Chapter 24

An Approach to Identify and Characterize a Subunit Candidate *Shigella* Vaccine Antigen

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Abstract

Shigellosis remains a serious issue throughout the developing countries, particularly in children under the age of 5. Numerous strategies have been tested to develop vaccines targeting shigellosis; unfortunately despite several years of extensive research, no safe, effective, and inexpensive vaccine against shigellosis is available so far. Here, we illustrate in detail an approach to identify and establish immunogenic outer membrane proteins from *Shigella flexneri* 2a as subunit vaccine candidates.

Key words *Shigella flexneri* 2a, Outer membrane proteins, Protective immune response, Macrophages, T cell, B cell

1 Introduction

Shigellosis or bacillary dysentery, an acute intestinal infection caused by bacteria of genus *Shigella*, is a leading cause of childhood morbidity and mortality particularly in developing countries where it is estimated that over 163 million cases occur annually, leading to possibly one million deaths per year worldwide [1]. While control and treatment of shigellosis outbreaks with antibiotics is feasible, the high cost of antibiotics and the constant emergence of antibiotic resistant *Shigella* species, even to the newest antibiotics, stress the prerequisite for an effective vaccine to combat against shigellosis in the developing regions of the world [2].

Numerous strategies to develop vaccines targeting *Shigella* have been explored over several decades; nevertheless, a licensed vaccine is not accessible so far. Some of the important approaches of *Shigella* vaccines include live attenuated vaccines [3, 4], delivery of *Shigella* LPS or O polysaccharides with carriers such as proteosomes [5], tetanus toxoid [6], or ribosomes [7], conjugate vaccines, in which *Shigella* O-specific polysaccharide (O-Ag) is conjugated with protein from other strains [8], a hybrid vaccine, in which attenuated *Shigella* bacteria are used as vectors for expressing

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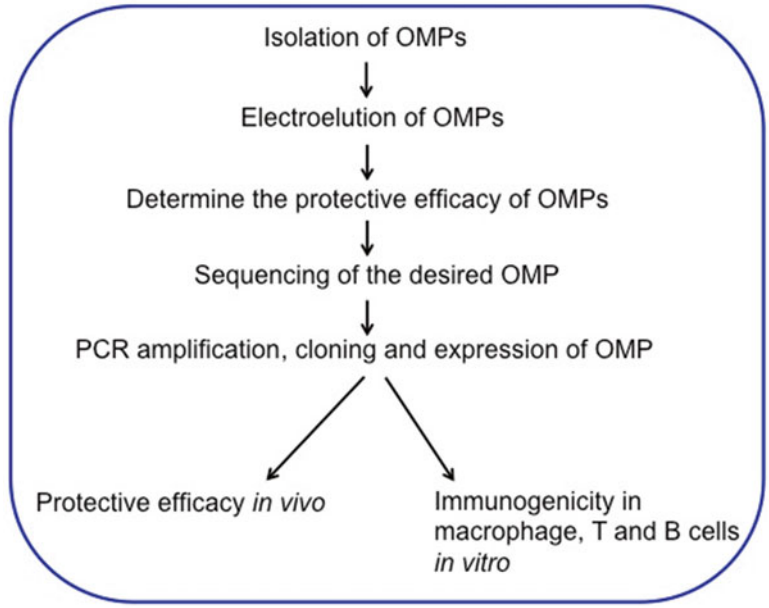

Fig. 1 Flowchart for identification of an effective vaccine antigen

enterotoxigenic *Escherichia coli* (ETEC) antigen [9], invasion proteins [10], etc. However, it has been observed that the current vaccine candidates are not immunogenic enough [11, 12], implying the identification of novel protective antigens capable of triggering robust immune response.

Recent studies have shown that bacterial outer membrane proteins (OMP) are attractive vaccine antigens [13, 14] and we have established outer membrane protein A (OmpA) of *S. flexneri* 2a as an immunogenic and efficacious protective subunit vaccine candidate against shigellosis [15–20]. Here we describe in detail of how OMPs of *S. flexneri* 2a can be identified and characterized as fruitful protective subunit vaccine antigens against shigellosis (Fig. 1).

