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Immunology

Recombinant unpurified rETX^{H106P}/CTB-rETX^{Y196E} protects rabbits against *Clostridium perfringens* epsilon toxin

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ABSTRACT. Epsilon toxin (ETX), produced by *Clostridium perfringens* types B and D, has been touted as a potential biological weapon and is known to induce fatal enterotoxemia in a variety of livestock animals. For the efficient production of recombinant proteins with the objective of investigating the effects of different recombinant vaccines against ETX, a bicistronic design (BCD) expression system including the ETX coding sequence with mutation of amino acid 106 from Histidine to Proline (ETX^{H106P}) in the first cistron, followed by Cholera Toxin B (CTB) linked with the ETX coding sequence with mutation of amino acid 196 from Tyrosine to Glutamic acid (ETX^{Y196E}) in the second cistron, was generated under the control of a single promoter. Rabbits were immunized twice with five inactivated recombinant *Escherichia coli* (*E. coli*) vaccines containing 100 µg/ml of the recombinant mutant rETX^{H106P}/CTB-rETX^{Y196E} proteins mixed with different adjuvants. Apart from rETX^{H106P}/CTB-rETX^{Y196E}-IMS1313-vaccinated rabbits, the neutralizing antibody titers of rETX^{H106P}/CTB-rETX^{Y196E}-vaccinated rabbits were higher after the initial immunization than those administered the ETX toxoid or current commercial vaccines. rETX^{H106P}/CTB-rETX^{Y196E} mixed with ISA201 induced the highest neutralizing antibody titer of 120 after the first immunization, suggesting that 0.1 ml of pooled sera could neutralize 120× mouse LD_{100} (100% lethal dose) of ETX. Following the second vaccination, rETX^{H106P}/CTB-rETX^{Y196E} mixed with ISA201 or GR208 produced the highest neutralizing titer of 800. Rabbits from all vaccinated groups were completely protected from a 2× rabbit LD_{100} of ETX challenge. These results show that these novel recombinant proteins can induce a strong immune response and represent potential targets for the development of a commercial vaccine against the *C. perfringens* epsilon toxin.

KEY WORDS: bicistronic design system, *Clostridium perfringens*, epsilon toxin, recombinant vaccine

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Clostridium perfringens is a ubiquitous, spore-forming, Gram-positive bacterium that is present in soil and in the gastrointestinal tract of humans and animals [12, 14]. Toxin types B and D of *C. perfringens* both produce epsilon toxin (ETX) [3, 12]. ETX is the third most potent toxin produced by *Clostridium* spp. and is coded by the plasmid-encoded gene *etx*. The latter gene product is translated as a 32.98-kDa protoxin and then activated by trypsin and chymotrypsin proteases. ETX is highly lethal to humans and animals and is considered a potential biowarfare threat according to the Centers for Disease Control and Prevention (CDC) [4]. Since curative treatment is difficult, control predominantly relies on preventive measures such as vaccination. Mutated rETX has been developed for the purposes of vaccination and has been tested for its ability to confer protective immunity. The *C. perfringens* epsilon toxin with mutation of amino acid 106 from Histidine to Proline (ETX^{H106P}) is non-toxic and has been validated as safe for the development of a vaccine antigen against enterotoxemia [1, 5, 7, 11]. In a previous study, mice vaccinated with 15 µg of the recombinant C-terminal region of *C. perfringens* epsilon toxin with mutation of amino acid 196 from Tyrosine to Glutamic acid (rETX^{Y196E}-C) exhibited protection after being challenged with 500× median lethal dose (LD_{50}) [15].

In this current study, we developed a bicistronic design (BCD) expression system that includes the ETX^{H106P} coding sequence (first cistron) followed by Cholera Toxin B (CTB) linked with the ETX^{Y196E} coding sequence (second cistron) under the control of a single promoter. CTB usually acts as a nontoxic vaccine adjuvant to improve immunity; thus, the CTB encoding gene was introduced into the BCD system. The present study evaluated whether the recombinant *C. perfringens* epsilon toxins were capable of inducing higher protective neutralizing antibody titers than the epsilon toxoid or current commercial vaccines in rabbits.

