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Necrotic Enteritis Potential in a Model System Using *Clostridium perfringens* Isolated from Field Outbreaks

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SUMMARY. Necrotic enteritis is an enteric disease of avian species caused by the anaerobic bacterium *Clostridium perfringens*. The disease is regularly controlled in the broiler chicken industry with antimicrobials in feed but is reemerging in areas such as Europe where there is a ban on antimicrobials as growth promoters. To study prospective therapies, researchers must be able to reproduce this disease in a controlled environment, but this is not always possible because of differences in the pathogenicity of *C. perfringens* strains. Our objective was to test the potential of five isolates (SNECP43, 44, 47, 49, and 50), taken from field cases of necrotic enteritis, at recreating the disease in a controlled challenge experiment. SNECP43 and 50 were derived from a common clone, with SNECP50 passed *in vivo* and SNECP43 subcultured *in vitro*. Four hundred birds were divided into 16 pens, with three pens each receiving one of five treatments, with one control pen. Day-old birds were raised on a high wheat-based diet to promote necrotic enteritis development and were challenged with between 3.4×10^9 and 3.2×10^{11} colony-forming units (cfu) of *C. perfringens* in feed for a period of 24 hr starting on day 13 of the challenge experiment. Lesion scores were assessed on two birds per pen sacrificed on day 17 and on any dead birds during the 25-day study. Growth performance was assessed up to 25 days, and mortality recorded throughout. Only SNECP50 produced necrotic enteritis mortalities significantly different ($P \leq 0.05$) from the control. The five isolates were also typed using pulsed-field gel electrophoresis to assess their genetic relatedness. All epidemiologically unrelated isolates were deemed genetically unrelated, whereas SNECP43 and 50 differed by only a single minor band. Toxin type was assessed using polymerase chain reaction (PCR), which was also used for the detection of the gene encoding the β2-toxin.

RESUMEN. Potencial de enteritis necrótica en un modelo utilizando *Clostridium perfringens* aislado de brotes de campo.

La enteritis necrótica es una enfermedad entérica de las especies aviares causada por la bacteria anaeróbica *Clostridium perfringens*. En la industria del pollo de engorde la enfermedad se controla regularmente con antimicrobianos en el alimento, pero la enfermedad esta emergiendo en áreas como Europa donde existe un veto en la utilización de antimicrobianos como promotores del crecimiento. Para estudiar posibles terapias, los investigadores deben ser capaces de reproducir la enfermedad en un ambiente controlado, sin embargo, debido a las diferencias en la patogenicidad de las cepas de *C. perfringens*, esto no es siempre posible. Nuestro objetivo fue estudiar el potencial de cinco aislamientos (SNECP43, 44, 47, 49 y 50) tomados de casos de enteritis necrótica en el campo para recrear la enfermedad en un experimento de desafío controlado. Los aislados SNECP 43 y 50 fueron derivados de un clon común (SNCCP50 con pasajes *in vivo* y SNECP43 subcultivado *in vitro*). Se dividieron 400 aves en 16 corrales, con tres corrales recibiendo uno de cinco tratamientos y un corral control. Aves de un día de edad se criaron con una dieta con alto contenido de trigo para promover el desarrollo de enteritis necrótica y se desafiaron comenzando el día 13 del experimento con entre 3.4×10^9 y 3.2×10^{11} unidades formadoras de colonia de *C. perfringens* en el alimento durante las 24 horas del día. Se evaluaron las lesiones en dos aves por corral, sacrificadas el día 17 y en cualquier ave muerta durante los 25 días del estudio. La tasa de crecimiento se evaluó hasta los 25 días y se registró la mortalidad a lo largo del experimento. Solo el aislamiento SNCCP50 produjo mortalidades por enteritis necrótica significativamente diferentes al control ($P \leq 0.05$). Los cinco aislamientos fueron a su vez tipificados utilizando electroforesis en gel de campo pulsante para evaluar su asociación genética. Todos los aislamientos no relacionados epidemiológicamente resultaron no relacionados genéticamente, mientras que los aislamientos SNECP43 y 50 difirieron solo en una pequeña banda. El tipo de toxina se evaluó utilizando la prueba de reacción en cadena por la polimerasa, que también fue utilizada para la detección del gen que codifica para la toxina β2.

Key words: *Clostridium perfringens*, broiler chicken, necrotic enteritis, pulsed-field gel electrophoresis, disease model

Abbreviations: CFU = colony forming units; MLF = Maple Leaf Foods; OD_{600} = optical density at 600 nm; PCR = polymerase chain reaction; PFGE = pulsed-field gel electrophoresis; TE = 10 mM Tris, 100 mM EDTA buffer