2 Materials

Prepare all reagents and solutions using ultrapure water and store at room temperature unless stated otherwise. Please note we do not employ sodium azide to the reagents.

2.1 Components for Isolation of Outer Membrane Proteins

2.1.1 Bacterium

Shigella flexneri 2a (N.Y-962/92).

2.1.2 Culture Medium

Tryptic soy broth; Suspend 30 g of dehydrated media in 1 L of ultrapure water. Sterilize at 121 °C for 15 min. Cool to 45–50 °C. Mix gently and dispense into sterile culture tubes and store the tubes at 4 °C.

2.1.3 Buffer and Detergent

HEPES (N-2 hydroxyethyl piperazine N′-ethanesulfonic acid); 1 M HEPES, pH 7.0. Weigh 238.30 g HEPES and transfer to a glass beaker. Add ultrapure water to a volume of 900 ml. Mix and adjust pH with sodium hydroxide. Finally, bring the final volume to 1 L with ultrapure water. Use filtration for sterilization.

10 % N-lauroylsarcosine sodium salt; Dissolve 10 g of N-lauroylsarcosine sodium salt in 100 ml of 100 mM HEPES, pH 7.

2.2 Components for Evaluating Immune Response in Rabbit and Electroelution of Proteins

2.2.1 Animal

For immunization experiments use outbred New Zealand white rabbits of either sex weighing between 1.7 and 2.5 kg. Acclimatize all the animals in laboratory for a week before performing the experiments.

2.2.2 Preparation of Heat-Killed Bacteria

Grow *S. flexneri* 2a in TSB with 0.6 % yeast extract overnight at 37 °C with shaking (200 rpm). Harvest bacteria by centrifugation at 10,000 × *g* for 10 min, wash the bacterial pellet twice with phosphate buffer saline (PBS) and adjust to a concentration of 10^{10} bacteria per ml. Expose the bacterial cells under steaming condition for 1 h at 100 °C in an autoclave under normal atmospheric pressure.

2.2.3 Preparation of Working Solutions for SDS-PAGE

Solution A: 30 % acrylamide stock solution [29.2 % (w/v) acrylamide and 0.8 % N,N′-methylene bis-acrylamide]; Solution B: 1.5 M Tris–HCl, pH 8.8; Solution C: 0.5 M Tris–HCl, pH 6.8; Solution D: 10 % (w/v) ammonium persulfate (prepare just prior to use); 10 % sodium dodecyl sulfate solution.

2.2.4 Preparation of Electrophoresis Buffer (pH 8.3)

Glycine—14.4 g; Tris–HCl—3.0 g; 0.1 % SDS—1.0 g; distilled water—1000 ml.

2.2.5 Preparation of 5× Sample Buffer (10 ml)

0.6 ml 1 M Tris–HCl, pH 6.8; 5 ml 50 % glycerol; 2 ml 10 % SDS; 0.5 ml 2-mercaptoethanol; 1 ml 1 % bromophenol blue; 0.9 ml distilled water.

Stable for weeks in the refrigerator or for months at –20 °C.

2.2.6 Solutions for Preparing SDS-PAGE Resolving Gels

↓Components/Gel %→	5 %	7.5 %	10 %	12.5 %	15 %	20 %
Water	10.5	9.00	7.50	6.00	4.50	1.50
Solution A (ml)	3.00	4.50	6.00	7.50	9.00	12.00
Solution B (ml)	4.50	4.50	4.50	4.50	4.50	4.50
Solution D (ml)	0.08	0.08	0.08	0.08	0.08	0.08
10 % SDS (ml)	0.10	0.10	0.10	0.10	0.10	0.10
TEMED (ml)	0.01	0.01	0.01	0.01	0.01	0.01

2.2.7 Solutions for Preparing 5 % SDS-PAGE Stacking Gel

Water—3.60 ml; Solution A—0.90 ml; Solution C—1.50 ml; Solution D—0.20 ml; 10 % SDS—0.10 ml; TEMED—0.01 ml.

