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Pathogenesis and toxins

TpeL-producing strains of *Clostridium perfringens* type A are highly virulent for broiler chicks

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ABSTRACT

Clostridium perfringens type A and type C are causative agents of necrotic enteritis (NE) in poultry. TpeL, a recently-described novel member of the family of large clostridial cytotoxins, was found in *C. perfringens* type C. Others have since reported TpeL in type A isolates from NE outbreaks, suggesting that it may contribute to the pathogenesis of NE. The virulence of TpeL-positive and -negative *C. perfringens* strains from cases of NE was examined by challenge of broiler chicks. Gross lesions typical of NE were observed in all challenged birds, and those inoculated with $TpeL^{pos}$ strains had higher average macroscopic lesion scores than those inoculated with a $TpeL^{neg}$ strain. Infection with $TpeL^{pos}$ strains may yield disease with a more rapid course and higher case fatality rate. Thus, TpeL may potentiate the effect of other virulence attributes of NE strains of *C. perfringens*. However, $TpeL^{pos}$ and $TpeI^{neg}$ strains compared here were not isogenic, and definitive results await the production and testing of specific TpeL mutants.

1. Introduction

Clostridium perfringens, a Gram-positive, anaerobic, spore-forming bacterium, produces various toxins and enzymes and is responsible for a wide range of diseases in humans and animals [1]. The species is divided into five toxinotypes, based on the production of major toxins alpha, beta, epsilon, and iota [2]. *C. perfringens* is often isolated from the intestinal tract of healthy birds, but can also cause outbreaks of a poultry disease known as necrotic enteritis (NE), which occurs most commonly in broiler chickens and turkeys [3]. Clinical signs of NE include depression, reluctance to move, and ruffled feathers. Affected birds may be somnolent, diarrheic, inappetent, anorexic, and dehydrated [4].

NE is associated mainly with *C. perfringens* type A, but there are reports of type C strains [5,6]. There is limited knowledge of the roles of specific virulence factors in pathogenesis of NE. A recently-discovered pore-forming toxin, NetB, is likely a critical virulence attribute among NE strains [7].

Recently, a novel toxin named TpeL was found in *C. perfringens* type C [8]. It is a member of the family of large clostridial cytotoxins (LCTs), which comprise the largest bacterial protein toxins, ranging in size from 250 to 308 kDa [9]. The 5 LCTs described to date include toxins A (TcdA) and B (TcdB) of *Clostridium difficile*, the hemorrhagic (TcsH) and the lethal (TcsL) toxins of *Clostridium sordellii*, and alpha-toxin (Tcnα) of *Clostridium novyi*. LCTs share primary amino acid sequence identities ranging from 36% to 90% [10].

Since discovery of *tpeL* in *C. perfringens* type C strains, it has been reported in type A NE isolates [11], suggesting that it may contribute to the pathogenesis of some cases of type A-induced NE. This, and the similarity of TpeL to other LCTs, led us to characterize NE resulting from infection with $TpeL^{pos}$ strains of *C. perfringens* type A.

2. Methods and materials

2.1. Strains and cultivation

In each challenge trial, the test strains (Table 2) were examined in company with positive and negative (uninoculated) controls. Challenge inocula were prepared by serial, alternate passage through cooked meat medium (Becton, Dickinson, Sparks, MD) and thioglycollate broth (Becton, Dickinson) (twice each), with incubation at 37 °C for 18 h in each case.

2.2. Challenge of broiler chicks

Newly-hatched Cornish × Rock chicks were randomly divided into experimental groups (n = 15–17 per group) and fed an

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Table 1
Strains.

Strain	Characteristics	Source	Genotype
JGS4143 (TpeL^{neg})	Chicken NE; positive control	JGS collection	A cpb2 netB
JGS5252 (TpeL^{neg})	Chicken NE; positive control	JGS collection	A cpb2 netB
JGS5529 (TpeL^{neg})	Chicken NE; positive control	Keyburn	A cpb2 netB
JGS4140 (TpeL^{pos})	Chicken NE	CAHFS Davis	A cpb2 netB
JGS1651 (TpeL^{pos})	Chicken NE	CAHFS Davis	A cpb2 netB
JGS4102 (TpeL^{pos})	Turkey NE	University of Georgia	A netB
JGS1870 (TpeL^{pos})	Chicken NE	CAHFS Davis	A cpb2 netB
JGS5369 (TpeL^{pos})	Chicken NE	CAHFS Davis	A cpb2 netB

Table 2
Necrotic enteritis in birds inoculated with TpeL^{pos} and TpeL^{neg} strains.