Clostridium perfringens is a gram-positive, spore-forming, anaerobic bacillus that is found in soil and in the intestines of humans and animals (26). It is considered to be the causative agent of necrotic enteritis in birds, a disease that is characterized by reduced growth performance, decreased feed efficiency, and depression in its mild form and by anorexia, severe morbidity, and significant mortality at its worst (2,26). Because of its effect on growth performance and mortality, necrotic enteritis is a disease of economic significance to the broiler chicken industry (1). Factors that predispose commercial flocks to an outbreak of necrotic enteritis appear to be high protein, high fiber, or wheat-based diets and infection with coccidia (3,30). Control of necrotic enteritis has historically consisted of minimizing predisposing factors and application of antimicrobial agents in bird feed or water at levels shown to be preventive (5,9,13,14,18,23,24). Recently, inclusion of antimicrobial agents in animal feeds has come under considerable scrutiny, with many countries legislating against the practice (6). As a result, outbreaks of necrotic enteritis have become more frequent under antibiotic-free conditions than when antibiotics were fed at subtherapeutic levels, and it has again become an important disease in the poultry industry (29). Much research has been devoted to alternative, nondrug preventive therapies, which have required model methods to reliably reproduce the disease (16,20,21,30). How representative an isolated strain of *C. perfringens*

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used in a disease model is of field strains that cause significant disease and mortality in the field has yet to be examined. The objective of this study was to identify the potential of *C. perfringens* strains isolated from commercial flocks raised without antibiotics to cause necrotic enteritis under typical Canadian commercial broiler flock conditions and to investigate any genetic differences and similarities between these strains using pulsed-field gel electrophoresis (PFGE). Toxin typing of the *C. perfringens* strains was performed by polymerase chain reaction (PCR), including the β2-toxin gene *cpb2* (10). *Clostridium perfringens* plasmids are known to carry many of the significant toxin genes (22), and so, plasmid preparations of SNECP43 and 50 were also compared.

MATERIALS AND METHODS

***C. perfringens* isolates.** Five isolates of *C. perfringens* (SNECP43, 44, 47, 49, and 50) were recovered from the surfaces of the intestinal mucosa of birds that died of necrotic enteritis during four different outbreaks in commercial antibiotic-free flocks in the southwestern Ontario area between 2001 and 2005. They had been isolated from birds with intestinal lesions typical of necrotic enteritis and with symptoms ranging from minimal to significant mortality.

SNECP44, 47, and 49 isolates were cultured from epidemiologically unrelated cases of necrotic enteritis at three different farms in 2005. SNECP43 and 50 were derived from an isolate of a field case in 2001. The original isolate was passaged several times *in vitro* and kept frozen at −60 C as SNECP43; this isolate is identical to the isolate known as CP4 described in a study by Thompson *et al.* (28). CP4 has also been recently used in an immunology study to test the recognition of *C. perfringens* antigens (19). SNECP50 was isolated from a bird successfully challenged with the original strain in 2004. The other three isolates were chosen for their association with typical necrotic enteritis lesions shown in their respective field cases and were intended to give a high probability of effectively recreating the disease. All strains were subcultured and frozen at −20 C in a cooked meat medium (Difco, Becton Dickinson, Sparks, MD) until the start of the project.

Positive controls AHL155 and AHL156 for PCR toxin typing, *cpe* and *cpb2* detection, were obtained from the Animal Health Laboratory at the University of Guelph.

Experimental design. The study used a completely randomized design with a one-way comparison of five treatments. Sixteen pens of 25 birds each were used. Three pens were administered one of the following strains: SNECP43, SNECP44, SNECP47, SNECP49, or SNECP50. One pen was not treated and served as the control. Statistical comparisons were made between the 15 treatment pens and a single control pen using key variables for comparison, i.e., cumulative mortality by day, cumulative mortality due to necrotic enteritis by day, and average lesion score on day 17.

Study system. A total of 400 male day-old broiler chickens were assigned to treatment, with 25 chicks placed in each pen on day 0. Birds were vaccinated for Marek's disease and bronchitis (Mildvac-M®, Intervet Canada Ltd., Whitby, Canada) at the hatchery. Birds that died or were culled before noon on day 5 were replaced with extra birds, and the total number of birds purchased from a commercial hatchery allowed for replacement of early mortality.

The poultry research facility at Maple Leaf Foods (MLF) Agresearch in Burford, Ontario, Canada, was used to conduct the study. The 16 pens, each providing about 13.7 m^2 of floor space, were randomly assigned by block to treatment groups. Birds were randomly assigned to pens by block, such that each hatchery box contributed an approximately equal number of birds to each treatment within each block.

Each pen had a concrete floor and nylon-mesh partitions supported by a polyvinyl chloride (PVC) frame. A solid 12-inch-high plastic barrier at bird level separated adjacent pens. A welded-wire fence with 2.54-cm^2 openings was located on top of all barriers. Each pen was permanently identified with a number and provided about 0.55 m^2/bird. Five natural-gas heaters that were equally spaced and positioned to warm incoming air at the north wall of the building heated the barn, and fans located on the south-facing wall of the building exhausted air. Each pen contained four nipple-type drinkers that provided clean drinking water *ad libitum*. Dry feed was provided *ad libitum* in tube-type feeders (1/pen) of 20-kg capacity. The barn was cleaned, washed, and disinfected before placement of new chopped-straw bedding. The lighting program, barn temperature, litter type, and other management practices were typical of commercial broiler chicken producers in the local geographic area. Birds that were moribund and unable to reach feed and water were culled and euthanatized by asphyxiation with carbon dioxide gas. This protocol was approved by the MLF Agresearch Animal Care Committee.