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MATERIALS AND METHODS

Animals

All animal experiments were approved by the Animal Ethics Committee of the China Institute of Veterinary Drug Control and were performed in accordance with the Guidelines for Animal Experiments of the China Institute of Veterinary Drug Control (Beijing, China).

Recombinant plasmid construction

The BCD expression system containing the ETX^{H106P} coding sequence (first cistron), followed by CTB linked to the ETX^{Y196E} coding sequence (second cistron) under the control of a single promoter, was synthesized, digested with *Eco*RI and *Xho*I, and ligated to a pBLUE-T Simple vector. The pBLUE-T Simple vector harboring the synthesized gene was subsequently cleaved with *Eco*RI and *Xho*I enzymes, and then the target gene was subcloned into the pET-32a (+) plasmid (Novagen, Darmstadt, Germany) previously treated with the same restriction enzymes. The sequence of the cloned gene was determined; nucleotide and protein sequence comparisons were then performed using GenBank BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Protein expression

The plasmid harboring pET32a-ETX^{H106P}/CTB-ETX^{Y196E} was transformed into *E. coli* BL21 (DE3). The transformants were grown in Luria Broth (LB) medium containing 100 µg/ml of ampicillin at 37°C. Upon reaching an optical density at a wavelength of 600 nm of 0.7, 0.5 mM isopropyl-β-thio-D-galactopyranoside (IPTG) was added and the cultures were further grown for an additional 24 hr at 15°C or 4 hr at 37°C. The cells were harvested via centrifugation at 4,000 rpm for 15 min, and the pellet was lysed by sonication for further analysis. To assess expression, the bacterial culture was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using an anti-His monoclonal antibody (Mouse). For protein quantitation, known quantities of bovine serum albumin (KPL, Inc., Gaithersburg, MD, USA) were included in the SDS-PAGE, and the concentration of recombinant proteins was estimated using the Bradford method.

Immunization and sera collection

A total of eight vaccines (Table 1) were tested in this study. Five inactivated recombinant *E. coli* vaccines were utilized, each containing 100 µg/ml of the recombinant mutant ETX^{H106P}/CTB-ETX^{Y196E} proteins mixed with different adjuvants. One *C. perfringens* formaldehyde-inactivated ETX toxoid and two commercial clostridial vaccines were also included in the study as controls to evaluate the efficacy of the recombinant proteins. Fresh culture of *clostridium perfringens* type D was added with trypsin for activation at 37°C for 1 hr. The mixture was then centrifuged and filtered to remove bacteria. The filtrate was native ETX. The native ETX was inactivated by adding 2.8 mg/ml of formaldehyde at 37°C for 7 days to produce ETX TOXOID. Two commercial clostidial vaccines, BSE (Braxy, Struck and Enterotoxaemia Vaccine) and ULT (UltraChoice^{TM}8), were purchased. The adjuvants included an aluminum hydroxide adjuvant, $Al(OH)_3$, VAC 20 HA (SPI Pharma, Septemes Les Vallons, France); two w/o/w oil adjuvants, GR208 (GloryBio, Changsha, China) and MONTANIDETM ISA 201 VG (SEPPIC SA, Puteaux, France); a polymeric adjuvant based on a dispersion of a high molecular weight polyacrylic polymer in water, MONTANIDETM GEL O2 PR (SEPPIC SA); and an aqueous adjuvant based on nanoparticles associated with an immunostimulating compound, MONTANIDETM IMS 1313 VG NST (SEPPIC SA).

