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Live attenuated vaccine-based control of necrotic enteritis of broiler chickens

D.R. Thompson, V.R. Parreira, R.R. Kulkarni, J.F. Prescott *

Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ont., Canada N1G 2W1

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Abstract

A vaccine for necrotic enteritis (NE) of chickens would reduce the current need to prevent or treat the disease in broiler chickens with antimicrobial drugs. The objective of this study was to understand aspects of immunity to the disease. The first experiment examined the virulence of six strains of *Clostridium perfringens* isolated from cases of NE in broiler chickens. Using a 5-day experimental oral infection of 2-week-old broiler chickens, four of the six strains were found to be virulent. Pulsed-field gel electrophoresis and PCR showed that virulence was not associated with a plasmid encoding the beta2 toxin gene, *cpb2*, since this was present in virulent and one of the two avirulent strains.

In the second experiment, two virulent and one avirulent strains were tested for their ability to immunize (''infection-immunization'') chickens through the oral route. The procedure used experimental infection for 5 days followed by bacitracin treatment for 9 days, and then re-challenge 2 days later with a virulent strain, CP4. Infection-immunization with the virulent isolates protected chickens from subsequent virulent challenge, whereas the infection-immunization with the avirulent isolate did not. In a third experiment, two of four alpha-toxin-negative mutants of CP4 protected birds from experimental NE after oral immunization. These two mutants were also attenuated for virulence. We conclude that it is possible to immunize chickens successfully against NE and that immunogen(s) other than alpha-toxin are important in protective immunity against oral infection.

Keywords: *Clostridium perfringens*; Necrotic enteritis; Vaccination; Alpha-toxin mutants

1. Introduction

Clostridium perfringens is a Gram-positive, spore-forming anaerobe that is found in the soil and in the intestine of humans and animals. *C. perfringens* synthesizes and secretes up to 17 different extracellular toxins, 4 of which (alpha, beta, epsilon and iota) are major mouse-lethal toxins used to distinguish five toxin types: A, B, C, D or E (Songer, 1996). Necrotic enteritis (NE) is an enteric disease of birds that is caused predominantly by *C. perfringens* type A and to a lesser extent type C strains (Songer, 1996),

* Corresponding author. Tel.: +1 519 824 4120x54453; fax: +1 519 824 5930.
E-mail address: Prescott@uoguelph.ca (J.F. Prescott).

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and is an economically significant disease in broiler chickens. The disease is thought to occur when *C. perfringens*, which is part of the normal intestinal flora, overgrows in the small intestine and produces extracellular, particularly alpha, toxins that damage the intestine. Predisposing factors include infection with coccidia, as well as a high protein, high fibre and/or a wheat-based diet (Truscott and Al-Sheikhly, 1977; Al-Sheikhly and Al-Saieg, 1980; Riddell and Kong, 1992). Necrotic enteritis is commonly controlled in a preventative manner by incorporation of antimicrobial drugs in the feed or water, but this practice is increasingly criticised or has been banned in some jurisdictions. Since NE is such a common disease of broiler chickens, there is a need to investigate alternative approaches to its effective control. Despite the importance of NE, there is very little known about the basis of immunity to this infection, although immunization is an obvious approach to control.

The purposes of this study were therefore to examine aspects of immunity to NE in chickens, specifically: firstly, whether it is possible to immunize broiler chickens against NE; secondly, whether both virulent and avirulent strains of *C. perfringens* are immunogenic; and thirdly, whether immunity might depend on antigens other than the alpha-toxin.

2. Materials and methods

2.1. Bacterial strains and plasmids

Six strains of *C. perfringens* (CP1–CP6) were obtained from D.A. Barnum, Department of Pathobiology, University of Guelph. These strains were isolated from different field cases of necrotic enteritis in Ontario and were stored frozen at −70 °C in cooked meat medium (CMM) (Difco, Detroit, MI, USA) within one to two passages in vitro following isolation. Strains Cpa $^{-}$1, Cpa $^{-}$2, Cpa $^{-}$3, Cpa $^{-}$4 are alpha-toxin negative derivatives of CP4 identified by the absence of a zone of turbidity surrounding the colonies grown on egg yolk medium (Section 2.10).

C. perfringens reference strain types B and EE, used as controls for toxin typing and positive for *cpb* and both *cpe* and *cpb2* genes, respectively, were obtained from J.G. Songer, Department of Veterinary Science and Microbiology, University of Arizona, Tucson, AZ, USA.

Escherichia coli DH5α was used as the host for gene cloning and protein expression. Plasmid vector pGEM-T Easy (Promega Corporation, Madison, WI) was used as a cloning vector when antibiotic selection was necessary, the growth medium was supplemented with ampicillin (100 μg/ml). All strains were stored at −70 °C in LB broth containing 15% glycerol.

2.2. DNA and RNA manipulations

DNA manipulations, such as DNA ligation, restriction digestion and Southern blotting were performed by standard methods as outlined by Sambrook et al. (1989). Plasmid DNA was purified with a Plasmid Mini Kit (Qiagen, Mississauga, Ont., Canada). DNA fragments were extracted from agarose gel slices and purified with the QIAquick Gel Extraction Kit (Qiagen). For digoxigenin (DIG) labeling of PCR products, a PCR DIG probe synthesis Kit (Roche, Laval, Que., Canada) was used according to the manufacturer's instructions. DNA ligations were performed with enzymes supplied by New England Biological Laboratories (Mississauga, Ont., Canada). DNA transformations of bacteria were performed by the heat shock method described by Nishimura et al. (1990).

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, except that a 60-min on-column DNA digestion with 60 U of RNase-free DNase I (Qiagen) was performed. After elution, 4 U of RNase-free Dnase was incubated with 5 μg of total RNA according to the manufacturer's instructions (Ambion Inc., Austin, TX, USA). Genomic DNA contamination was tested by PCR with same primers and conditions used for RT-PCR. In case of a positive result, another RNase-free DNase treatment was performed and genomic DNA contamination tested again, until a negative result was obtained.

2.3. PCR toxin gene typing

The toxin type (A, B, C, D or E) of each *C. perfringens* isolate was determined by a modified version of Meer and Songer (1997). Briefly, 4 μl of template was prepared by the rapid DNA extraction