One feed, MLF Agresearch Standard nonmedicated starter chick feed in crumble form, was fed through the entire study (formulation confidential). The diet contained 25% wheat to encourage necrotic enteritis development and was based on corn–soybean meal. The diet was formulated to commercial nutritional standards for chicks from 0 to 25 days of age.

***C. perfringens* challenge.** Frozen cultures of *C. perfringens* were thawed and 0.5 ml was used to inoculate 43 ml of reduced thioglycolate media (Difco) and incubated overnight at 37 C. Each vial of thioglycolate was then added to 430 ml cooked-meat medium (Difco) and, again, incubated overnight under the same conditions. Finally, 13 liters of thioglycolate + 1% starch (Difco) was inoculated with the entire cooked-meat media preparation and held at 37 C for 8 hr until added to the feed. Final concentrations in colony-forming units (CFU) per milliliter at the time of their addition to feed were determined with serial dilutions on blood agar plates. Mean CFUs of bacteria consumed were calculated as total inoculum consumed per pen divided by the number of birds in each pen, multiplied by the bacterial concentration values determined from the serial dilutions, resulting in the following per-treatment: SNECP43, 3.4×10^9; SNECP44, 6.9×10^{10}; SNECP47, 3.2×10^{11}; SNECP49, 1.3×10^{11}; and SNECP50, 1.8×10^{10}. Feed was withdrawn from all birds for approximately 8 hr before the first introduction of challenge. Inoculum was administered to birds *via* feed *ad libitum* in trough-type, disposable feeders commencing the afternoon of day 13 and ending the afternoon of day 14. The feeder in the control pen was not removed during the challenge period. When administration of the challenge was completed for all pens assigned to the challenge, the disposable feeders were removed, and regular feed returned to the challenged pens. Inoculated feed remaining was weighed to ascertain amount of challenge feed consumed.

Growth performance, mortalities, and lesion scoring. Pen average body weights were recorded at the beginning and end of the experiment on days 0 and 25, respectively. Feed consumption was recorded from day 0 to 25 as well, and feed conversion derived by dividing average feed consumed per pen by average weight gain per pen.

All birds that died after noon on day 5 were tagged with a unique number that was recorded, along with date, bird weight and pen number. Necropsies were performed to diagnose cause of death in all mortalities. Blood and clinical analyses were not performed but would be of interest to complement these data in future investigations. Any birds that were diagnosed with necrotic enteritis were scored grossly for necrotic enteritis (4,24) and coccidiosis (15). Lesion scoring was performed as follows:

> 0 = Small intestine was grossly normal.
>
> 1 = Small intestine wall was grossly thinner than normal and broke or tore easily under mild tension but had no gross evidence of mucosal necrosis or other abnormalities.
>
> 2 = One or more focal round or oval areas of acute full thickness mucosal necrosis of the small intestine. These foci varied in diameter from approximately 1 to 5 mm. The surface of these lesions was generally raised above the surrounding tissue and consisted of grey or white necrotic debris. Alternatively, if the superficial necrotic material had been removed, the lesions were slightly depressed and were grey or white.

3 = Irregularly shaped confluent areas of full thickness mucosal necrosis of the small intestine >5 mm in diameter but affecting <25% of the small intestine surface area. The surface of these lesions was generally raised above the surrounding tissue and consisted of orange/brown necrotic debris. In some cases, where portions of the superficial necrotic material had been removed, the lesions were slightly depressed from surrounding tissue.

4 = Large confluent areas of full thickness mucosal necrosis of the small intestine affecting 25% or more of the small intestinal surface area and involving the entire internal circumference of the affected small bowel. The surface of these lesions was generally raised above the surrounding tissue and consisted of orange/brown necrotic debris.

For all birds that died or were sacrificed postchallenge, the intestine was opened and examined for lesions and were scored if present. A portion of the unopened intestine next to the intestinal tissue containing lesions was removed and placed in a separate preweighed and labeled sterile plastic sample bag. The air was removed from each sterile sample bag before being chilled at about 4 C until cultured anaerobically to verify the presence of *C. perfringens*.

***C. perfringens* culture.** Unopened intestine was removed from the sterile plastic container, opened, and the intestinal contents removed. An area with necrosis was selected, sampled with a sterile loop, and the sample cultured anaerobically on blood agar media and incubated at 37 C for 18 hr; the sterile loop sample was streaked on the blood agar plate into four successive quadrants, sterilizing the loop between quadrants.

Identification and quantification of *C. perfringens*. A sample from the same necrotic area as that cultured on blood agar was streaked onto a microscopic slide and stained using Gram stain. The number of gram-positive rods (magnification 1000×) were counted in a single microscopic field and graded as 0, no gram-positive rods; 1, 1–10 gram-positive rods; 2, 11–20 gram-positive rods; 3, 21–30 gram-positive rods; 4, >30 gram-positive rods.