Japanese White rabbits (male, approximately 1.5–2.0 kg, 80–100 days old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and randomly divided into nine groups (four rabbits per group). Rabbits from each treatment group were immunized subcutaneously with 100 µg of antigen per rabbit; this was performed for $rETX^{m}$-$Al(OH)_3$,

Table 1. Vaccines used in this study

Vaccines	Antigen components	Adjuvant	Antigen: Adjuvant	Target antigen concentration
$rETX^{m}$-$Al(OH)_3$	Inactivated *Escherichia coli* containing the recombinant mutant ETX^{H106P}/CTB-ETX^{Y196E} proteins.	$Al(OH)_3$	4:1 (v/v)	100 µg/ml
$rETX^{m}$-GR208		GR208	5:6 (v/v)	
$rETX^{m}$-ISA201		ISA201	5:6 (v/v)	
$rETX^{m}$-GEL02		GEL02	17:3 (v/v)	
$rETX^{m}$-IMS1313		IMS1313	1:1 (v/v)	
ETX TOXOID	ETX toxoid.	$Al(OH)_3$	4:1 (v/v)	100 µg/ml
BSE	A commercial vaccine from China containing inactivated *Clostridium Septicum-Perfringens* Types C&D Bacterin-Toxoid.	UN	UN	UN
ULT	A commercial vaccine from USA containing inactivated *Clostridium Chauvoei-Septicum-Haemolyticum-Novyi- Sordellii-Perfringens* Types C&D Bacterin-Toxoid.	UN	UN	UN
Saline	Saline only, no antigen.	NA	NA	NA

BSE, braxy, struck and enterotoxaemia vaccine; ULT, UltraChoice^{TM}8; CTB, cholera toxin B; ETX, epsilon toxin; NA, not applicable; UN, unknown.

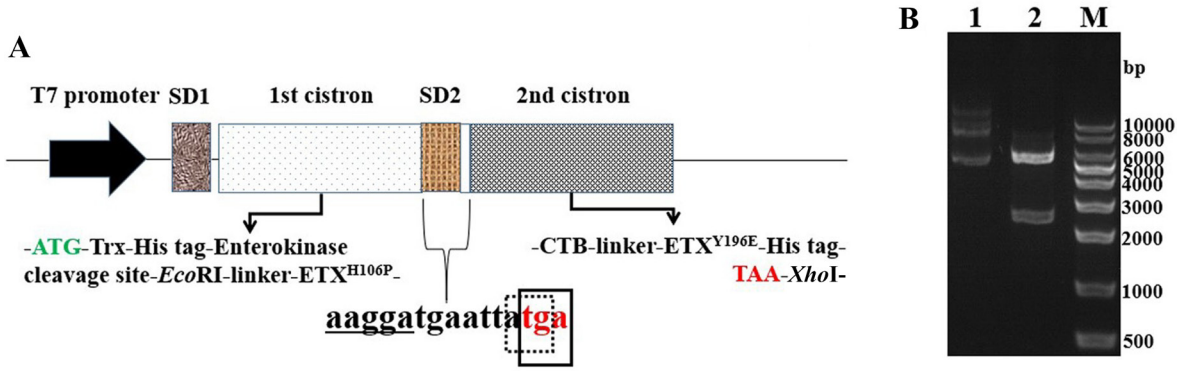

Fig. 1. Construction of the recombinant plasmid with bicistronic design system for epsilon toxin (ETX) expression. (A) Schematic diagram of a bicistronic design (BCD) expression system for ETX gene expression. First cistron, second cistron, second SD sequence (SD2) and associated junction with the Cholera Toxin B (CTB) gene for the BCD system are shown. The sequence of SD2 is underlined, and the solid and dashed boxes in the DNA sequences indicate the stop codon of the first cistron and the start codon of the second cistron, respectively. (B) Restriction enzyme digestion of the recombinant plasmid. Lane 1: Undigested recombinant plasmid, Lane 2: The recombinant plasmid digested with *Eco*RI and *Xho*I, Lane M: standard molecular weight marker.

rETX^{m}-GR208, rETX^{m}-ISA201, rETX^{m}-GEL02, rETX^{m}-IMS1313, ETX TOXOID, BSE, or the ULT vaccine (where necessary this was performed in accordance with the manufacturer's instructions); sterile saline was used as the negative control group. The rabbits were given an intramuscular booster of the same type of vaccine approximately 21 days after the first immunization. After the animals were anesthetized, blood samples were collected prior to vaccination (D0) and at 21 (D21) and 35 days (D35) after primary immunization. Serum was collected and kept at −20°C until analysis.

