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Identification and cloning of two immunogenic *Clostridium perfringens* proteins, elongation factor Tu (EF-Tu) and pyruvate:ferredoxin oxidoreductase (PFO) of *C. perfringens*

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ABSTRACT

Clostridium-related poultry diseases such as necrotic enteritis (NE) and gangrenous dermatitis (GD) cause substantial economic losses on a global scale. Two antigenic *Clostridium perfringens* proteins, elongation factor Tu (EF-Tu) and pyruvate:ferredoxin oxidoreductase (PFO), were identified by reaction with immune sera from commercial meat-type chickens with clinical outbreak of *Clostridium* infections. In addition to the genes encoding EF-Tu and PFO, *C. perfringens* alpha-toxin and necrotic enteritis B-like (NetB) toxin were also expressed in *Escherichia coli* and their corresponding recombinant proteins were purified. Using the four recombinant proteins as target antigens in ELISA immunoassays, high serum antibody titers were observed not only in chickens with clinical signs of *Clostridium* infections, but also in apparently healthy animals from the same disease-endemic farm. By contrast, no antibodies against any of the proteins were present in the serum of a specific pathogen-free bird. In ELISA using recombinant proteins of *C. perfringens*, the levels of anti-bacterial protein antibodies were also higher in chickens which were experimentally induced to show NE clinical signs after co-infection with *C. perfringens* and *Eimeria maxima* compared with uninfected controls. These results show that two antigenic *C. perfringens* proteins, EF-Tu and PFO can be useful detection antigens for *C. perfringens*-afflicted infections in commercial poultry.

1. Introduction

Necrotic enteritis (NE) and gangrenous dermatitis (GD) are among the most important *Clostridial* poultry diseases in the US (Smith and Helm, 2008). NE is caused by *Clostridium perfringens* type A, a Gram-positive, anaerobic, spore-forming, rod-shaped bacterium (Williams, 2005), whereas GD is primarily associated with infection by *C. perfringens* type A or *Clostridium septicum* with underlying predisposing factors (Clark et al., 2010). Typically, NE occurs at 17–18 days post hatching, while GD develops later at 4–7 weeks of age (Wages and Opengart, 2003; McDevitt et al., 2006). Apart from the fact that both NE and GD can be caused by the same pathogen, no apparent link between these clinically distinct diseases has been reported, and it is currently unknown what other factors (bacterial, host, or environmental) influence the incidence and character of *Clostridium*-associated poultry diseases. Because of lack of reliable animal models and scarcity of basic research, our understanding on NE versus GD is relatively limited. Recent studies from our laboratory on host immune response to NE (Park et al., 2008) and GD (Li et al., 2010a,b) indicated heightened local inflammatory responses in the gut and skin, respectively, in *Clostridium*-affected chickens compared with their healthy counterparts. Better understanding of host–pathogen interaction will lead to the development of effective and practical control strategy against NE.

Two major *C. perfringens* virulence factors in chickens are alpha-toxin and the NetB (necrotic enteritis B-like) toxin, both of which are implicated in the pathogenesis of NE (Awad et al., 2001; Keyburn et al., 2006), and presumably GD (Li et al., 2010a,b). Considerable insights into NE pathogenesis was provided recently by Lepp et al. (2010) who suggested that NE is caused by several novel virulence factors that are encoded by cluster of genes on a pathogenicity loci, and some of which are plasmid-born in nature. Additional *C. perfringens* proteins that may be involved in bacterial pathogenesis and host protective immunity including pyruvate:ferredoxin oxidoreductase (PFO), elongation factor G (EF-G), perfringolysin O, glyceraldehyde-3-phosphate dehydrogenase,

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fructose 1,6 biphosphate aldolase, phosphoglyceromutase, and endo-beta-N-acetylglucosaminidase were previously reported (Kulkarni et al., 2006; Jiang et al., 2009). Some of these proteins induced protective immunity against experimental challenge infection with *C. perfringens* (Kulkarni et al., 2007, 2008, 2010; Jiang et al., 2009). However, their utility as poultry vaccines against *Clostridium* field infections was not validated and remains to be realized. The current study was undertaken to identify *C. perfringens* proteins which are recognized by chickens from a field *Clostridium* disease outbreak. For this purpose, we used serum from *Clostridium*-afflicted broiler chickens from a recent field outbreak (Li et al., 2010a,b) to identify immunogenic *C. perfringens* proteins. In this paper, we describe cloning, protein expression and characterization of two immunogenic proteins of *C. perfringens* we initially identified using sera from broiler chickens with clinical signs of GD and/or NE.

2. Materials and methods

All experimental studies in this study were approved by the Institutional Animal Care and Use Committees of the Beltsville Agricultural Research Center.

2.1. C. perfringens

C. perfringens (ATCC 13124, American Type Culture Collection, Manassas, VA), a type A strain gas gangrene isolate, was cultured anaerobically for 18 h at 37 °C in brain heart infusion broth containing 0.5% glucose, 0.5% yeast extract, 2% polypeptone, and 0.05% sodium thioglycolate, pH 7.2 (Sigma, St. Louis, MO). Bacteria were harvested by centrifugation at 3000 rpm for 15 min, washed three times in sterile PBS, and resuspended in PBS containing an EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). A bacterial lysate was prepared by sonication on ice (6 pulses of 30 s with 30 s intervals) (Misonix, Farmingdale, NY) and clarified by centrifugation at 3000 rpm for 20 min at 4 °C.

