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Potential protective immunogenicity of recombinant *Clostridium perfringens* α–β2–β1 fusion toxin in mice, sows and cows

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ABSTRACT

Clostridial toxins are main pathogenic virulence of *Clostridium perfringens* that have been associated with a wide range of diseases in both humans and domestic animals. Genetically engineered toxoids have been shown to function as potential vaccine candidates in the prevention of *Clostridium* derived infectious diseases. In this study, we have developed recombinant α-toxin (CPA), β2/β1-fusion toxin (CPB2B1) and α/β2/β1 trivalent fusion-toxin (CPAB2B1) as vaccine candidates that may be used to vaccinate against *C. perfringens* α, β1 and β2-toxins. Mice immunized with these recombinant toxoids demonstrated a strong protective immunological response when administered a lethal dose of *C. perfringens* type C culture filtrate with high titers of neutralizing antibodies to the toxins in the sera, as well as the intestinal mucosal s-IgA level. Specific neutralizing antibodies to the toxins were also detected in the sera and colostrum of sows and cows vaccinated with the toxoids. Furthermore, the CPA and CPB2B1 recombinant toxoid cocktail was capable of stimulating relatively higher levels of immune responses compared to that of CPA, CPB2B1 and CPAB2B1 alone. The CPAB2B1 trivalent fusion toxoid also displayed increased immunogenicity relative to CPA and CPB2B1 alone. These results suggest that recombinant toxoids are potential vaccine candidates against *Clostridial* toxins; the use of mixed cocktails and/or multivalent recombinant toxoids against different types of toxins may be an effective approach in the prevention of diseases caused by toxins produced by *C. perfringens*.

1. Introduction

Clostridium perfringens, a Gram-positive, rod-shaped, spore-forming, anaerobic bacterium, is part of the normal intestinal microbiota flora in humans and other mammals. This bacterium has been associated with a variety of diseases in both humans and domestic animals including: lamb dysentery, enterotoxemia, gas gangrene, hemorrhagic enteritis, food poisoning and many other severe enterotoxemic diseases [1,2]. Toxins produced by this microbe are main pathogenic virulence. This pathogen can be subdivided into five toxinotypes (A–E) based on the production of four major toxins (α, β, ξ, ι) [3]. However, there are as many as ten other toxins produced that (β1, β2, γ, η, ο, θ, η, λ, μ and ν) have also been reported in the *C. perfringens* [4,5]. *C. perfringens* type C is classified by the production of α toxin (Phospholipase C, PLC, also referred to as CPA) and β toxin (CPB), although other lethal toxins, including perfringolysin O (PFO), β2 toxin (CPB2), and/or enterotoxin (CPE), produced by this species have been reported as well [6–8].

Type C isolate is a common cause of disease in domestic animals worldwide, most commonly in neonatal livestock, mainly responsible for a variety of diseases in newborns including "Sudden Death Syndrome" in lamp and hemorrhagic diarrhea (blood stained dysentery) in piglet [7]. The CPA is produced by all five types of *C. perfringens*, whereas the production of CPB is currently found in strains of type B and type C. The disease caused by type C strain is very rare in a healthy adult animal with normal digestive function. However, a high incidence of the disease occurs in the early neonatal period of animals when they are lacking intestinal proteases. CPB has been suggested to be the key pathogenic virulence in type C isolates as supported by CPB susceptibility to proteolysis and protease inhibitors elevating the effects both in vitro and in vivo [7–10]. CPB is a lethal necrotizing toxin produced by type B and type C of *C. perfringens*, which plays critical roles in the tissue damage by targeting endothelial cells. The death caused by *C. perfringens* type C infection is largely due to the effects of intestinal damage

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and toxemia where it has been detected in the intestinal contents and peritoneal fluid of infected animals [11,12]. In addition to CPB (now known as β1 toxin, or referred as CPB1), type C isolates also produce β2 toxin (referred as CPB2). CPB2 is produced by most or all swine type C isolates and shares similar biological activity with CPB1 even though they are encoded by different microorganism plasmids and amino acid sequences [13,14]. CPB1 and CPB2 are both important in the virulence of necrotic enteritis in humans and animals, particularly in piglets [13,15,16]. Pigs inoculated with *C. perfringens* type C isolates developed typical lesions in the gut loops, but inoculation with CPB1 alone did not cause necrosis [17]. This suggests CPB2 may play a important role in the pathogenesis of *C. perfringens* infection [11].

Immunization is universally advocated to prevent infectious diseases in livestock. Animals vaccinated with toxoids have shown to be protected against *C. perfringens* type C infections. Recombinant vaccines against many types of *C. perfringens* toxins have been widely studied and were able to induce protective responses, particularly against the α-toxin [18–24] and ε-toxin [25–28]. β-Toxins (CPB1 and CPB2) are the major virulent factors of *C. perfringens* type C isolates that have been shown to be essential to intestinal virulence for disease isolate CN3685 in a rabbit ileal loop model, as well as the cause of necrotizing enteritis in pigs, calves, goats and humans [8,10,12,16,29–31]. However, vaccines against CPB have received much less attention, as no recombinant vaccine against CPB2 has been reported [32–34]. In this study, we evaluated the immunogenicity of recombinant CPA toxoids, fusion toxins of CPB2B1 and CPAB2B1 in both mouse models and livestock. Our results demonstrate that multivalent and/or mixed recombinant vaccines against CPA, CPB1 and CPB2, not only increase the survival rate of mice challenged with lethal doses of *C. perfringens* type C culture filtrate, but also stimulate the production of neutralization antibodies in the sera and colostrum of immunized mice, sows or cows.