Colonies on blood agar plates were identified as *C. perfringens* if they exhibited double-zone hemolysis (11). The number of colonies meeting these criteria were counted within each quadrant, and the density was scored as 4+, 1 or more colonies in the fourth quadrant; 3+, 0 colonies in the fourth quadrant, 1–10 in the third quadrant; 2+, 0 colonies in the third and fourth quadrants, 1–10 colonies in the second quadrant; 1+, 0 colonies in the second, third, and fourth quadrants, 1–10 in the first quadrant; 0, no colonies in any quadrant.

Pulsed-field gel electrophoresis of isolates. SNECP43, 44, 47, 49, and 50 strains were grown overnight on blood agar plates, at 37 C under anaerobic conditions. A homogenous suspension of bacteria was embedded in 0.8% SeaKem Gold Agarose (Cambrex Bio Science Rockland, Rockland, ME) 1-mm-thick plugs to obtain a final optical density at 600 nm (OD_{600}) of 1.25. Plugs were incubated in 10 mM Tris, 100 mM EDTA (TE) buffer with 50 mg/ml lysozyme (Roche Applied Science, Mannheim, Germany) with gentle shaking at 37 C for 5 hr. They were then rinsed for 15 min in TE buffer and subsequently incubated overnight in EDTA 0.5 M, 1% sarkosyl (Fisher Scientific, Fair Lawn, NJ), 2 mg/ml proteinase K (Roche Applied Science), pH 8.0 at 50 C with gentle shaking. They were then rinsed five times each for 30 mins in 10 mM Tris, 1 mM EDTA, pH 8.0 to remove traces of proteinase K.

One plug per isolate was equilibrated in 200 µl restriction buffer at room temperature for 20 min and was then incubated at room temperature for 5 hr in 200 µl fresh digestion buffer containing 100 U of the restriction enzyme *Sma*I (New England BioLabs, Ipswich, MA) following the manufacturer's recommendations. Electrophoresis was performed in a 1% SeaKem Gold Agarose gel with TE buffer. Gels were run in 0.5× Tris-borate-EDTA (Fisher Scientific) containing 200 µM thiourea (Fisher Scientific) at 14 C for 19 hr. Pulse times started at 4 sec and ended at 38 sec with linear ramping and a field of 6 V/cm and an angle of 120° in a Bio-Rad (Hercules, CA) CHEF-III electrophoresis unit. Gels were stained with ethidium bromide (Fisher Scientific) in Tris–borate–EDTA buffer and analyzed using BioNumerics software v4.0 (Applied Maths, Austin, TX). Band matching was performed using a 0.5% position tolerance, and cluster analysis was performed using the Dice similarity coefficient and unweighted pair group method with arithmetic mean.

Table 1. Effect of *C. perfringens* strain on least-square mean body weights (kg) and feed conversion (kg feed/kg gain) of broiler cockerels shown with standard error of the mean (SEM) and the *P* value of the effect.

Treatment	Number of pens	Weight		Feed conversion day 0–25
		Day 0	Day 25	
0 (control)	1	0.040	1.05[ab]	1.72
1 (SNECP43)	3	0.047	1.07[a]	1.56
2 (SNECP44)	3	0.043	1.00[bc]	1.58
3 (SNECP47)	3	0.050	1.05[a]	1.60
4 (SNECP49)	3	0.040	1.04[ab]	1.60
5 (SNECP50)	3	0.047	0.97[c]	1.67
SEM for group 0		0.004	0.03	0.04
SEM for groups 1–5		0.003	0.02	0.02
P value		0.164	0.013	0.066

[a–c]Means within the same column that have different letters are significantly different according to Student *t*-test at $P \leq 0.05$

PCR and plasmids. Each *C. perfringens* isolate was toxin typed according to Yoo and collaborators (31). In addition, detection of the *cpb2* gene was performed according to Herholz and collaborators (12). Plasmid preps of SNECP43 and SNECP50 were obtained using a QIAGEN Plasmid Mini Kit (QIAGEN Inc., Valencia, CA) with a supplementary initial lysozyme treatment (50 mg/ml) for 3 hr with shaking at 37 C. Plasmid preps were compared visually using a 0.6% UltraPure agarose gel (Invitrogen, Carlsbad, CA).

Statistical analysis. Body weight, lesion scores, and mortality data were analyzed using one-way analysis of variance with *C. perfringens* strain as the sole source of variation using the statistical analysis package JMP Version 5.1.1 (SAS Institute, Cary, NC). Feed conversion (kg feed/kg gain) was analyzed with and without body weight at 25 days included as a covariant in the analysis of variance. Mortality data were transformed in Excel (Microsoft Corporation, Redmond, WA) using arcsin $(\text{mortality})^{0.5}$ as recommended by Steel and Torrie (27) for proportions over a wide range of values. Means within sources of variation that were significant ($P \leq 0.05$) were examined for differences using Student *t*-tests. Linear relationships between measurements were determined using Pearson correlations.

RESULTS

Growth performance. Mean body weights between the treatments were not significantly different at day 0 but were at day 25 ($P = 0.013$) (Table 1). The effect of treatment on feed conversion from 0 to 25 days was close to significant ($P = 0.066$), with the control birds and treatment 5 (SNECP50) tending to require more feed per kg gain than the treatment 1 birds (SNECP43) (Table 1). Inclusion of body weight at 25 days as a covariant in the analysis of variance reduced the significance of this trend but did not eliminate it completely ($P = 0.16$).