Strain	% Gross lesions (Score)	% Microscopic lesions (Score)	Number of Replicates (n)
JGS4143 (TpeL^{neg})	100 (1.8)	100 (1.8)	3 ($n = 20$)
JGS5252 (TpeL^{neg})	75 (1.8)	N/A	3 ($n = 20$)
JGS5529 (TpeL^{neg})	97 (1.9)	94 (1.9)	3 ($n = 39$)
JGS4140 (TpeL^{pos})	100 (2.3)	100 (3.2)	3 ($n = 29$)
JGS1651 (TpeL^{pos})	100 (2.1)	100 (3.1)	3 ($n = 37$)
JGS4102 (TpeL^{pos})	100 (2.5)	90 (2.3)	1 ($n = 10$)
JGS1870 (TpeL^{pos})	100 (2.1)	81 (2.1)	1 ($n = 11$)
JGS5369 (TpeL^{pos})	100 (2.5)	75 (3.6)	1 ($n = 13$)

antibiotic-free chick starter diet (containing 16% protein) for 7 days. On day 8, the diet was changed to a wheat-based feed containing 60% fishmeal and zinc at 400 ppm. On Day 14, feed was withdrawn for 20 h. Beginning on day 15, birds were fed (every 12 h for 4 days) a 1.25:1 feed: culture mixture. Birds were assessed every 6 h for presence or progression of disease signs. Surviving birds were necropsied when moribund or on the first post-challenge day (day 19). Gross lesions were evaluated throughout the small intestine. Lesions were counted and graded as 0 (no gross lesions), 1 (thin-walled or friable small intestine), 2 (focal necrosis or ulceration), 3 (large patches of necrosis), and 4 (severe or extensive necrosis typical of field cases). Intermediate scores of 1.5, 2.5, and 3.5 were utilized to more accurately assess levels of damage when necessary. Specimens were collected in 10% phosphate-buffered formalin for histopathology and fresh for bacteriologic culture.

2.3. Bacteriologic culture

Jejunal segments ($n = 1–2, 2–3$ inches in length) were collected from necropsied chicks on day 4 of challenge (samples taken this day only if birds were moribund) or post-challenge day 1. Segments of jejunum were opened aseptically on sterile foil and the mucosa removed by scraping with sterile microscope slides. Areas with visible gross lesions were cultured separately from the remaining intestinal contents. This material was plated on tryptose agar (Difco, Sparks, MD) with 5% citrated bovine blood and incubated overnight under anaerobic conditions (5% H_2:5% CO_2:90% N_2) at 37 °C. All isolated colonies with typical morphology and hemolytic pattern were picked, streaked for purity on blood agar, and confirmed as *C. perfringens* (Gram-positive, anaerobic rods, double-zone hemolysis).

2.4. Genotyping

Genotyping was carried out by a standard procedure. Briefly, isolates ($n = 4–8$) obtained from intestine samples from each bird (those inoculated with TpeL^{pos} strains, TpeL^{neg} strains, and uninoculated controls) and were passed on blood agar. Isolated colonies were suspended in 200 μl of sterile water, boiled for 20 min and centrifuged at 15,000× *g* for 5 min. The supernatant fluid (9.5 μl aliquots) was used as template. Genotype was determined by a multiplex PCR assay [12], in a 25 μl reaction mixture containing 2× GoTaq® Green Master Mix (Promega, Madison, WI, USA), and 50 pM of primers. PCR conditions included denaturation at 94 °C for 5 min and 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; a final extension step (72 °C for 7 min) was also included. PCR products were detected by electrophoresis in 1.5% agarose gels, staining with ethidium bromide, and examination via UV transillumination. Presence of a 324 bp amplification product indicated the presence of CPA, confirming that the isolates were *C. perfringens*. Absence of amplification products of *cpb*, *etx*, and *itxAB* indicated that strains were of genotype A.

PCR to detect TpeL was performed subsequently with 50 pM of primers (forward; 5′-ATATAGAGTCAAGCAGTGGAG-3′, reverse; 5′ GGAATACCACTTGATATACCTG-3′) and 9.5 μl template solution. PCR conditions included denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, and with a final extension step at 72 °C for 7 min. PCR products were fractionated by electrophoresis in 1.5% agarose gels and the target 466 bp product was detected by staining with ethidium bromide and examination by UV transillumination.

2.5. Histopathology

Jejunal segments containing gross lesions (when present) were collected at necropsy and fixed overnight in 10% phosphate-

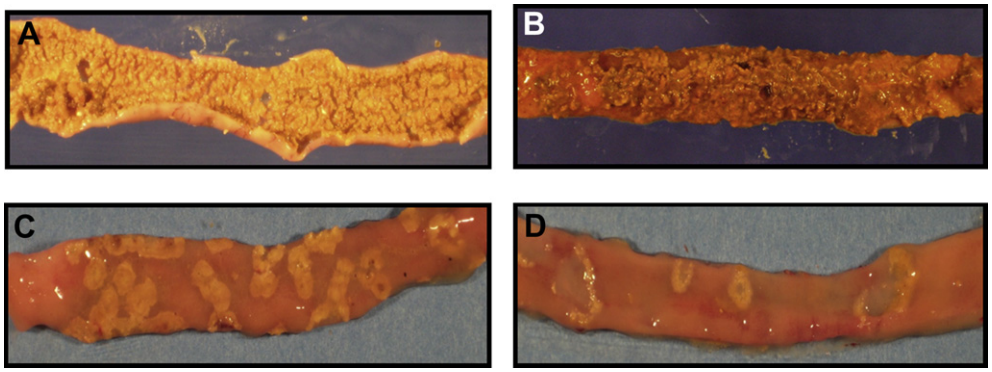

Fig. 1. Macroscopic lesions in birds inoculated with TpeL^{pos} and TpeL^{neg} strains. Extensive pseudomembrane formation (Panel A) and hemorrhage (Panel B) were common in birds inoculated with TpeL^{pos} strains. Focal areas of ulceration and necrosis (Panels C and D) were common in birds inoculated with TpeL^{neg} strains.