Fusion Expression and Immunogenicity of EHEC EspA-Stx2A1 Protein: Implications for the Vaccine Development

Yan Cheng[†], Youjun Feng[#†], Ping Luo[†], Jiang Gu, Shu Yu, Wei-jun Zhang, Yan-qing Liu, Qing-xu Wang, Quan-ming Zou[*], and Xu-hu Mao[*]

Department of Clinical Microbiology and Immunology, College of Medical Laboratory, The Third Military Medical University, Chongqing 400038, P. R. China
[#]*Present address: Department of Microbiology, University of Illinois at Urbana-Champaign (UIUC), USA*

Shiga toxin 2 (Stx2) is a major virulence factor for enterohemorrhagic *Escherichia coli* (EHEC), which is encoded by λ lysogenic phage integrated into EHEC chromosome. Stx2A1, A1 subunit of Stx2 toxin has gathered extensive concerns due to its potential of being developed into a vaccine candidate. However, the substantial progress is hampered in part for the lack of a suitable *in vitro* expression system. Here we report use of the prokaryotic system pET-28a::*espA-Stx2A1*/BL21 to carry out the fusion expression of Stx2A1 which is linked to *E. coli* secreted protein A (EspA) at its N-terminus. Under the IPTG induction, EspA-Stx2A1 fusion protein in the form of inclusion body was obtained successfully, whose expression level can reach about 40% of total bacterial protein at 25°C, much higher than that at 37°C. Western blot test suggested the refolded fusion protein is of excellent immuno-reactivity with both monoclonal antibodies, which are specific to EspA and Stx2A1, respectively. Anti-sera from Balb/c mice immunized with the EspA-Stx2A1 fusion protein were found to exhibit strong neutralization activity and protection capability *in vitro* and *in vivo*. These data have provided a novel feasible method to produce Stx2A1 in large scale *in vitro*, which is implicated for the development of multivalent subunit vaccines candidate against EHEC O157:H7 infections.

***Keywords*: EHEC O157:H7, EspA, Stx2A1, fusion protein, vaccine**

Enterohaemorrhagic *Escherichia coli* (EHEC), an important zoonotic pathogen, can give rise to a wide spectrum of food and water-born infectious diseases including diarrhea, hemorrhagic colitis (HC) and even hemolytic-uremic syndrome (HUS) (Ritchie *et al.*, 2003). Serotype O157:H7 of EHEC populations, Shiga-like toxin producing strains, is frequently found more related to HUS, the life-threatening complication following its infection (Tarr *et al.*, 2005; Bielaszewska *et al.*, 2007). EHEC infections have challenged the global public health each year, since it was discovered to cause HUS in USA in 1982 (Karmali *et al.*, 1983; Riley *et al.*, 1983). In particular, a large-scale epidemic of EHEC O157:H7 emerged and lasted for nearly seven months in East China, 1999, in which over 20,000 persons were infected with 177 death case (www.chinacdc.net.cn). Recently, Food and Drug Administration (FDA) announced an outbreak of EHEC O157:H7 happened in USA, in the fall of 2006. This unsuspected epidemic was triggered by the contaminated Spinach, and caused 205 human cases with 3 deaths included (http://www.fda.gov/bbs/topics/NEWS/2006). Unfortunately, effective treatments against EHEC infections (esp. HUS) can not be available so far (Mukherjee *et al.*, 2002).

EHEC colonization in human colon is a key determinant for virulence manifestation, featured attaching, and effacing (A/E) lesions (Ritchie *et al.*, 2003). The genes necessary for the formation of A/E lesions are located in the locus of enterocyte effacement (LEE) pathogenicity island (Perna *et al.*, 1998; Roe *et al.*, 2003). Among them, *E. coli* secreted protein A (EspA) is a component of type 3 secretion system (T3SS) encoded by this LEE locus (Kato *et al.*, 2005) and plays roles in the signal transduction between bacteria and host cells (Kenny *et al.*, 1996) and even in molecular pathogenesis (La Ragione *et al.*, 2006; Dziva *et al.*, 2007). On the other hand, EHEC produces Shiga like toxins (Stx) with two major subtypes: Stx1 and Stx2 (Ritchie *et al.*, 2003; Tarr *et al.*, 2005), of them Stx2 is mostly correlated with HUS (Louise and Obrig, 1992; Russmann *et al.*, 1994; Yoshimura *et al.*, 2000; Tarr *et al.*, 2005). Both Stx1 and Stx2 are heteropolymers which are composed of an active (A) domain and five binding (B) subunits (Louise and Obrig, 1992; Zhang *et al.*, 2002; Kuczius *et al.*, 2004). The A subunit of the Stxs can be proteolytically cleaved at a susceptible site into two portions: A1 (28 kDa) and A2 (4 kDa) (Fraser *et al.*, 2004). The A1 portion possess the N-glycosidase catalytic activity, it acts by removing the adenine group from position 4324 in the eukaryotic 28S rRNA of the 60S ribosomal subunit, which leads to the cessation of protein biosynthesis. Stx2 mediates HUS via endothelial cell injury, particularly the cell injury within kidneys. More importantly, Stx2A1, a fragment