Mortality and lesion scores. Culture and smear scores for mortalities from treatments SNECP43, 44, 47, and control were all zero. SNECP49 mortalities had an average culture and smear score of 1.3, whereas SNECP50 had average culture and smear scores of 3.6. Birds that received SNECP50 (treatment 5) had a greater percentage of mortality due to necrotic enteritis than all other treatments ($P = 0.0005$), which did not differ from each other (Table 2). Lesion scores of the necrotic enteritis mortalities revealed

Table 2. Effect of *C .perfringens* strain on mean lesion score of sacrificed birds and mortality [% adjusted with arcsin (percent mortality)$^{0.5}$] of broiler cockerels shown with the standard error of the mean (SEM) and the *P* value of the effect.

Treatment	Number of pens	Lesion score	Necrotic enteritis mortalities (%)	Total mortality (%)
0 (control)	1	1.00[ab]	0[a]	0[a]
1 (SNECP43)	3	0.33[b]	0[a]	7.7[a]
2 (SNECP44)	3	1.17[ab]	0[a]	3.8[a]
3 (SNECP47)	3	0.33[b]	0[a]	3.8[a]
4 (SNECP49)	3	0.33[b]	3.8[a]	7.7[a]
5 (SNECP50)	3	1.83[a]	33.7[b]	35.8[b]
SEM for group 0		0.48	6.5	7.4
SEM for groups 1 to 5		0.26	3.8	4.3
P value		0.017	0.0005	0.002

[a,b]Means that have different superscripts within the same column are significantly different according to Student *t*-test at $P \leq 0.05$.

that birds that died after receiving SNECP50 had a mean lesion score of approximately 3.5. Mortalities for the SNECP43, 44, 47 treatments and the control had mean lesion scores of zero, whereas SNECP49 had an average of 1.3 (because of a single lesion score of 4). Sacrificed birds receiving SNECP50 (treatment 5) had greater mean lesion scores than those that received inoculums SNECP43, 47, and 49 but similar to those of the control and SNECP44 birds (Table 2). Treatment 1 with isolate SNECP43 did not produce lesions or mortalities significantly different from that found in the unchallenged birds (control). SNECP44, 47, and 49 also did not produce lesions significantly different from the control birds.

Correlations. Pearson correlations between body weights, feed conversion, mortality, and lesion scores indicated that increased lesion scores were related to reduced body weight at 25 days and increased mortality, that feed conversion increased as mortality and lesion scores increased, and that total mortality was positively correlated with lesion score and necrotic enteritis mortality (Table 3).

Identification and quantification of *C. perfringens*. All necrotic enteritis mortalities had culture and smear scores equal to or greater than 3, indicating that large amounts of *C. perfringens* were present in the intestine of the birds that died.

PFGE typing. The PFGE profiles of SNECP44, 47, and 49 differed significantly from each other by at least eight bands (Fig. 1) and were not considered closely genetically related. SNECP43 and 50 differed by only a single minor band and are, therefore, considered genetically related. Three repeated restriction enzyme digests of these two isolates were performed using three different plug preparations, and this single low-intensity band difference was consistently visible in all replicates.

PCR and plasmids. All 5 isolates were identified as toxin type A by PCR analysis. The β2-toxin gene (*cpb2*) was not detected in any of the isolates. Lastly, there was no discernable difference in plasmid profile between SNECP43 and 50 after agarose gel electrophoresis (data not shown).

DISCUSSION

Two treatments, the control and SNECP50, tended to have increased feed conversion ratios, indicating that the birds with these treatments consumed more feed relative to each kg weight gained than birds in the other treatments ($P = 0.066$). An increase in the feed conversion ratio was expected in broiler chickens with necrotic enteritis because inflammation of the intestine during the infection slows transfer of nutrients across the gut lining and reduces growth. This was supported by the correlations observed between feed conversion, body weight at 25 days, lesion score, and mortality (Table 3). The trend for the control and SNECP50-treated birds to have increased mean feed conversion ratios was not because of body weight because it remained even after the inclusion of body weight at 25 days as a covariant in the analysis ($P = 0.16$). The increased feed conversion ratio of the birds administered strain SNECP50 was likely indicative of the increased virulence of the strain. However, the increased feed conversion ratio of the control birds could possibly reflect a natural necrotic enteritis infection, as one of the control birds exhibited ileal necrotic enteritis lesions, or could also be due to other etiological factors within the group. Unfortunately, *C. perfringens* isolates from these birds were not available for PFGE typing to confirm this hypothesis.

Mean lesion scores for birds sacrificed from the SNECP43, 47, and 49 strain treatments were the lowest at 0.33 and were significantly lower than the mean lesion score of birds sacrificed from SNECP50 (Table 2); however, the mean lesion score of sacrificed birds from SNECP50 was not different from that of the controls because one of the two control birds exhibited necrotic lesions from a natural infection. Having only two samples in the control group, one of which had a natural infection of necrotic enteritis, skewed the mean lesion score for the control birds upwards. The lesion scores of the necrotic enteritis mortalities was the best indicator of strain virulence and revealed that birds that died after receiving SNECP50 had a mean lesion score of about 3.5.