2.2. C. perfringens immune sera

C. perfringens infection of 34 day old broiler chickens (Ross/Ross) in a GD-endemic commercial poultry farm was confirmed by PCR amplification of the alpha-toxin gene from skin, muscle, and intestinal tissues, as well as by demonstration of *C. perfringens*-reactive serum antibodies by enzyme-linked immuosorbent assay (ELISA) as described (Li et al., 2010a). To prepare an experimental antiserum against *C. perfringens*, bacteria were cultured for 18 h, resuspended in isotonic saline, and inactivated with 0.4% formalin. One milliliter of the killed cell suspension containing approximately 1×10^9 cells was mixed with an equal volume of Montanide™ Gel adjuvant (Seppic, Puteaux, France) and injected into 4-week-old specific pathogen-free Ross/Ross broilers (Longenecker's Hatchery, Elizabethtown, PA). Identical booster immunizations were given at 1 and 2 weeks post-primary immunization. One week following boosting, the chickens were euthanized by cervical dislocation, blood was obtained by cardiac puncture, serum was prepared by centrifugation at 1000 rpm for 20 min at 4 °C, and stored at −20 °C until use.

2.3. Characterization of antigenic C. perfringens proteins by Western blotting

C. perfringens lysate was mixed with an equal volume of 0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.004% bromophenol blue, heated for 5 min at 100 °C, and proteins were resolved on 12% SDS–acrylamide gels. Separated proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) and blocked for 1 h at 22 °C with Superblock T20 (PBS) (Thermo Fisher Scientific, Rockford, IL). Membranes were sequentially incubated for 1 h at 22 °C with pooled sera ($n = 9$) from chickens with clinical signs of GD from the disease-endemic farm (1:50 dilution in blocking buffer), peroxidase-conjugated goat anti-chicken IgG secondary antibody (1:1000, Sigma), and 10 min with 4-chloronaphthol substrate (Sigma).

2.4. Identification of C. perfringens proteins by liquid chromatography/mass spectrometry

Simultaneous with Western blotting, *C. perfringens* proteins were separated by SDS–PAGE, the gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad, Richmond, CA), de-stained, rinsed with distilled water, and selected protein bands were excised. Gel pieces were sequentially washed with 30% methanol, water, and 30% methanol in 100 mM NH_4HCO_3, pH 7.8, de-hydrated with acetonitrile, and dried in a vacuum centrifuge. Proteins were reduced *in situ* for 1 h at 56 °C with 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (Pierce, Rockford, IL) in 100 mM NH_4HCO_3, followed by alkylation for 45 min at 22 °C in the dark with 55 mM iodoacetamide (Sigma) in 100 mM NH_4HCO_3. Gel pieces were rehydrated with 50 μl of 100 mM NH_4HCO_3, 0.75 μg of sequencing grade TPCK-trypsin (Promega, Madison, WI) was added, and incubated for 16 h at 37 °C. Peptides were resuspended in formic acid:acetonitrile (5:95, vol:vol), vacuum dried, desalted on PepClean C-18 spin columns (Pierce), and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) at the University of Maryland Greenebaum Cancer Center Proteomics Core Facility (Baltimore, MD). LC was performed using the Xtreme Simple chromatography system (Micro-Tech Scientific, Vista, CA). MS analysis was performed using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). MS/MS spectra were searched against the National Center for Biotechnology Information *C. perfringens* strain 13 genomic database using Sorcerer-SEQUEST software (Sage-N Research, Milpitas, CA). Peptide and protein assignments were assessed using PeptideProphet and ProteinProphet software. Proteins with ProteinProphet probabilities $\geqslant$90% and manually verified peptide assignments were accepted as positive identifications.

2.5. Cloning, expression, and purification of recombinant C. perfringens proteins

Oligonucleotide primers were synthesized for PCR amplification of the *C. perfringens* genes encoding elongation factor Tu (EF-Tu), pyruvate:ferredoxin oxidoreductase (PFO), alpha-toxin, and necrotic enteritis B-like (NetB) toxin (Table 1). DNA of a virulent *C. perfringens* strain (Park et al., 2008) was isolated using the High Pure PCR Template Preparation Kit (Roche), PCR was performed as described (Li et al., 2010a), and amplicons were isolated on ethidium bromide-stained 1% agarose gels. PCR products were purified using the Gel Extraction Kit (Qiagen, Valencia, CA), cloned into the pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), and transformed into *Escherichia coli* DH5a. Nucleotide sequences of cloned genes were verified by DNA sequence analysis. Coding regions of cloned genes were amplified by PCR using GoTaq flexi DNA polymerase (Promega) with primers containing EcoRI, BamHI, or XhoI restriction endonuclease sites (Table 2). Amplified products were digested with restriction enzymes, ligated to the pET32a(+) vector incorporating a polyhistidine epitope tag (Novagen EMD, Gibbstown, NJ), and transformed into *E. coli* Top10 (Invitrogen). Bacterial clones were screened by PCR and inserts were verified by DNA sequence analysis. Transformed *E. coli* were cultured for 16 h at 37 °C and induced for 5 h