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† These authors contributed equally to this work.
* To whom correspondence should be addressed.
(Tel) 86-23-68752315; (Fax) 86-23-68752315
(E-mail) mxh95xy@mail.tmmu.com.cn

derived from Stx2A, contributes greatly to the clinical manifestation. Increasingly accumulated evidence suggested that intervention with Stx-specific antibody seems to be useful for EHEC O157:H7 infections (Matise *et al.*, 2001; Mukherjee *et al.*, 2002; Akiyoshi *et al.*, 2005; Ma *et al.*, 2008). Therefore, it is necessary for developing an efficient method to produce Stx2A1 protein *in vitro*.

In this paper, we report a fusion protein EspA-Stx2A1 which was obtained by linking genetically N-terminus of Stx2A1 to EspA. Furthermore, this fusion protein was confirmed to be of immunogenicity and can be potentially applied into the development of vaccines candidate.

Materials and Methods

Bacterial strains, plasmids, and antibodies
Strain 44828 of EHEC O157:H7 was kindly provided by National Institute for the Control of Pharmaceutical & Biological Products (NICPBP) of China. Both *E. coli* strains [DH5α & BL21 (DE3)] were kept in our laboratory. pMD-18T (TaKaRa, USA) and pET-28a(+) (Novagen, USA) were used here. EspA-specific McAb and Stx2A1 McAb were prepared in our laboratory as described previously (Yu *et al.*, 2007). EspA protein was expressed and purified as described in the protocol recommended by manufacture. HeLa cells and Vero cells were kept in our laboratory.

Construction of *espA-stx2A1* fusion gene
Overlapping PCR was applied to develop the *espA-stx2A1* fusion gene (GenBank Accession No.**AE005174** and GenBank Accession No.**AY6527**) using two pairs of specific primers: *espA*-F; 5'-CG**CCATGG** ATA CAT CAA ATG CAA CAT C-3' and *espA*-R; 5'-CG**CTTGTCGTCATCGTC** TTT ACC AAG GGA TAT T-3': *stx2A1*-F; 5'-AA**GACGATGACGAC AAG** CGG GAG TTT ACG ATA G-3' and *stx2A1*-R; 5'-GC**AAGCTT** ATT CAG TAT AAC GGC CAC AGT C-3'. The cleavage sites of *Nco*I (in *espA*-F) and *Hin*dIII (in s*tx2A1*-R) were highlighted with underlined letters. The underlined sequences in *espA*-R & *stx2A1*-F, which is designed to be translated as enterokinase site into five amino acids (Asp-Asp-Asp-Asp-Lys). The amplified PCR products of both *espA* and *stx2A1* were mixed equally to serve as templates to generate the overlapped chimeric gene of *espA-stx2A1*. The acquired chimeric gene was cloned into pMD-18T vector for direct DNA sequencing. Finally, this insert was transferred from pMD-18T vector into pET-28a(+) prokaryotic expression vector via *Nco*I and *Hin*dIII cleavage sites, generating the recombinant expression plasmid, designated pET-28a::*espA-stx2A1*.

Expression and purification of EspA-Stx2A1 fusion protein
E. coli BL21 strains carrying the recombinant plasmid of pET-28a::*espA-stx2A1* were inoculated in LB media supplemented with 50 μg/ml of Kanamycin and then induced by 1 mM IPTG at 25°C and 37°C, respectively. After sonication, bacterial supernatants and pellets were separated on 12% SDS-PAGE to determine existing forms of the interested proteins. Subsequently, UVP gel scanner was applied to roughly estimate the expression level.

Disruption, wash, and isolation of inclusion bodies
After the bacteria were harvested by centrifugation at 800×g for 15 min, about 40 g of pellet was collected. The pellets were resuspended in 360 ml TE (50 mM Tris-HCl, 0.5 mM EDTA, pH 8.0), and disrupted under a pressure of 130 MPa six times at a flow rate of 130 ml/min in ice-water bath using homogenizer (An Invernsys Group, Denmark), then followed by differential centrifugation (500×g, 25 min; 10,000×g, 40 min), and pellets containing the inclusion bodies were collected. The pellets were washed two times with 500 ml 1% TritonX-100, twice with 500 ml of 2 M urea solution, to remove the contaminants out of the insoluble precipitate. Inclusion bodies were resuspended in guanidine hydrochloride denaturing buffer (6 M guanidine hydrochloride, 50 mM Tris-HCl, 0.15 M NaCl), stirred slowly at 4°C overnight, and centrifuged at 10,000×g for 30 min at 4°C. The supernatant was the dissolved inclusion body.

