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Quantification of Cell Proliferation and Alpha-Toxin Gene Expression of *Clostridium perfringens* in the Development of Necrotic Enteritis in Broiler Chickens[▽]

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Cell proliferation and alpha-toxin gene expression of *Clostridium perfringens* in relation to the development of necrotic enteritis (NE) were investigated. Unlike bacitracin-treated chickens, non-bacitracin-treated birds exhibited typical NE symptoms and reduced growth performance. They also demonstrated increased *C. perfringens* proliferation and alpha-toxin gene expression that were positively correlated and progressed according to the regression model $y = b_0 + b_1X - b_2X^2$. The average *C. perfringens* count of 5 $\log_{10}$ CFU/g in the ileal digesta appears to be a threshold for developing NE with a lesion score of 2.

Necrotic enteritis (NE) is an enteric disease in poultry caused predominantly by *Clostridium perfringens* type A strains and to a lesser extent type C strains (6, 13, 19). Type A strains produce only alpha-toxin as the major toxin, and alpha- and beta-toxins are the two major toxins produced by type C strains (19). The disease is thought to occur when a pathogenic strain of *C. perfringens*, which is normally part of intestinal microbiota, overgrows in the small intestine and produces extracellular toxins that damage the intestine (9, 12, 18). NE can appear in 2- to 5-week-old broilers (7), but outbreaks typically happen around 17 to 18 days of age (17). Although substantial evidence supports the role of alpha-toxin in the pathogenesis of NE (1, 2, 8), a recent report with alpha-toxin-defective mutants has questioned the importance of alpha-toxin (10). Therefore, more studies are required to clarify the importance of alpha-toxin and other factors in NE pathogenesis. The present study was undertaken to quantify the relationship between cell proliferation and alpha-toxin gene expression of *C. perfringens* in the development of NE. To this end, a specific quantitative PCR assay, including reverse transcription (RT) and real-time PCR (Q-RT-PCR), was developed to measure alpha-toxin gene expression, and an experimental model for chicken infection with *C. perfringens* (3, 4, 5) was used.

Bacteria. A type A strain of *C. perfringens* routinely used to induce NE in broiler chickens at Nutreco Canada Agresearch by following an established NE infection model (3) was used for this study. The bacterium was grown in Mueller-Hinton broth or on Mueller-Hinton agar containing 5% (vol/vol) sheep blood at 37°C under an anaerobic atmosphere (85% N_2, 10% CO_2, and 5% H_2). *Escherichia coli* DH5α harboring plasmid pGEM-T with an alpha-toxin gene (*cpa*) from type A *C. perfringens* strain D32124 was from J. Prescott (University of Guelph, Guelph, Canada).

Chicken trial. Broiler chickens (Ross × Ross) were reared by following the guidelines of the Canadian Council on Animal Care (15). Six-hundred-day-old chicks in 12 pens (50 birds/pen) were assigned in equal proportions to one of two dietary treatments: (i) a typical all-vegetable starter diet (Shur-Gain; Nutreco Canada) with zinc bacitracin (55 mg/kg) or (ii) the same diet without bacitracin. The first day of the trial was designated day 0. On day 18, birds were challenged for 16 h with C. *perfringens* (10^7 CFU/ml) through the diet after 8 h of starvation (3). Before the challenge, 12 birds from each treatment group (two birds/pen) were randomly selected and euthanized with CO_2. The sampling was repeated after the challenge for 4 days (days 19 to 22). Ileal digesta were collected from each bird. Digesta (0.25 g) for RNA extraction was mixed, immediately after dissection, with 1.75 ml RNAlater (Ambion, Austin, TX) and stored on ice for subsequent processing. NE lesion in the small intestine was monitored and scored for the last 3 days (days 20 to 22) of the trial as described elsewhere (16). Chicken growth performance, including body weight and feed intake, was recorded weekly prior to clostridial challenge and daily after the challenge. *C. perfringens* in the digesta was enumerated by plating (12).

RNA preparation. Each digesta sample in RNAlater was aliquoted into two Eppendorf tubes (1 ml each), and the volume was brought to 2 ml with phosphate-buffered saline buffer (pH 7.4). The digesta were recovered by centrifugation (20,000 relative centrifugal force, 20 min), quickly frozen in liquid nitrogen, and then stored at −80°C until extraction. Total RNA was extracted from ileal digesta with a RiboPure bacterial RNA isolation kit (Ambion) according to the manufacturer's instructions except for using 1 ml of lysis buffer in each tube, the application of bead beating to lyse bacterial cells as

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TABLE 2. Relationships of cell proliferation and alpha-toxin gene expression of *C. perfringens* in the ileal digesta of chickens on diets with or without bacitracin during the development of NE[a]

Diet	Post challenge day[b]	No. of infected birds[c]	Log CFU/g (avg ± SD)[d]	No. of birds with lesions	Score (avg ± SD)	Alpha-toxin mRNA (log N/μg; avg ± SD)[e]
Nonmedicated	0	2	0.8 ± 2.0	ND	ND	0
	1	12	7.3 ± 1.7	ND	ND	5.1 ± 3.4
	2	10	6.7 ± 3.2	8	1.3 ± 1.0	7.4 ± 3.4
	3	10	6.2 ± 3.1	5	0.9 ± 1.2	6.2 ± 3.6
	4	6	4.1 ± 4.4	5	0.8 ± 1.0	3.7 ± 4.4
Medicated	0	1	0.5 ± 1.8	ND	ND	0
	1	0	0	ND	ND	0
	2	1	0.4 ± 1.3	0	0	0
	3	2	0.8 ± 1.3	1	0.1 ± 0.3	0
	4	3	1.1 ± 1.9	0	0	0

[a] Samples were collected from 12 birds for each treatment. The nonmedicated diet contained no antibiotics; the medicated diet contained zinc bacitracin (55 mg/kg). ND, not determined; SD, standard deviation.
[b] Chickens were challenged with *C. perfringens* on day 18. On postchallenge day 0, samples were collected just before the challenge.
[c] Birds were regarded as infected when *C. perfringens* was detected in the digesta.
[d] Log_{10} CFU per gram of ileal digesta.
[e] N, copy numbers of alpha-toxin gene mRNA (regarded as 0 when the mRNA was not detected).

described previously (11), and two chloroform extractions during RNA preparation. RNA extract was purified using a Turbo DNA-free kit (Ambion).