Challenge with native ETX

Two weeks after the second immunization, rabbits immunized with each of the vaccines under analysis were intravenously challenged with 2× rabbit LD_{100} of ETX. The rabbits that received saline were intravenously challenged with 1× rabbit LD_{100} of ETX. The survival of all rabbits was recorded daily for five days.

Toxin neutralization assay

ETX (200 µl) at doses of 6×, 9×, 10×, 20×, 40×, 50×, 60×, 120×, 250×, 300×, 600×, and 800× mouse LD_{100} of ETX was incubated with 100 µl of pooled polyclonal antiserum from immunized rabbits for 40 min at 37°C. Groups of two 16–18 g female CD-1® (ICR) IGS mice were injected intravenously via the tail vein with 300 µl of the mixed solution. The survival status of all mice was observed and recorded for 24 hr. Survival of both mice in the group indicated that the sera neutralized the associated ETX dose. The neutralizing antibody titer was defined as the maximum dose of mouse LD_{100} of ETX, which was neutralized by 0.1 ml of pooled sera.

RESULTS

Construction of the BCD system recombinant plasmid for ETX expression

To enhance the production of recombinant protein, a BCD expression system (Fig. 1A) was developed for ETX expression. The full length (bp) for the first cistron and the second cistron were 1,671 and 1,287 bp, respectively, and the expected protein molecules were 556 and 428 amino acids in size, respectively. To improve the immunogenicity of the protein product, a mucosal adjuvant CTB coding sequence followed by the ETX^{Y196E} sequence was included in the second cistron. In the BCD expression system, SD2 was located at the 3′-end of the first cistron and served as a ribosome binding site for the translation of the second cistron. A 1-bp frame shift was designed between the stop codon of the first cistron and the start codon of the second cistron so that the second cistron would be expressed under the same promoter by translational coupling (Fig. 1A). The resultant recombinant plasmid was confirmed to be a 2,459-bp product following digestion with *Eco*RI and *Xho*I (Fig. 1B). DNA sequencing was followed by nucleotide and protein sequence comparisons with BLAST; this analysis revealed that the inserted sequence and the reading frame were correct.

Expression of the recombinant proteins in E. coli

The results of the SDS-PAGE (Fig. 2A) and Western blot (Fig. 2B) analyses indicated that induced recombinant protein expression resulted in a 61.2-kDa polypeptide expressed from the first cistron and a 47.1-kDa polypeptide expressed from the second cistron. Moreover, after induction at 15°C for 24 hr with 0.5 mM IPTG, the recombinant proteins were overexpressed at a high level (41.7%) with respect to the percentage of total cell protein, and 35% of the two expressed proteins were in soluble form. The expression level of the recombinant bacteria was evaluated to be 20 mg/l, and the final yield of the recombinant proteins was estimated to be 7 mg/l following a one-step purification strategy.