Treatment with SNECP50 provided the highest number of necrotic enteritis mortalities, 33.7% (Table 2). This isolate proved most capable of reproducing the disease in a challenge experiment, whereas SNECP43, 44, and 47 produced no mortalities clearly attributable to necrotic enteritis. The difference in lethality between the SNECP43 and 50 clones is highly significant and displays the strong effect that *in vitro* subculturing *vs. in vivo* passage had on attenuating the virulence of this particular strain. It remains unclear in necrotic enteritis outbreaks what the most important factors are for inducing disease, but differences in the genetic regulation of many genes including potential virulence factors may play a role; this has been recently supported by Keyburn and collaborators (17) who found that the alpha toxin is not necessarily the most important

Table 3. Correlation coefficients for correlations between growth performance parameters, lesion score, and mortality (*P* values of correlations in parentheses).

	Variable	BW0	BW25	FC	LS	NEM
BW0	Body weight (kg) 0 days					
BW25	Body weight (kg) 25 days	−0.047 (0.864)				
FC	Feed conversion (kg feed/kg gain)	−0.249 (0.352)	−0.38 (0.147)			
LS	Lesion score	−0.089 (0.744)	−0.627 (0.009)	0.336 (0.204)		
NEM	NE mortality %	−0.013 (0.962)	−0.532 (0.034)	0.581 (0.018)	0.643 (0.007)	
TM	Total mortality %	0.177 (0.513)	−0.458 (0.074)	0.402 (0.123)	0.65 (0.006)	0.926 (<0.0001)

