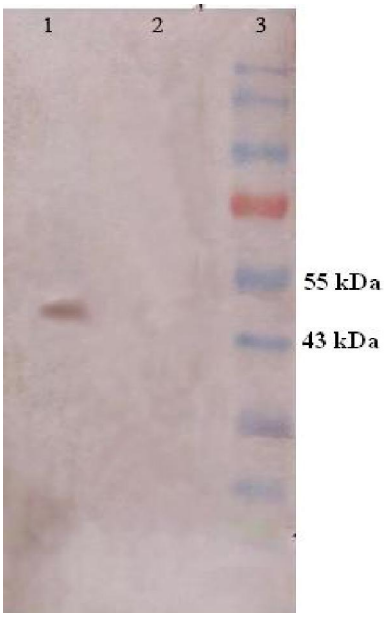

Figure 5. Western blot analysis. Lane 1, Purified recombinant IpaC. Lane 2, from *E. coli* clone without pET28/*ipa*C. Lane 3, Protein Marker SM0671.

All guinea pigs immunized (test) and non immunized (control) were challenged intraocularly with virulent *S. dysenteriae* Type 1(6×10^8 CFU/eye) according to Sereny test. After 24-48 h all control animals were fully developed in keratoconjunctivitis with purulence whereas in immunized animal low Sereny reaction or mild irritation was observed (Figure 6).

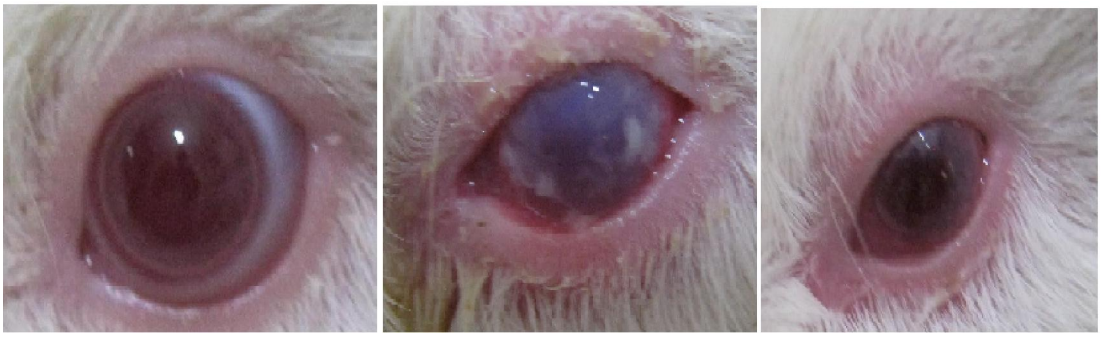

Figure 6. Clinical results of challenge with *S. Flexneri* 2a. Left picture: eye guinea pig in normall condition, middle picture: the eye of negative control after challenge, right eye: eye immunized guinea pig after challenge.

DISCUSSION

In this study we constructed a new recombinant candidate vaccine against shigellosis. Our analyses on purified recombinant protein showed a candidate vaccine that can be used in further studies to become standardized for a trial phase. However the method of delivery for this vaccine remains to be addressed. Although various types of vaccines against *Shigella* have been under development, nowadays only oral live attenuated vaccine of *flexeneri 2a and sonnei* (FS) are in use (5,18,19).

This study is the first report of evaluating the cloning, expression, immunogenicity and animal challenge of recombinant IpaC of *Shigella dysenteriae* strains from Iran. Regarding the numerous reports of mortality by shigellosis, antibiotics resistance and lack of a robust vaccine against this disease, developing a more effective vaccine for shigellosis is necessary. Vaccine development for invasive mucosal pathogens such as *Shigella* requires consideration of both the pathogenic mechanisms as well as the host immune responses upon infection. In both humans and monkeys, infection with *Shigella* induces an immune response against LPS and the protein invasive IpaB and IpaC protein (11).

In general, various studies have indicated that pathogenic and immunogenic role of IpaC is significant and this protein can be used as a candidate vaccine (12,13). Edwin et al. investigated Invaplex vaccine and showed that the vaccine had successfully primed the immune system (14). These reports support our opinion for selection of IpaC as a candidate vaccine. In 2012, Martinez-Becerra reported that intranasal immunization with IpaB/D can evoke a protective immune response only in combination with dmLT (20). Strikingly, we showed that the protection can be achieved with intraperitoneal administration of IpaC. In this study, the gene encoding *Ipa*C was isolated from local isolate of *Shigella dysenteriae* that causes Shigellosis in humans. DNA fragment of the expected size (near 1092 bp) was the most important domain of this gene (21-23). Similar to this study, previous studies reported that the purification had to be carried in the presence of urea. Therefore, IpaC was prepared by urea and then dialysis was used to remove urea (24,25). Immunogenecity assay for IpaC recombinant protein showed acceptable efficacy (80-90%) in the immunized animals with very low dose of purified protein.

In conclusion, we propose more standardized procedures to be carried on this protein for production of a new generation of recombinant vaccines against shigellosis.

ACKNOWLEDGEMENTS

We thank Dr. Ali Mohammad Latifi and all colleagues in the Applied Biotechnology Research Center from Baqiyatallah Medical Sciences University.