Purification and renaturation of the fusion proteins
The guanidine HCl solubilization buffer was replaced with buffer containing 8 M urea, 0.15 M NaCl, and 50 mM Tris-HCl, pH 8.0 via dialysis, then the concentration of protein was adjusted to 0.1~0.2 mg/ml (determined using a lowry assay). The protein solution was slowly dripped into 40 volume of refolding buffer (50 mM Tris-HCl, 1 mM arginine monohydrochloride, 1 mM EDTA, 1 mM reduced form, and 0.5 mM oxidized form of glutathione, pH 8.0) under vigorous stirring at 4°C for 20 h. Firstly, the refolding fusion protein was purified by anion chromatography. Briefly, the refolding fusion protein was subjected to a Pharmacia HiTrap SP Sepharose Fast Flow column equilibrated in 50 mM Tris buffer (pH 8.0). The bound protein was subsequently eluted by a linear salt gradient (0.05~1.0 M NaCl in 50 mM Tris-HCl, pH 8.0). After identification by SDS-PAGE, the target fractions were refined by affinity chromatography fixed with EspA-specific McAb. A pharmacia chelating Sepharose Fast column (XK16/10) was preloaded with EspA-specific McAb and equilibrated with 50 mM Tris buffer (pH 8.0). The protein solution was subsequently loaded onto the column at a flow rate of 0.4 ml/min; the impurity was removed by elution with low concentration imidazole (less than 50 mM). Finally, the fusion protein was eluted using 50 mM Tris-HCl, 0.15 M NaCl, and 0.5 M imidazole (pH 8.0). 12% SDS-PAGE was also used for testing protein purity.

Removal of endotoxin from EspA-Stx2A1 and EspA
Endo-toxin was removed from the purified EspA-Stx2A1 and EspA as described previously (Marcato *et al.*, 2001; Marcato *et al.*, 2005). The cytotoxicity of EspA-Stx2A1 and EspA without endotoxin contamination was evaluated using Vero cells.

Western blotting
Western blotting was performed as Feng *et al.* (2007) described with minor modifications. Briefly the refolded EspA-Stx2A1 fusion protein was subjected to 12% SDS-PAGE, electro-transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech), and blocked with 5% skim milk in TBST (50 mM Tris-HCl; pH 7.3, 150 mM NaCl, 0.1% Tween 20) at 37°C for 2 h. The membrane was incubated

with McAb against EspA (or Stx2A1-specific McAb) in a 1:5,000 dilution in TBST at 37°C for 1 h, which was followed by the incubation with HRP-conjugated rabbit anti-mouse antibody (Sigma) in a 1:10,000 dilution in TBST at 37°C for 1 h.

ELISA analyses

To determine the antigenicity of the fusion protein, Balb/c mice (4~5 weeks) were inoculated with the protein of EspA-stx2A1 or EspA in the dose of 100 μg/mouse/time. This step was repeated for triplicates with interval of 1 week (0, 1st, 2nd week). The control group was injected with 200 μl mixture of PBS and Complete Freund's Adjuvant at a ratio of 1:1 at the same schedule. The collected sera were subjected to ELISA-based antibody titer assays in a series of dilution. EspA protein and Stx2 toxin were used with BSA as negative control.

ELISA analysis was carried out as Feng *et al*. (2007) described. Briefly, ninety-six micro-tube ELISA plates were coated overnight at 4°C with 100 ml/well of EspA protein and Stx2 toxin at an appropriate concentration in carbonate buffer. After three washes with TBST, the plates were blocked with 5% skim milk in TBST for 1 h at 37°C. Next, sera in a series of dilution were added to appropriate wells in triplicate at 100 ml/well to incubate for 1 h at 37°C, and washed three times. Then, the bound antibodies were detected with HRP-conjugated rabbit anti-mouse IgG (Sigma, USA) in a 1:20,000 dilution in TBST for 1 h. These plates were developed with O-phenylenediamine as a substrate (Amresco, USA) and H_2O_2 (Sigma) as the oxidation agent; 1 M sulfuric acid was used to stop the reaction. The absorbance score was measured at 490 nm in a micro-plate reader.

Immunized with EspA-Stx2A1 protein or its control and Stx2 toxin challenge protocols

Balb/c mice (4~5 weeks) were subcutaneously inoculated with the fusion protein of EspA-Stx2A1 or EspA protein in the dose of 100 μg/mouse/time emulsified with an equal amount of Freund's complete adjuvant three times on days 0, 7, and 14, as described previously (Takahashi *et al.*, 2006). Two weeks after the last administration, the immunized mice were challenged intra-peritoneally with 50 μg of Stx2 toxin and the number of dead mice was determined every day. PBS-treated animals were regarded as negative controls.