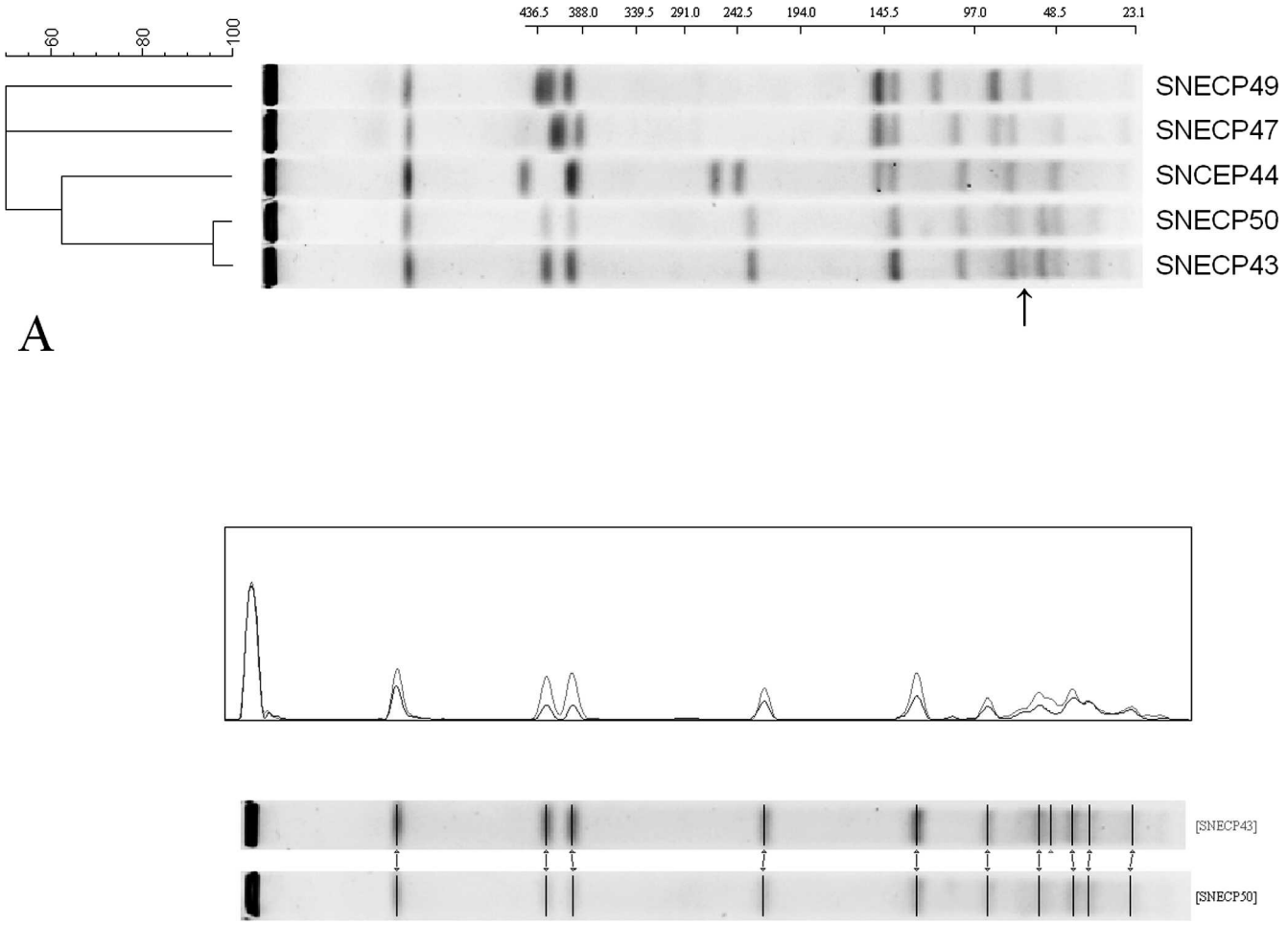

Fig. 1. Genetic fingerprinting of the five challenge strains of *C. perfringens*, using PFGE (arrow indicates the single band difference between SNECP43 and 50) in both (A) banding patterns and (B) densitometric curves; approximate molecular weight displayed above figures in kilobase pairs.

factor for necrotic enteritis development. It is not possible on the basis of the PFGE patterns to determine whether a critical virulence factor was lost from SNECP43 or whether it is simply a matter of gene regulation that created the difference in lethality. The small genetic variation discovered between these two isolates was a single weak band of approximately 60 kb appearing only in the SNECP43 restriction digest. This difference reflects a genetic modification between the isolates, and it is unclear whether the mutation was responsible for the differences in pathogenicity observed. However, PFGE analysis suggests that closely related isolates of the same clone may strongly vary in their pathogenicity potential and that *in vitro* passage *vs. in vivo* passage may be responsible for this difference.

Because most *C. perfringens* toxins are known to be plasmid borne, differences in plasmid profile were assessed, but no visible difference was observed. It has been well documented that plasmids can be lost through subculturing in a laboratory environment, which can lead to the loss of genes encoding toxins and bacteriocins, as well as antimicrobial resistance genes (25). To the best of our knowledge, there has been no research involving the attenuation of *C. perfringens* through *in vitro* subculture passages and assessing changes in virulence using *in vivo* models. The mechanisms for any loss of virulence through subculturing of *C. perfringens* are, therefore, still not well understood.

There is a body of evidence to suggest that the β2-toxin of *C. perfringens* is associated with many animal (8,10) and human (7) gastrointestinal diseases. However, a PCR specific for *cpb2* yielded negative results for all five isolates from necrotic enteritis outbreaks in this study, including SNECP50. Furthermore, this gene was found in a large number of *C. perfringens* isolates from healthy broiler chickens performed in our laboratory (unpubl. data). These findings, in agreement with results from Thompson and collaborators (28), give evidence that the newly described *cpb2* gene may not be a necessary factor for necrotic enteritis in broiler chickens.

In summary, only one of the five *C. perfringens* challenge isolates (SNECP50) from necrotic enteritis outbreaks was able to consistently reproduce the disease in this experimental model. This isolate caused 33.7% necrotic enteritis-associated mortalities in the challenged birds, and produced an average lesion score of 3.5 in the diseased birds. All of the other isolates taken from field cases of necrotic enteritis were unable to produce significant mortalities and would not be suitable for use in future challenge studies with this model when mortality is a parameter of interest. PCR and PFGE

analysis helped to further characterize these isolates and revealed the absence of the *cpb2* toxin gene as well, thus questioning its importance in necrotic enteritis development. The difference in virulence between SNECP43 and SNECP50 demonstrates that a high degree of genetic relatedness does not necessarily imply analogous virulence in a challenge model. Thus, because of the current absence of other specific virulence attributes, the virulence level of *C. perfringens* strains should not be predicted based on genetic relatedness to known virulent strains but only upon testing in an adequate *in vivo* challenge model.