REFERENCES

1 Simona J, Wahida R, Maciel M, Pickingb J, Kotloff K and Levinea M. Antigen-specific B memory cell responses to lipopolysaccharide (LPS) and invasion plasmid antigen (Ipa) B elicited in volunteers vaccinated with live-attenuated *Shigella flexneri 2a* vaccine candidates. Vaccine 2009; 27:565-72.
2 Yayue L, Boyang Cao B, Xia Peng J, David A, Bastin Lu, Feng and Lei W. Molecular detection of all 34 distinct O-antigen forms of Shigella. Journal of Medical Microbiology 2009*;* 58:69-81.
3 Clinical microbiology reviews. 2008; 21:134-56.
4 Simona B, Zineb B, Armelle P and Philippe S. Functional Analysis of the *Shigella flexneri* IpaC Invasin by Insertional Mutagenesis. Infection and immunity 1997; 65:1599-603.
5 Gunnar N and Schroeder. Intracellular type III secretion by cytoplasmic *Shigella flexneri* promotes caspase-1-dependent macrophage cell death. Microbiology 2007; 153:2862-76.
6 Mary E, Marquart W, Picking and William D. Soluble invasion plasmid antigen C (IpaC) from *Shigella flexneri* elicits epithelial cell responses related to pathogen invasion. *Infection and immunity* 1996; 4182-7.
7 Musa S, Anne B, Claude P, Philippe S, Egbert J and Abdelmounaaim A. IpaD is localized at the tip of the Shigella flexneri type III secretion apparatus. Biochimica et Biophysica Acta. 2007; 1770:307-11.
8 Robert K, Kaminski W, Chao W, Ching and Edwin V. Mucosal Adjuvanticity of a *Shigella* Invasin Complex with DNA-Based Vaccines. Clinical and vaccine immunology 2009; 16:574-86.
9 Lisa A, John O, Jeff B, Wendy L, Baran E, William D and Russle C. Structure-function analysis of invasion plasmid antigen C (IpaC) from *Shigella flexneri*. The journal of biological. 2003; 278:2792-9.
10 Christina M, Wendy L, Picking E, Birket I, Kelly F, Bryce M, et al. The C-terminus of IpaC is required for effector activities related to Shigella invasion of host cells. Microbial Pathogenesis. 2008; 45:282-9.
11 Ross T, Robert W, Kaminski E. Immunogenicity Efficacy of highly purified invasin complex vaccine from Shigella flexneri 2a. Vaccine. 2007; 1353-64.
12 Malabi M, Venkatesan M and Dennis J. Characterization of invasion plasmid antigen genes (ipaBCD) from Shigella flexneri. Microbiology. 1988; 85:9317-21.
13 Simona B, Annick F, Philippe S, Armelle P. Induction of a local anti-IpaC antibody response in mice by use Of a *Shigella* flexneri 2a vaccine candidate: Implications for use of IpaC as a protein carrier. Infection and Immunity. 1996;1190-6.
14 Edwin V, Oaks K, Ross T. Development and evaluation of a *Shigella flexneri* 2a and *S. sonnei* bivalent invasion complex (Invaplex) vaccine. Vaccine. 2006; 24:2290-301.
15 Sambrook and Russell. Molecular cloning: a laboratory manual. 2nd Edition. Plainview: Cold Spring Harbor Laboratory press, New York. Volume 2001; 3: 1500-12.
16 John E, Ben M, David W and Paul T. Current Protocols in Protein Science. Copyright © 2007; 430-1700.
17 Bollag DM, Michel DR, Edelstein SJ. Protein methods. 2nd ed. New York: Wiley-Liss 1996.
18 Amy V and Jennison K. *Shigella flexneri* infection: pathogenesis and vaccine development. FEMS Microbiology Reviews. 2004; 28:43-58.
19 DeRoecka D, Clemensa J, Nyametea A and Mahoney R. Policymakers' views regarding the introduction of new-generation vaccines against typhoid fever, shigellosis and cholera in Asia. Vaccine 2005; 23:2762-74.
20 Martinez-Becerra F, Kissmann J, Diaz-McNair J, Choudhari S, Quick A, Mellado-Sanchez G, et al. Broadly Protective *Shigella* Vaccine Based on Type III Secretion Apparatus Proteins. Infection & Immunity. 2012; 80:1222-31.
21 Rebecca D, Mary E, Damien L, William D. protein-protein interaction in the assembly of *Shigella flexneri* invasion plasmid antigens IpaB and IpaC into protein complexes. Biochemica et Biophysica Acta. 1998; 1429:45-56
22 Geyter C. Purification of IpaC, a protein involved in entry of *Shigella flexneri* into epithelial cells and characterization of its interaction with lipid membrane. FEBS Letters. 1997; 400:149-54.
23 Swapan N. Shigellosis. The journal of microbiology. 2005*;* 43:133-43.
24 Amy F, Preiszner J, Shirley N and Robert I. Expression of His-tagged *Shigella* IpaC in *Arabidopsis*. Bolla Journal of Biotechnology. 2004; 112: 247-53.
25 Wingfield PT, Palmer I, Liang SM. Folding and purification of insoluble (inclusion-body) proteins from Escherichia coli. Curr Protoc Protein Sci. 1995; 1:651-7.