3 Methods

3.1 Preparation of Outer Membrane Proteins

Prepare outer membrane proteins according to the method of Fillip et al. [21].

1. Grow *S. flexneri* 2a (N.Y-962/92) in tryptic soy broth at 37 °C for 18 h under shaking condition.
2. Harvest the cells by centrifugation at 10,000 × *g* (Beckman) for 10 min and then wash twice in 100 mM HEPES (N-2 hydroxyethyl piperazine N′-ethanesulfonic acid, pH 7.0) buffer.
3. Suspend the harvested cells in 50 ml of HEPES buffer, pH 7.0 and disrupt the cells with an ultrasonic disintegrator (MICROSON) at 4 °C with intermittent bursts of 1 min.
4. Remove the unbroken cells by centrifugation at (10,000 × *g*) for 10 min at 4 °C.
5. Treat the envelope fraction with 0.5 % (w/v) N-lauroylsarcosine sodium salt for 20 min at room temperature to selectively solubilize the inner membrane part. Recover the insoluble outer membrane protein (OMP) fraction by centrifugation at 100,000 × *g* for 1 h at 4 °C.
6. Wash the OMP fraction twice with HEPES buffer, pH 7.0 and use this fraction immediately or preserve at −20 °C for further processing.

3.2 Identification and Electroelution of Major Outer Membrane Protein

In order to identify the immunogenic major outer membrane proteins, rabbits were immunized with heat-killed *S. flexneri* 2a and the serum was collected to measure OMP-specific antibodies. Additionally, the OMP fraction was immunoblotted with the raised

serum to profile the major outer membrane proteins (MOMP). The individual MOMP was electroeluted and protective efficacy of each MOMP was assessed in rabbit model of shigellosis.

3.2.1 Identification of Outer Membrane Proteins as Immunogens

1. Prepare heat-killed *S. flexneri* 2a as described in Subheading 2.
2. Immunize adult male/female white New Zealand rabbits (2–2.5 kg) on days 0, 7, 14, and 21 with heat-killed bacteria or PBS.
3. Orally administer 10 ml of heat-killed bacteria (containing 10^{11} bacteria) through orogastric tube following a dose of 5 % sodium bicarbonate to neutralize the gastric acidity.
4. Collect non-immunized and immunized serum 6 days after each dose of immunization.
5. Perform ELISA to determine the antibody level (IgG and IgA) against the OMP fraction.
6. Carry out immunoblotting with the serum to identify the MOMP using OMP as antigen.

3.2.2 Electroelution of MOMPs

1. Mix the OMP fraction with equal volume of sample buffer, boil for 5–7 min at 100 °C, cool on ice, gently vortex, and centrifuge the sample.
2. Resolve the sample on 12.5 % SDS-PAGE at a constant voltage of 150.
3. Visualize the protein bands by placing the gel in chilled 1 M KCl.
4. Excise each band corresponding to MOMP using clean surgical blade.
5. Electroelute each MOMP via electroelution (Bio-Rad).
6. Dialyze each protein fraction against 1× PBS at 4 °C, change the buffer at least three times each with 6 h interval.

3.2.3 Protective Efficacy of the Gel Cut MOMPs

1. Homogenize individual electroeluted MOMP in PBS, pH 7.2.
2. Immunize rabbits with each of the MOMP or PBS with five intramuscular injections at 15 days intervals.
3. Anesthetize the animals with intravenous injection of pentothal sodium (at a dose of 30 mg/kg of body weight), open the abdomen and make a tie at 3–5 cm proximal to the ceco-colic junction to completely obstruct the cecum while maintaining continuity between ileum and colon.
4. Flush the colon with NaCl (0.9 %) warmed at 37 °C, then inject 10 ml of virulent *S. flexneri* 2a culture into the colon, at about 10 cm distal to the ceco-colic junction and close the abdomen.
5. Observe the rabbits for development of diarrhea (bloody mucoid stools characteristics of shigellosis) or protection from the disease (normal pellet stool) up to 96 h.