In vitro neutralization assay

The Stx2 toxin was found to be toxic to HeLa cells less than 50 pg/ml. HeLa cells in log phase growth with the density of 1.4×10^4 were plated in each well of 96-well plates in McCoy's 5A medium (Mediatech, Inc., USA) containing 10% fetal bovine serum (Harlan Bioproducts for Science, Inc., USA) followed by incubation overnight at 37°C in 5% CO_2. Stx2 at a dilution of killing 70% of HeLa cells was pre-incubated with anti-sera collected from mice immunized with EspA-Stx2A1 fusion protein (or EspA protein) for 1 h at 37°C in 5% CO_2, and then the mixture was added to the cells and incubated overnight at 37°C in 5% CO_2. Cells were washed, fixed, and stained with crystal violet, and the absorbance at 690 nm was determined.

Fluorescence actin staining (FAS)

Fluorescence actin staining test was carried out to detect the formation of "attaching and effacing" (A/E) lesion and the adherence between O157:H7 and HeLa cells in the presence of anti-EspA-Stx2A1 sera. Briefly, HeLa cells were incubated at 1.4×10^4/well on 96 well plates in McCoy's 5A medium (Mediatech, Inc., USA) supplemented with 10% fetal bovine serum (Harlan Bioproducts for Science, Inc., USA) overnight at 37°C in 5% CO_2. FAS test was divided into two groups. In group 1, whole bacteria of O157:H7 was pre-incubated with anti-EspA-Stx2A1 sera for 1 h at 37°C and then added to the HeLa cells, and the mixture were incubated for an additional 4 h at 37°C in 5% CO_2. In group 2, anti-EspA-Stx2A1 sera were added and incubated for 1 h again, after the mixture of bacterial suspensions and HeLa cells was incubated for 4 h at 37°C in 5% CO_2. The plates were added the three antibodies below subsequently: FITC-conjugated goat anti-mouse IgG, rabbit anti-β-actin antibody, and TRITC-conjugated goat anti-rabbit IgG. After three times of wash in PBS, cover slips were blocked with 50% glycerol. Specimens were examined with confocal laser scanning microscope.

Results

### Molecular cloning of *espA-stx2A1* fusion gene

A 1,319 bp of DNA fragment was obtained by overlapped extension PCR using the specific *espA*-F and *stx2A1*-R primers. The resultant cDNA fragment was cloned into T-A cloning vector. The recombinant plasmid pMD-18T::*espA-stx2A1* was digested by restriction endonucleases *Nco*I and *Hin*dIII and the insert was subcloned into the likewise digested pET-28a(+) expression vector. The finally constructed pET-28a(+)::*espA-stx2A1* was verified by restriction enzymes analysis and direct sequencing (Fig. 1).

In vitro expression of EspA-Stx2A1 fusion protein

The fusion proteins were expressed at 37°C and at 25°C under the induction of 1 mM IPTG. The expression level
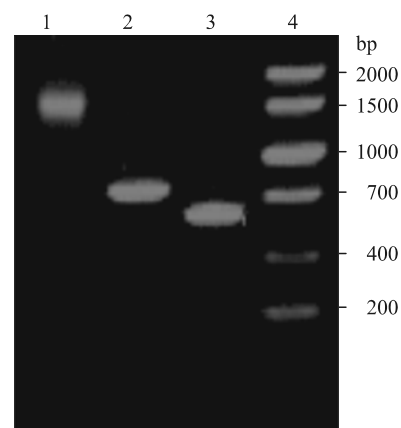
Fig. 1. Electrophoresis analysis of PCR products. Lanes: 1, *espA-stx2A1* fusion gene of PCR amplification; 2, *stx2A1* gene of PCR amplification; 3, *espA* gene of PCR amplification; 4, Marker

(A)