REFERENCES

1. Agriculture and Agri-Food Canada. Poultry marketplace—poultry at a glance. Agriculture and Agri-Food Canada, Ottawa, Ontario. p. 1. 2005.
2. Al-Sheikhly, F., and R. B. Truscott. The interaction of *Clostridium perfringens* and its toxins in the production of necrotic enteritis of chickens. Avian Dis. 21:256–263. 1977.
3. Branton, S. L., B. D. Lott, J. W. Deaton, W. R. Maslin, F. W. Austin, L. M. Pote, R. W. Keirs, M. A. Latour, and E. J. Day. The effect of added complex carbohydrates or added dietary fiber on necrotic enteritis lesions in broiler chickens. Poult. Sci. 76:24–28. 1997.
4. Brennan, J., G. Moore, S. E. Poe, A. Zimmermann, G. Vessie, D. A. Barnum, and J. Wilson. Efficacy of in-feed tylosin phosphate for the treatment of necrotic enteritis in broiler chickens. Poult. Sci. 80:1451–1454. 2001.
5. Brennan, J., J. Skinner, D. A. Barnum, and J. Wilson. The efficacy of bacitracin methylene disalicylate when fed in combination with narasin in the management of necrotic enteritis in broiler chickens. Poult. Sci. 82:360–363. 2003.
6. Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52:159–161. 2003.
7. Fisher, D. J., K. Miyamoto, B. Harrison, S. Akimoto, M. R. Sarker, and B. A. McClane. Association of beta2 toxin production with *Clostridium perfringens* type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene. Mol. Microbiol. 56:747–762. 2005.
8. Garmory, H. S., N. Chanter, N. P. French, D. Bueschel, J. G. Songer, and R. W. Titball. Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. Epidemiol. Infect. 124:61–67. 2000.
9. George, B. A., C. L. Quarles, and D. J. Fagerberg. Virginiamycin effects on controlling necrotic enteritis infection in chickens. Poult. Sci. 61:447–450. 1982.
10. Gibert, M., C. Jolivet-Reynaud, and M. R. Popoff. Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. Gene 203:65–73. 1997.
11. Hansen, M. V., and L. P. Elliott. New presumptive identification test for *Clostridium perfringens*: reverse CAMP test. J. Clin. Microbiol. 12:617–619. 1980.
12. Herholz, C., R. Miserez, J. Nicolet, J. Frey, M. Popoff, M. Gibert, H. Gerber, and R. Straub. Prevalence of beta2-toxigenic *Clostridium perfringens* in horses with intestinal disorders. J. Clin. Microbiol. 37:358–361. 1999.
13. Hofacre, C. L., T. Beacorn, S. Collett, and G. Mathis. Using competitive exclusion, mannan–oligosaccharide and other intestinal products to control necrotic enteritis. J Appl. Poult. Res. 12:60. 2003.
14. Jackson, M. E., D. M. Anderson, H. Y. Hsiao, G. F. Mathis, and D. W. Fodge. Beneficial effect of beta-mannanase feed enzyme on performance of chicks challenged with *Eimeria* sp. and Clostridium perfringens. Avian Dis. 47:759–763. 2003.
15. Johnson, J., and W. M. Reid. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. Exp. Parasitol. 28:30–36. 1970.
16. Kaldhusdal, M., M. Hofshagen, A. Lovland, H. Langstrand, and K. Redhead. Necrotic enteritis challenge models with broiler chickens raised on litter: evaluation of preconditions, *Clostridium perfringens* strains and outcome variables. FEMS Immunol. Med. Microbiol. 24:337–343. 1999.
17. Keyburn, A. L., S. A. Sheedy, M. E. Ford, M. M. Williamson, M. M. Awad, J. I. Rood, and R. J. Moore. Alpha-toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens. Infect. Immun. 74:6496–6500. 2006.
18. Kondo, F. In vitro lecithinase activity and sensitivity to 22 antimicrobial agents of *Clostridium perfringens* isolated from necrotic enteritis of broiler chickens. Res. Vet. Sci. 45:337–340. 1988.
19. Kulkarni, R. R., V. R. Parreira, S. Sharif, and J. F. Prescott. *Clostridium perfringens* antigens recognized by broiler chickens immune to necrotic enteritis. Clin. Vaccine Immunol. 13:1358–1362. 2006.
20. McReynolds, J. L., J. A. Byrd, R. C. Anderson, R. W. Moore, T. S. Edrington, K. J. Genovese, T. L. Poole, L. F. Kubena, and D. J. Nisbet. Evaluation of immunosuppresants and dietary mechanisms in an experimental disease model for necrotic enteritis. Poult. Sci. 83:1948–1952. 2004.
21. Olkowski, A. A., C. Wojnarowicz, M. Chirino-Trejo, and M. D. Drew. Responses of broiler chickens orally challenged with *Clostridium perfringens* isolated from field cases of necrotic enteritis. Res. Vet. Sci. 81:99–108. 2006.
22. Petit, L., M. Gibert, and M. R. Popoff. *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol. 7:104–110. 1999.
23. Prescott, J. F. The prevention of experimentally induced necrotic enteritis in chickens by avoparcin. Avian Dis. 23:1072–1074. 1979.
24. Prescott, J. F., R. Sivendra, and D. A. Barnum. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. Can. Vet. J. 19:181–183. 1978.
25. Rood, J. I., and S. T. Cole. Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol. Rev. 55:621–648. 1991.
26. Songer, J. G. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 9:216–234. 1996.
27. Steel, R. G. D., and J. H. Torrie. Principles and procedures of statistics, second edition. McGraw-Hill Book Company, New York, New York. p. 236. 1980.
28. Thompson, D. R., V. R. Parreira, R. R. Kulkarni, and J. F. Prescott. Live attenuated vaccine-based control of necrotic enteritis of broiler chickens. Vet. Microbiol. 113:25–34. 2005.
29. Van Immerseel, F., J. De Buck, F. Pasmans, G. Huyghebaert, F. Haesebrouck, and R. Ducatelle. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathol. 33:537–549. 2004.
30. Williams, R. B., R. N. Marshall, R. M. La Ragione, and J. Catchpole. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis, coccidiosis and anticoccidial vaccination of chickens. Parasitol. Res. 90:19–26. 2003.
31. Yoo, H. S., S. U. Lee, K. Y. Park, and Y. H. Park. Molecular typing and epidemiological survey of prevalence of *Clostridium perfringens* types by multiplex PCR. J. Clin. Microbiol. 35:228–232. 1997.

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