3.3 Sequencing, Cloning, and Expression the OMP

Among the different MOMP, which showed discernible protective activity in rabbit model of shigellosis was sequenced, identified from database, amplified, cloned in expression vector and finally expressed in *Escherichia coli*.

3.3.1 Sequencing of the OMP

1. Perform electrophoresis of the OMP fraction harvested from *S. flexneri 2a*, visualize protein bands by Coomassie brilliant blue staining, destain, excise the concerned MOMP from gel, and send it for full-length sequencing (MALDI-TOF MS).
2. Carry out in-gel protein digestion, concentrate the resulting peptides on a ZipTip micropurification column and elute onto an anchorchip target for analysis on MALDI-TOF MS instrument.
3. Analyze the peptide mixture in positive reflector mode for accurate peptide mass determination and select five to ten of the peptides for analysis by MS/MS fragmentation for partial peptide sequencing.
4. Combine the MS and MS/MS spectra and use for a database search in an in-house protein database by the Mascot software.

3.3.2 Cloning and Expression of the OMP

1. Retrieve the sequence of the concerned OMP from the GenBank and design a set of primer using the sequence to amplify the respective coding sequence from *S. flexneri* 2a genome using PCR.
2. Resolve the PCR amplified product in 1 % agarose gel by electrophoresis and analyzed using Gel-Doc and ligate the PCR amplified product of OMP gene to commercial pET100/D-TOPO® linearized vector (Invitrogen).
3. Transform the ligated product into One Shot® TOP10 chemically competent *E. coli* cells by heat shock, select the recombinant transformants using ampicillin (100 mg/ml) on LB agar plates and perform DNA sequencing using the resulting colonies to confirm the sequence identity and proper cloning orientation of the amplified product (OMP gene).
4. Isolate the plasmid from the correct transformant from an overnight culture of recombinant *E. coli* Top 10 cell using alkaline lysis protocol, transform the plasmid into BL21 Star™ (DE3) One Shot® Chemically Competent *E. coli* cells by heat shock and use the transformed *E. coli* BL21 (DE3) cells harboring the recombinant plasmid in the expression study.
5. Inoculate the entire transformation reaction into 10 ml of LB broth containing 100 mg/ml ampicillin and incubate at 37 °C with shaking (200 rpm) until the OD at 600 nm is 0.5–1.2.
6. Prepare the big batch of bacteria culture for protein expression. Add an aliquot of the starter culture into 1.5 L of sterile LB

medium (containing 100 mg/ml ampicillin) and incubate at 37 °C with shaking (200 rpm).

7. Once an optical density at 600 nm of the cultures reach 0.5–0.8 (mid log), induce the cells to express protein by adding appropriate amount of isopropyl thiogalactoside (IPTG). Caution: IPTG is light sensitive and also cannot be freeze-thawed too often. Typically prepare about 0.5 ml aliquots of 0.5 M each and freeze at –20 °C, freeze thaw aliquots only 2–3 times.
8. After adding IPTG, incubate the cultures at 37 °C with shaking (200 rpm) for appropriate time. After induction, centrifuge the bacterial culture at 4000 × *g* for 15 min in 4 °C. Freeze the cell pellet at –80 °C if necessary.

3.4 Purification of Recombinant OMP and Removal of Endotoxin

The recombinant outer membrane protein expressed in *E. coli* was purified from the cells using Ni-NTA (nickel-nitrilotriacetic acid) affinity chromatography and any lipopolysaccharide present in the expressed protein was removed in order to eliminate the possibility that the immunogenicity of the protein is due to LPS contamination as LPS can evoke protective immunity in shigellosis.

3.4.1 Purification of the Recombinant OMP

1. After induction, lyse the recombinant cells (0.5 g) by gentle stirring in lysis buffer (8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris–HCl; pH 8.0) for 30 min at room temperature and then centrifuge at 10,000 × *g* for 30 min at 4 °C.
2. Mix the lysate with 50 % Ni-NTA slurry (4:1) and kept at 4 °C for 1 h. Load the Ni-NTA slurry on the column and finally elute the protein using elution buffer (8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris–HCl; pH 4.5) after washing with wash buffers (8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris–HCl; pH 6.3 and 5.9).