(B)
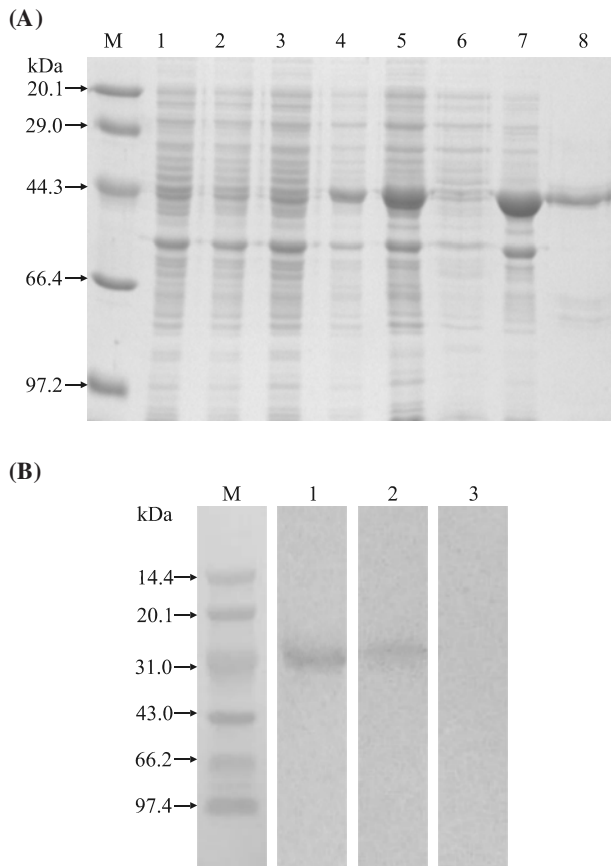
Fig. 2. The expression and purification of induced fusion protein analyzed by SDS-PAGE and Western blotting. The expression and purification of fusion protein analyzed by SDS-PAGE and Western blotting. (A) Lanes: M, protein molecular weight markers; 1, whole cell lysate of BL21 induced at 37℃; 2, whole cell lysate of pET-28a(+)/BL21 induced at 37℃; 3, whole cell lysate of pET-28a(+)-stx2A1/BL21 induced at 37℃; 4, whole cell lysate of pET-28a(+)::espA-stx2A1/BL21 induced at 37℃; 5, whole cell lysate of pET-28a(+)::espA-stx2A1/BL21 induced at 25℃; 6, ultrasonic supernatant of recombinant strain induced by IPTG; 7, ultrasonic sediment of recombinant strain induced by IPTG; 8, the purified fusion protein EspA-Stx2A1. (B) Lanes: M, a low molecular weight standard; 1 and 2 were both applied with purified EspA-Stx2A1 fusion protein, and incubated with anti-EspA-McAb and anti-Stx2A1-McAb, respectively; 3 was applied with EspA-Stx2A1 fusion protein, and incubated with BSA.

of EspA-Stx2A1 at 25℃ was significantly higher than that at 37℃. SDS-PAGE of cell lysates showed a major protein band of the expected 45 kDa size. The recombinant protein was shown to be expressed in the form of inclusion bodies in *E. coli* host strain BL21(DE3) under different temperatures. The expression level was more than 40% of the total cell lysate at 25℃ (Fig. 2).

Purification and refolding of EspA-Stx2A1 fusion protein
Inclusion bodies were washed extensively two times with detergents and two times with 2 M urea, and then solubilized by a 6 M guanidinium hydrochloride solution. The

(A)

(B)
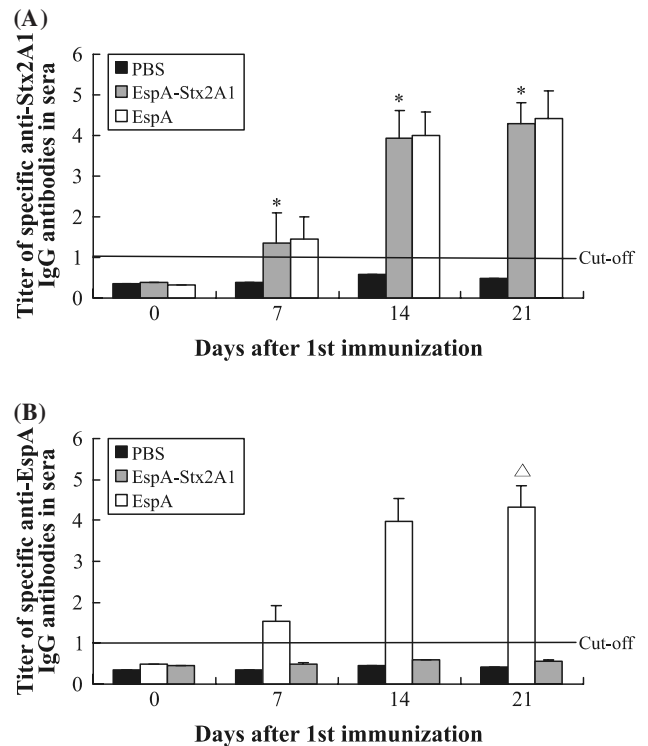
Fig. 3. ELISA analysis of EspA-specific and Stx2A1-specific IgG titers of sera from mice immunized with EspA-Stx2A1 or EspA protein. Balb/c mice were immunized with 100 μg EspA-Stx2A1 or EspA protein there times on days 0, 7, and 14. PBS-treated mice were included as negative control. Serum samples were collected 7 days after every immunization. Micro-well plates were coated with 100 μl EspA protein or Stx2 toxin (10 μg/ml) and then reacted with sera collected from mice immunized with EspA-Stx2A1 (n=20), EspA (n=20), or PBS (n=20). The absorbance score was measured at 490 nm in a micro-plate reader. The results of three independent experiments were displayed as the mean antibody titer (Log$_{10}$ titers)±SD. The horizontal line represents cut off value. (*) $P > 0.05$ vs. control group of EspA, (△) $P < 0.05$ vs. EspA-Stx2A1 group (immunization once).