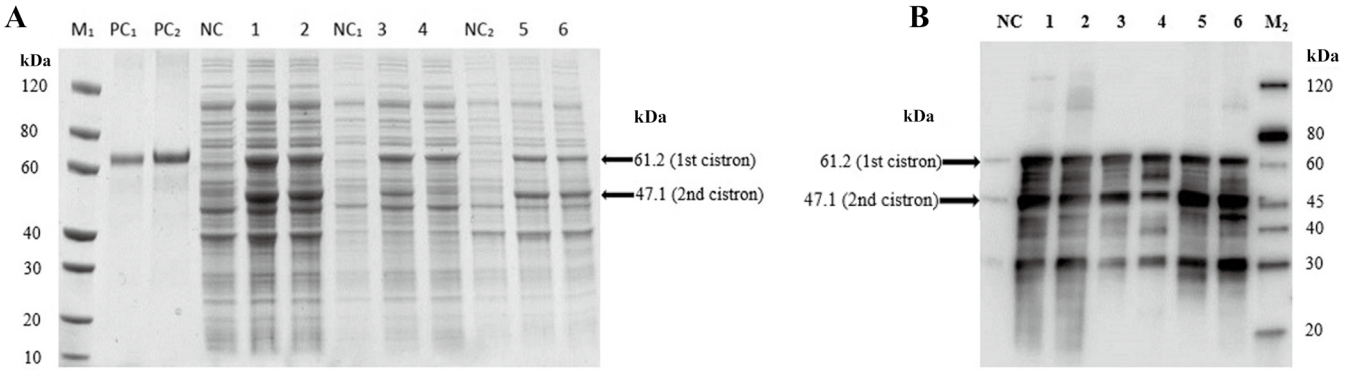

Fig. 2. (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and (B) Western blot analysis of the recombinant proteins expressed in *Escherichia coli* (*E. coli*) BL21 (DE3). Recombinant bacteria (DE3) were ultrasonically disrupted after isopropyl-β-thio-D-galactopyranoside (IPTG) induction at 15°C for 24 hr (Lanes 1, 3, and 5) or 37°C for 4 hr (lanes 2, 4, and 6). Lanes 1 and 2 are crude cell lysate; lanes 3 and 4 are supernatant of cell lysate; and lanes 5 and 6 are pellets after removal of the supernatant. Crude cell lysate without IPTG induction for Lane negative control (Lane NC), its supernatant for (Lane NC_1), and its pellet after removal of the supernatant (Lane NC_2) were used as controls, and 1 µg for Lane positive control 1 (Lane PC_1) and 2 µg (Lane PC_2) of Bovine Serum Albumin (BSA) were used as indicators of protein content. Lanes M1 and M2: standard molecular weight markers. The primary antibody for Western blot was anti-His antibody.

Table 2. The number of mouse LD_{100} (100% lethal dose) of ETX (epsilon toxin) that can be neutralized per 0.1 ml of vaccinated rabbit sera and the number of surviving vaccinated rabbits challenged with 2× rabbit LD_{100} of ETX

Groups	× mouse LD_{100} of *Clostridium perfringens* epsilon toxin neutralized by 0.1 ml of pooled sera		Alive/total
	Primary immunization	Second immunization	
$rETX^m$-$Al(OH)_3$	50	300	4/4
$rETX^m$-GR208	50	800	4/4
$rETX^m$-ISA201	120	800	4/4
$rETX^m$-GEL02	60	600	4/4
$rETX^m$-IMS1313	10	250	4/4
ETX TOXOID	20	120	4/4
BSE	6	40	4/4
ULT	9	50	4/4
Saline	0	0	0/4

BSE, braxy, struck and enterotoxaemia vaccine; ULT, UltraChoice™8.

Protective immunity in immunized rabbits

To examine the level of immunoprotection elicited by the recombinant ETX construct, we compared immunogenicity and efficacy with three established vaccines: ETX TOXOID, BSE, and ULT. Following a booster vaccination, all animals were challenged with ETX. Unvaccinated control rabbits challenged with 1× rabbit LD_{100} of ETX died within 18 hr. Compared to the negative control group, four out of four rabbits in the other groups survived (Table 2) after being challenged with 2× rabbit LD_{100} of ETX.

Toxin neutralization assay in mice

To test the *in vivo* ETX neutralization effect, mice were injected with a mixture of ETX and antiserum; the neutralizing antibody titers are shown in Table 2. All mice injected with mixtures of 1× mouse LD_{100} ETX and pre-immune serum died (data not shown). The results indicated that the neutralizing antibody titers of $rETX^m$-vaccinated rabbits (apart from those of $rETX^m$-IMS1313-vaccinated rabbits) were higher than those observed following vaccination with the ETX TOXOID, BSE or ULT vaccines. $rETX^m$ mixed with ISA201 oil adjuvant induced the highest neutralizing antibody titer of 120 after the initial immunization, i.e., 0.1 ml of pooled sera from the vaccinated rabbits could neutralize 120× mouse LD_{100} of ETX. Following the second vaccination, $rETX^m$ mixed with ISA201 or GR208 produced the highest neutralizing titer of 800.