3.4.2 Endotoxin Removal

The lipopolysaccharide present in the recombinant protein was removed by passing the protein through Detoxi-Gel endotoxin-removing resin and S3Δ peptide affinity gel columns, respectively.

1. Pass the purified recombinant protein through 1 ml of Detoxi-Gel endotoxin-removing resin, prepacked in a 5 ml disposable column by gravity.
2. Wash the column once with 5 ml of 1 % sodium deoxycholic acid, followed by 5 ml of 2 M NaCl and thrice with 5 ml each of pyrogen-free water before and after each lipopolysaccharide removal.
3. Under pyrogen-free conditions, pass the purified protein further through a column of 1 ml S3Δ peptide affinity gel to further remove traces of endotoxin, follow the same steps as for the Detoxi-Gel endotoxin-removing resin column.

4. Confirm the absence of traces of LPS in the purified protein by the *Limulus* amoebocyte lysate chromogenic assay with Kinetic-QCL® (Lonza) (*see* **Note 1**).

3.5 Determining the Immunogenicity of the Recombinant OMP In Vitro

Effective response to and control of microbial infection seems to require several levels of interactions between the innate and adaptive immune systems and hence an ideal subunit vaccine antigen should has the capacity to stimulate both innate and adaptive arms of the host immune system. The macrophage is a pivotal mediator of innate immunity and a precursor of the host response to tissue invasion. Once activated, macrophages produce an enormous diversity of microbicidal effectors, immunoregulatory cytokines as well as express major histocompatibility complex and co-stimulatory molecules on their surface that are require for innate immunity and priming of the acquired immune response, namely activation of T and B cells [22].

3.5.1 Isolation of Murine Peritoneal Macrophages

1. Euthanize the mice with CO_2 inhalation followed by cervical dislocation and clean thoroughly with 70 % ethyl alcohol (*see* **Note 2**).
2. Inject 2 ml of the sterile DPBS in the peritoneal cavity to each mouse. Make an incision into the abdomen and then rise the peritoneal cavity 3–4 times through the opening with cold sterile DPBS.
3. Collect the peritoneal washing containing the macrophages on sterile petri dishes and incubate at 37 °C in 5 % CO_2 for 2 h. The cells of the monocyte macrophage lineage will adhere on the surface of the petri dishes to form a confluent cell monolayer during the incubation period.
4. Remove the non-adherent peritoneal cells by repeated washing of the plates with cold DPBS and harvest the adherent peritoneal cells from the surface with a rubber scraper.
5. Wash the cells by suspending in DPBS and subsequent centrifugation at 300 × *g* for 5 min in 4 °C. Resuspend the cell pellet in RPMI 1640 medium containing 10 % FBS and determine the viability and count of macrophages by trypan blue exclusion. (Macrophages should be 90–95 % viable).

3.5.2 Determining Activation of Macrophages by the Recombinant Protein

1. Culture macrophages (0.5×10^6 cells) in a final volume of 200 μl in round-bottomed 96-well plates in presence of the recombinant OMP or media alone for appropriate duration of time at 37 °C.
2. Harvest the cells and cell culture supernatants by centrifugation at 300 × *g* for 5 min at 4 °C (*see* **Note 3**).
3. Determine the level of antibacterial cytokines (IL1β, IL-6, TNF-α, IFN-γ, and IL-12p70) by ELISA in the culture supernatants.

4. Use the cells to check surface expression of TLR2, MHC-II, CD80, and CD86 (activation markers) by labeling with fluorochrome-conjugated anti-mouse TLR2, MHC-II, CD80, and CD86 antibodies for 30 min on ice and then examine the stained cells by flow cytometry.

3.5.3 Activation of T and B Cells

T and B cells are crucial players of adaptive immune response, T cells are involved in cell-mediated immune response while B cells regulate humoral immunity.

T Cell Activation

Activated Th1 cells secrete IL-2 and IFN-γ, which have been shown to regulate cellular immune responses by inducing Th1 differentiation [23].