2. Materials and methods

2.1. Animals, bacterial strains and plasmids

Male and female ICR mice that were 6- to 10-weeks old and 20–25 g were purchased from the Animal facility of Ningxia Medical University (Yinchuan, China). The sows and cows were breed at the Gupingpu Farm (Yinchuan, Ningxia, China) and the experiments using these animals were carried out in a quarantined zone of the farm. All procedures involving the use of animals were approved by the Committee for Animal Care and Use of Ningxia University. *C. perfringens* type C strain, China isolate C59-44 was purchased from the China Institute of Veterinary Drug Control (Beijing, China). Plasmid pET-28a (+) is a product of EMD Biosciences (San Diego, CA, USA). *Escherichia coli* strains of JM109 and BL21 (DE3) were lab stocks and were used as competent cells. *E. coli* strains were cultured on Luria-Bertani (LB) agar or in broth supplemented with 50 mg/ml ampicillin, if required for plasmid selection at 37 °C.

2.2. Restriction endonuclease, chemicals and antibodies

Restriction endonucleases and DNA modifying enzymes were products of Takara Biologicals (Japan), New England Biolabs (Ipswish, MA, USA) or Promega (Madison, WI, USA). Chemicals used in this study were products of Sigma (St. Louis, MO, USA) unless otherwise specified. Bacterial genomic DNA isolation kit, Plasmid mini-prep, RT-PCR and TA Clone kits and DNA ladders, prestained protein markers, and HRP-donkey anti-goat IgG antibodies were purchased from TianQen Biological Inc. (Beijing, China). Goat anti-CPA and CPB antibodies were products of the Lanzhou Institute of Biological Products (Lanzhou, China). Secretory IgA (s-IgA) enzyme-linked immunosorbent assay (ELISA) detection kit and s-IgA standard were purchased from CUSAbio Biotech Inc. (Wuhan, China).

2.3. Preparation of culture supernatants of C. perfringens type C

C. perfringens type C isolate C59-44 has been characterized for its genotype and toxin production at China Institute of Veterinary Drug Control. They reported that the microbe produces CPA, CPB1 and CPB2 under the following culture conditions. An isolated colony of C59-44 on SFP agar (Difco Laboratories) was inoculated in 10 ml FTG (Fluid Thioglycolate Medium, Difco Laboratories) and grown anaerobically overnight at 37 °C. A 0.1 ml aliquot of that overnight culture was then transferred to 30 ml of TGY (3% Tryptic soy broth, 2% glucose, 1% yeast extract, 0.1% thioglycolate, pH 7.5) media and grown at 37 °C for an additional 8–12 h. The resulting culture was transferred to the fresh TGY media at ratio of 1/100 (V/V) and grown at 37 °C until the culture reached the late log phase as determined by measuring the OD600 (the levels of lethal toxins expressed during vegetative growth were maximal in the late log phase). The culture was then chilled immediately on ice and centrifuged at 5000 rpm for 20 min. The resultant culture supernatants were filtered with 0.45-μm-pore-size filters and severed as the sterile vegetative supernatant filtrates containing a CPA, CPB1 and CPB2 crude toxin mixture (referred as culture filtrate in this study).

2.4. Molecular cloning of bacterial vectors expressing CPA, CPB2B1 and CPAB2B1 fusion proteins

Bacterial genomic DNA from the *C. perfringens* type C strain, China isolate C59-44 was isolated using a bacterial genomic DNA isolation kit (TianQen Biological Inc., China). Based on the DNA sequence of toxins described previously [13,33,35,36], the following primer sets were used to amplify the encoding regions of the CPA, CPB1 and CPB2 genes, respectively. CPA-F: CATG*CCATGG*CAATGAAAAGAAAGATTTGT and CPA-R: CCG*GAATTC*TTTTATATTATAAGTTGAATT (GenBank accession no.: L43548); CPB1-F: CGC*GGATCC*CCAATGATATAGGTAAAAC and CPB1-R: CCG*GAATTC*TTAATAGCTGTTACTTTGTG (GenBank accession no.: L13198); CPB2-F: CATG*CCATGG*CAAAAGAAATCGACCCTTAT and CPB2-R: CCC*GGATCC*GTCCACCATACCCTTCACCAAA (GenBank accession no: L77965). Appropriate restriction sites were added to the primers for subcloning of fusion genes in the pET28a (+) bacterial expression vector (underlined in the sequences above). The PCR amplified fragments were first cloned into a pMD-18T PCR TA cloning vector for sequencing. These fragments were then cloned into the pET28a (+) vector to generate the pET-CPA, pET-CPB2B1 and pET-CPAB2B1 vectors containing the CPA, CPB2-CPB1 and CPA-CPB2-CPB1 fusion toxins, respectively (Fig. 1A).

2.5. Inducing expression of the fusion proteins

The above pET-CPA, pET-CPB2B1 and pET-CPAB2B1 plasmids were transformed into *E. coli* BL21 (DE3) competent cells and selected with Amp antibiotics. Single bacterial colonies were isolated and cultured in the LB medium containing Amp (50 μg/ml for each) in a 37 °C incubator with shaking overnight. The cultured bacterial cells were used as seeds to induce protein expression. 1.0% (V/V) of the seed culture was inoculated into LB broth medium containing antibiotics with a 37 °C incubation to log phase. IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added at a final concentration of 0.4 mM and the mixture was cultured for an additional 4 h. Following the IPTG induction, the bacterial cells were harvested by centrifugation and the bacterial pellet was resuspended