contaminated proteins were removed by the use of 1% TritonX-100 and 2 M urea, while the fusion protein of EspA-Stx2A1 inclusion body not. SDS-PAGE assay showed that the fusion protein can reach about 60% purity (data not shown). After the conversion of two kinds of denaturation systems and isovolumic ultra-filtration with refolding buffer, the refolded fusion protein was loaded onto anion exchange chromatography for the purpose of removal of EDTA and preliminary purification. SDS-PAGE analysis revealed the fusion protein had a purity of over 85% (data not shown). The elution of interest was subjected to affinity chromatography fixed with EspA-specific McAb. This purification step has been proved to be efficient and it can result in high purity (95%) of EspA-Stx2A1 fusion protein (Fig. 2A lane 8). The yield of fusion protein was about 30 mg/L of culture.

Removal endotoxin from EspA-Stx2A1 and EspA
After endotoxin was eliminated from the purified EspA-

Table 1. The mortality of Balb/c mice immunized with EspA-Stx2A1 or its control challenged by Stx2 toxin

Groups	Challenge dose (μg/mouse)	Number of mice	Number of death mice		Number of surviving mice (within 15 days)	Mortality of mice	Protection ratio (%)
			Within 3 days	Within 6 days			
PBS	50	20	16	4	0	100%	0
EspA	50	20	12	8	0	100%	0
EspA-Stx2A1	50	20	0	1	19	5%	95%[a,b]

[a] $P<0.01$ vs. control group of PBS.
[b] $P<0.01$ vs. control group of EspA.

Stx2A1 and EspA, the lack of endotoxin contamination in them was confirmed by assaying cytotoxic activity in Vero cells. Results indicated that EspA-Stx2A1 and EspA were both completely nontoxic to Vero cells at a dose higher than 0.5 mg/ml.

Immunogenicity of EspA-Stx2A1 fusion protein
By the use of the McAb anti-EspA and anti-Stx2A1 western blotting was carried out to identify the specific reactivity of fusion protein EspA-Stx2A1. A band of approximate 45 kDa (Fig. 2B, lane 1 and 2) showed the strong and specific reaction between them. In contrast, no reactivity was observed in negative control (Fig. 2B, lane 3).

The levels of specific EspA and Stx2A1 IgG antibodies were determined by EspA-ELISA and Stx2-ELISA, respectively. As shown in Fig. 3A, specific IgG antibodies to EspA were detected as early as 7 days after the first immunization in the sera from mice immunized with EspA-Stx2A1 fusion protein or EspA protein. Compared with the PBS control, the titer of specific anti-EspA IgG antibodies significantly increased in these two groups after a booster immunization at day 7, which suggested strong humoral immuno-response was induced. There was no significant difference in antibodies to EspA in EspA-Stx2A1 group and EspA group ($P>0.05$). As described in Fig. 3B, the titer of specific anti-Stx2A1 IgG antibodies significantly increased in the sera of mice immunized with EspA-Stx2A1 fusion protein, moreover, 7 days after the last of the three vaccinations with EspA-Stx2A1, specific Stx2A1 IgG antibodies showed significantly higher titers than that from mice immunized once with this fusion protein. However, specific IgG antibodies to Stx2A1 were not detected in sera from mice immunized with EspA protein or PBS.

Immunized with EspA-Stx2A1 protein or its control and Stx2 toxin challenge protocols
When the mice immunized with the EspA-Stx2A1 fusion protein were challenged with 50 μg of crude Stx2 toxin, a lethal dose (LD) of Stx2 toxin, 19 of the 20 mice survived. However, in the PBS injected control group and EspA group, all mice died from challenge and showed toxic symptoms of O157:H7 infection before death (Table 1).

Neutralization activity of anti-EspA-Stx2A1 sera toward Stx2 *in vitro*
After Stx2 at a dilution that can kill 70% of HeLa cells has been mixed with serum at different concentration and incubated at 37°C for 1 h in 5% CO_2, the mixture was added to the HeLa cells. As shown in Fig. 4A, the neutralization