1. Immunize mice (BALB/c or C57BL/6) with the recombinant protein by intranasal route on days 0, 14, and 28.
2. Anesthetize the mice by intramuscular injection of a mixture of 0.3 mg of xylazine hydrochloride and 1.0 mg of ketamine hydrochloride in 50 μl of saline before each immunization.
3. Immunize each mouse intranasally with 3–5 μg of the recombinant protein. Deliver a total antigen volume of 25 μl in five to six small drops to the external nares with a micropipette. Immunize the control animals with 0.9 % saline.
4. One week after the final immunization, excise spleens from both immunized and nonimmunized mice and isolate $CD4^+$ T cells using $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec).
5. Stimulate the purified $CD4^+$ T cells in vitro with 2 μg/ml of plate-bound (96-well plate) anti-mouse CD3ε and 1 μg/ml of soluble anti-CD28 for 48 h.
6. Harvest the cell culture supernatants after 48 h of incubation by centrifuging the plate at 300 × *g* for 5 min at 4 °C. Measure secretion of IFN-γ and IL-2 in the culture supernatants by ELISA.

Activation of B Cells

Activated B cells upregulate the expression of MHC-II, CD80, or CD86 on the surface as well as proliferate and differentiate into antibody secreting cells [24].

1. Excise spleen from mice and isolate B cells from spleen by negative selection using mouse B cell isolation kit (Miltenyi Biotec). Resuspend the cells in DMEM medium containing 10 % FBS, count and assess the cell viability by trypan blue exclusion.
2. Stimulate B cells with 5 μg/ml of the recombinant OMP for 24 h. After stimulation stain the cells for surface expression of MHC-II, CD80, and CD86 and then analyze by flow cytometry.
3. For determining B cell proliferation, label the cells (1×10^6 cells) with 1 μM CFSE in prewarmed PBS at 37 °C for 15 min in dark.

4. Quench the staining by the addition of ice-cold complete medium, wash twice and then stimulate with the recombinant OMP for 96 h.
5. Analyze the cells every 24 h by flow cytometry.
6. To assess differentiation of B cells into antibody secreting cells (ASCs), culture B cells in presence of the recombinant protein into flat-bottom 96-well tissue culture plates for 72 h.
7. Transfer the cells to ELISPOT plates precoated with unlabeled anti-mouse Ig for 16–18 h at 37 °C, wash, incubate the plates with HRP-conjugated anti-IgM and anti-IgG Abs for 2 h at room temperature, developed with AEC Chromogen (BD Biosciences) and finally image and analyze the plates using Immunospot plate reader.

3.5.4 In Vivo Protective Immune Response by the Recombinant OMP

1. Prepare a frozen lot of *S. flexneri* 2a from the log phase of growth, which is the time of optimal invasiveness for *Shigellae* and then store in liquid nitrogen for the challenge experiment.
2. Immunize mice with the recombinant OMP intranasally as described above. Three weeks (day 49) after the final immunization, challenge all mice intranasally with a lethal dose of *S. flexneri* 2a (1×10^7 CFU/30 μl) as describe for the mouse lung model [25].
3. Bleed all mice on days 0, 28, 42, and 63, harvest the serum and store them in −80 °C for antibody ELISA.
4. Harvest pulmonary lavage by inflating the lungs with cold RPMI 1640 and by withdrawing the fluid through trachea. Remove the cellular debris from the lavage by centrifugation at $300 \times g$ for 5 min in 4 °C and then store the lavage fluids for antibody and cytokine ELISA at −80 °C.
5. Monitor all mice for weight loss, lethargy, fur ruffling, and death for 14 days after challenge.

4 Notes

1. After the final level of purification keep the recombinant protein in PBS and store at 4 °C (for short term storage) or −20 °C (for long term storage). For each lot of purification we usually perform CD spectroscopy in order to confirm that the secondary structure of the protein remains unaltered.
2. All the procedures should be performed under aseptic condition to avoid bacteria contamination, which can give false positive data.
3. The culture supernatants can be kept at −20 °C for short-term storage (2–3 months) or −80 °C for years.

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