DISCUSSION

rETX^{H106P} has previously been reported as a safe, non-toxic and immunoprotective vaccine antigen against enterotoxemia [1, 5, 7, 11]. rETX^{Y196E}-C has also been shown to exhibit low toxicity and can be used to protect mice at a dose of 500× LD_{50} of rETX [15]. Therefore, in this current study, the coding sequence of rETX^{H106P} was cloned into the first cistron of an appropriate expression system. Since CTB has been shown to be a nontoxic vaccine adjuvant [2, 6, 10, 13], the CTB gene was fused to the ETX^{Y196E} coding sequence in the second cistron to improve protein expression and immunogenicity. Mutalik *et al.* [9] successfully used synthetic BCD and appropriate promoters to facilitate controlled and reliable gene expression in *Escherichia coli* cells. The two recombinant proteins rETX^{H106P}/CTB-rETX^{Y196E} were successfully expressed after induction with IPTG; the ratio of target products to total *E. coli* proteins was as high as 41.7%. Thirty-five percent of the target proteins were expressed in soluble form and therefore ready-to-use in efficacy studies in rabbits or other animals.

rETX^{H106P}/CTB-rETX^{Y196E} were mixed with different adjuvants to make vaccines. These vaccines were then administered to rabbits to evaluate their efficacies and the results were compared with those resulting from the administration of ETX TOXOID and two commercial vaccines (BSE and ULT). All vaccinated rabbits were protected from 2× rabbit LD_{100} of ETX challenge. Notably, all rETX^{H106P}/CTB-rETX^{Y196E} vaccines except rETX^{m}-IMS1313 resulted in higher neutralizing antibody titers than ETX TOXOID or the two commercial vaccines, which may be partially due to the adjuvant effect of *E. coli* lipopolysaccharide (LPS). rETX^{m}-ISA201 induced the highest neutralizing antibody titers after the first vaccination, i.e., 0.1 ml of pooled sera from rETX^{m}-ISA 201-vaccinated rabbits could neutralize 120× mouse LD_{100} of ETX. This latter observation indicated that ISA 201 exhibited the best immune adjuvant effect. Furthermore, vaccination with the rETX^{m}-ISA201 and rETX^{m}-GR208 vaccines resulted in a 16- to 20-fold increase in neutralizing titers compared with those of commercial vaccines after the second vaccination. This was probably because of the characteristics of the adjuvant, i.e., oil adjuvants, such as GR208 and ISA201, have a stronger adjuvant effect than aluminum hydroxide adjuvants. However, two types of attenuated (or non-toxic) mutant ETXs can be simultaneously expressed in large quantities through the BCD expression system, and these mutant recombinant proteins can be effective vaccine antigens to protect rabbits against virulent ETX challenge. Since the use of purified antigen involves a laborious and time-consuming production process that makes recombinant antigens less commercially competitive than conventional toxoids, unpurified recombinant *E. coli* bacterins represent a simple alternative. Moreira *et al.* [8] demonstrated that recombinant bacterins and cell lysate fractions against *Clostridium botulinum* toxin serotypes C and D resulted in similar neutralizing antibody titers as purified antigens. Moreover, the latter study reported no side-effects from potential endotoxins (LPS) when guinea pigs were inoculated with unpurified antigens. In the current study, inactivated recombinant *E. coli* bacterin expressing rETX^{H106P} and CTB-rETX^{Y196E} conferred immune protection against ETX and resulted in superior neutralizing antibody titers. Therefore, these results indicate that rETX^{H106P}/CTB-rETX^{Y196E} might represent a promising subunit vaccine candidate.

To the best of our knowledge, this is the first report describing the expression of rETX^{H106P} and CTB-rETX^{Y196E} at high levels using the BCD system. In conclusion, our results demonstrate that the recombinant proteins developed in this study elicit higher levels of neutralizing antibodies than ETX toxoid or commercial vaccines, as evaluated by a toxin neutralization assay in mice. Thus, the developed recombinant proteins can be considered as candidates for the development of a vaccine against *C. perfringens* native epsilon toxin or the preparation of a hyperimmune antiserum.

CONFLICT OF INTEREST. The authors have nothing to disclose.

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