activity of sera from mice immunized with EspA-Stx2A1 fusion protein was significantly higher than that of sera from mice immunized with EspA protein or PBS *in vitro*. When the anti-EspA-Stx2A1 serum neutralized Stx2 toxin, the highest concentration was 5 μg/ml. When the lowest tested concentration reached 8 ng/ml, the inhibition of Stx2 cytotoxicity reached ~40%. Furthermore, we observed that Stx2 toxin can elicit typical morphological changes including crenation,
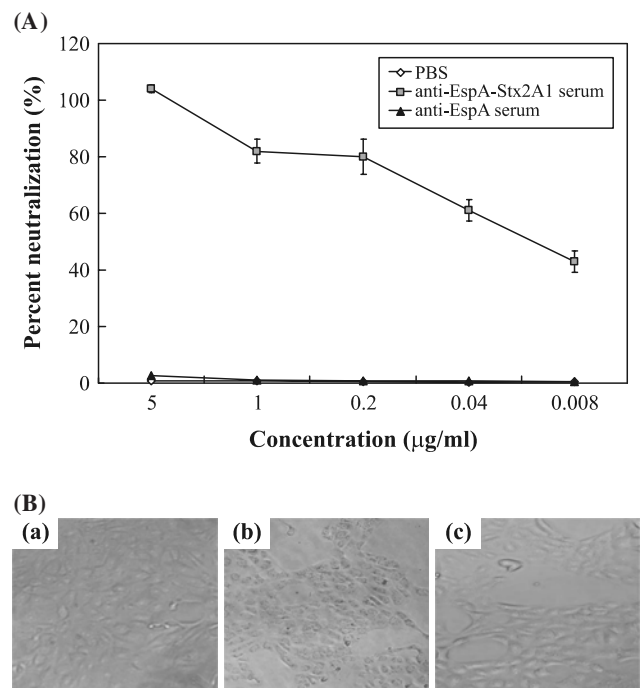
Fig. 4. Neutralization assay *in vitro*. (A) Detection of neutralization activity of anti-EspA-Stx2A1 serum toward Stx2 *in vitro*. The diluted Stx2 toxin (final 200-fold dilution) was mixed with anti-EspA-Stx2A1 sera at different concentration. After incubated at 37°C for 1 h, the mixture was added to HeLa cells. Plates were developed by crystal violet staining, and the absorbance [optical density (OD)] was read at 690 nm. The neutralization activity of anti-sera was calculated according to the following formula: [(OD$_{toxin+anti-sera}$ − OD$_{toxin~only}$)/(OD$_{no~toxin}$ − OD$_{toxin~only}$)]×100. Results of three separate experiments (20 mice per group) were expressed as the Mean±SE of percent neutralization. Significant differences ($P<0.01$) were observed in percent neutralization in various groups of immunized mice using ANOVA. (B) The morphological feature of HeLa cells *in vitro* neutralization assay. (a) HeLa cells without Stx2 toxin, (b) HeLa cells with Stx2 toxin only, (c) HeLa cells treated by Stx2 toxin preincubated with EspA-Stx2A1 polyclonal antibody *in vitro*.
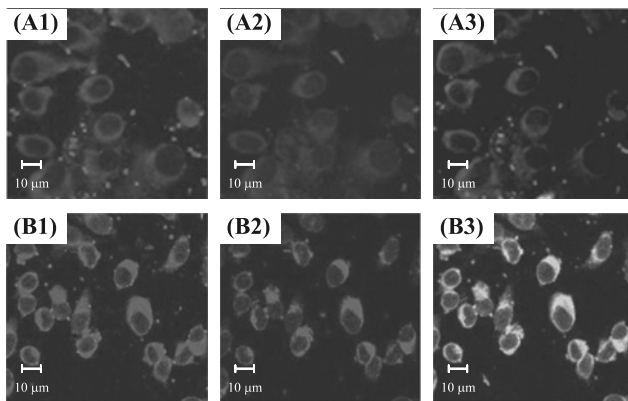
Fig. 5. Detection of actin aggregation and adherence between O157:H7 and HeLa cell in the presence of anti-EspA-Stx2A1 sera by fluorescent actin staining (FAS) test. (A) After O157:H7 was pre-incubated with anti-EspA-Stx2A1 sera *in vitro* for 1 h at 37°C in 5% CO_2, the mixture was added to the HeLa cells (A3 was superposition of A1 and A2). (B) After O157:H7 and HeLa cells were incubated together for 4 h at 37°C in 5% CO_2, anti-sera were added to the mixture (B3 was superposition of B1 and B2).

vacuole and defluxion [Fig. 4B(b)], whereas, the majority of HeLa cell treated with Stx2 toxin pre-incubated before with anti-sera *in vitro* can retain normal morphological feature [Fig. 4B(c)].

FAS analysis

FAS is a useful method for assessing the accumulations of actin and A/E lesion formation. If anti-EspA-Stx2A1 sera were not capable of inhibiting bacterial adherence to HeLa cells, green fluorescence could be observed. If polyclonal sera against EspA-Stx2A1 did not affect host cell actin rearrangement, red fluorescence in HeLa cell could be observed. As shown in Fig. 5A1 and B1, green fluorescence was localized at the HeLa cell periphery, suggesting anti-EspA-Stx2A1 sera could not block the adhesion between EHEC O157:H7 and HeLa cell. There were intense spots of red fluorescence at the cell surface in Fig. 5B2, whereas HeLa cells adherence with O157:H7 preincubated with anti-EspA-Stx2A1 sera *in vitro* [Fig. 5(A2)] could not display the same pattern of fluorescence staining, which suggested that anti-EspA-Stx2A1 sera had a marked effect upon O157:H7-induced HeLa cell actin rearrangement.

Discussion

Up to now, although many strategies have been proposed/advocated to control EHEC O157:H7 infection, effective therapeutics against EHEC O157:H7 infections are still in great demand. Antibiotics-based clinical therapy is relatively dangerous in part due to the fact that it can trigger bacterial break, subsequently increase the release of Shiga toxins, and finally the risk of the generation of severe complications (Mukherjee *et al.*, 2002). Increasingly accumulated investigations have been emphasized on the development and utilization of specific vaccines against EHEC O157:H7. Screening the protective subunit antigens is prerequisite for

vaccine designs. However, no single effector has been proved to be sufficiently protective thus far. La Ragione *et al.* (2006) have ever reported that anti-γ-intimin antibodies could not reduce adhesion of EHEC O157:H7 to host cells and host cell actin rearrangement. EspA, a key colonisation factor of EHEC O157:H7, can induce humoral immunity, but fail to confer protection against intestinal colonisation (Dziva *et al.*, 2007). The efficient combination of multiple protective antigens has been suggested to possess the potential to trigger much stronger immune response, which provides a broad prospect for vaccine development in the near future.

Stx with contribution to the EHEC O157:H7 pathogenesis, has also been demonstrated to exhibit the capability for stimulating the production of neutralizing antibody with nice protective immunity against EHEC O157:H7 (Wen *et al.*, 2006). Thus, it can be recognized as a vaccine antigen candidate. Previous study revealed that recombinant Stx2B subunit conjugated to keyhole limpet hemocyanin could elicit a protective immune response to Stx2 holotoxin in mice (Marcato *et al.*, 2005). It was reported by Ran *et al.* (2008) that they had carried out the fusion expression of Stx2B linked to the B subunit of *E. coli* heat-labile enterotoxin (LTB), and their study indicated that fusion protein possesses strong immunogenicity. However, the potential of Stx2A1 as a subunit vaccine for control of EHEC O157:H7 infection has not been previously assessed. Thus, Stx2A1 subunit was employed as one object of our studies. EspA filaments can be considered to be a bridge linking bacteria to the surface of host cells and can deliver effecter proteins to host cells (Sekiya *et al.*, 2001). Indeed, EspA as membrane protein has confirmed to be of nice immuno-reactivity and immunogenicity, becoming another promising vaccine antigen (Jarvis and Kaper, 1996; Karpman *et al.*, 2002).

Previously, we failed to abtain Stx2A1 protein in many prokaryotic expression vector: pET-22b, pQE-30, pET-28a(+), pET-11c. Through our continued efforts, we overcame the extreme difficulty in stx2A1's prokaryotic expression, and realized successful stx2A1 fusion expression at high level based on EspA protein. The EspA protein which was utilized in our design not only exhibits the capability for facilitating the expression of foreign protein in *E. coli*, but also functions as affinity tag for purification of the interested recombinant protein using column fixed with EspA-specific monoclonal antibody. Considering the recombinant protein contaminated with endotoxin would be unacceptable in human vaccine preparation, endotoxin was eliminated from the purified proteins. The cyto-toxicity result confirmed that the purified protein is safe and reliable. In this study, the immunogenicity of the fusion protein was addressed in mice. ELISA results demonstrated that subcutaneous administration of EspA-Stx2A1 fusion protein could elicite immune response against both EspA and Stx2A1 in mice. Most importantly, anti-Stx2A1 antibodies induced by fusion protein displayed high cytotoxicity neutralizing activities *in vitro* and could protect mice against Stx2 toxin challenge. EspA is one of the main components in EHEC O157:H7 required for formation of A/E lesions which is characterised by host cell actin rearrangement. Anti-EspA monoclonal antibodies have been demonstrated to block *E. coli* O157:H7-induced A/E lesions *in vitro* (La Ragione *et al.*, 2006). We therefore in-

vestigated whether polyclonal antibodies against EspA-Stx2A1 could block bacterial adherence to HeLa cells and reduce host cell actin rearrangement by FAS assays. FAS results suggested that anti-EspA-Stx2A1 sera could not block the adhesion between EHEC O157:H7 and HeLa cell but had a marked effect upon host cell actin rearrangement.

In summary, this study is the first demonstration of protective immunity induced by EspA-Stx2A1 fusion protein. The availability of the fusion protein not only provides the basis for the development of multiple-subunit vaccines, but also makes it possible to further investigate EspA physiological functions. Further research will concentrate on whether EspA and Stx2A1 subunits can be used in combination with other virulence factors to provide excellent protection from EHEC O157:H7 infection in human and animals. It is a major challenge to develop effective vaccination strategies for the control of EHEC O157:H7 infection in the future.

Acknowledgements

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