Q-RT-PCR. cDNA was synthesized from 0.5 μg purified RNA using a Retroscript reverse transcription kit (Ambion). cDNAs were verified by PCR with *cpa*-specific primers (14) and eubacterial universal primers (21).

Real-time PCR was performed on a Stratagene MX4000 thermal cycler with brilliant SYBR green Q-PCR Master Mix (Stratagene, La Jolla, CA). Previously published *C. perfringens cpa* gene-specific primers (cpaF, GCTAATGTTACTGCCGT TGA; cpaR, CCTCTGATACATCGTGTAAG) (14) were experimentally evaluated and used for real-time PCR. cDNA samples were diluted 10-fold, and 1 μl of each diluted sample (containing cDNAs equivalent to 2.5 ng of total RNA) was

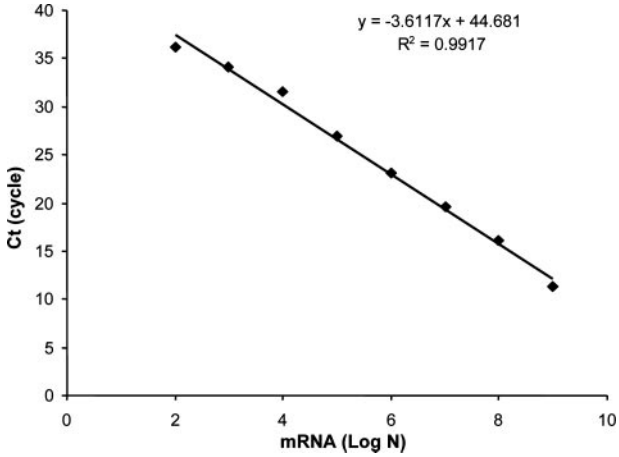

FIG. 1. Representative standard curve showing the relationship between real-time PCR amplification threshold cycle numbers (Ct) and copy numbers of *cpa*. PCR amplification efficiencies were 0.95. Log N, log_{10} copy numbers of alpha-toxin gene mRNA.

TABLE 1. Effect of bacitracin on body weight, daily gain, daily feed intake, and feed conversion ratio of pre- and postchallenge chickens[a]

Diet[b]	Body wt (kg)			Avg daily gain (kg)			Avg daily feed intake (kg)			Feed conversion ratio		
	Day 0[c]	Day 17[d]	Day 22[e]	Prech	Postch	Overall	Prech	Postch	Overall	Prech	Postch	Overall
Nonmedicated	0.045	0.573	0.786	0.031	0.053	0.035	0.047	0.103	0.075	1.436	1.617	1.484
Medicated	0.045 (0.440)	0.560 (0.110)	0.794 (0.400)	0.030 (0.110)	0.059 (0.010)	0.036 (0.410)	0.047 (0.160)	0.109 (0.010)	0.078 (0.069)	1.454 (0.011)	1.532 (0.009)	1.476 (0.340)

[a] Each of the data was generated from six replicates (pens). Values in parentheses are *P* values; *P* values equal to or less than 0.05 are considered significant. Prech, prechallenge; Postch, postchallenge.
[b] The nonmedicated diet contained no antibiotics, while the medicated diet contained zinc bacitracin at 55 mg/kg.
[c] The day that the chicken trial started (after hatch).
[d] The day before *Clostridium* challenge (challenge was performed on day 18).
[e] The last day of the chicken trial.

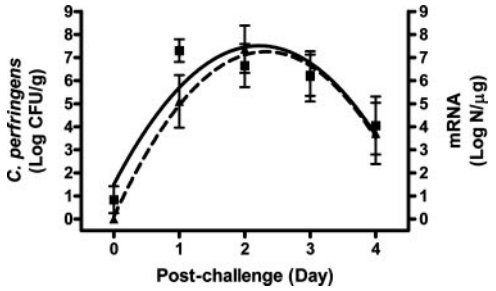

FIG. 2. Progress curves of *C. perfringens* cell proliferation and alpha-toxin gene expression after challenge. Log CFU/g, $\log_{10}$ CFU per gram of ileal digesta (■); N, copy number of alpha-toxin gene mRNA (▲). The solid line represents colony counts of *C. perfringens*, generated from a regression model: $y = 1.50694 + 5.41067x - 1.21876x^2$ ($r^2 = 0.3385$, $n = 60$). The dashed line represents mRNA copy numbers of the alpha-toxin gene, generated from a regression model: $y = 0.10793 + 6.15743x - 1.32615x^2$ ($r^2 = 0.3709$, $n = 58$). All slopes in the equations are significant ($P < 0.0001$). Data points are observed mean values with standard deviation bars.

added in a 25-μl reaction mixture which contained 1× Master Mix, 150 nM of each primer, and 30 nM ROX (6-carboxy-X-rhodamine). The program was 10 min at 95°C, then 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and finally 2 min at 72°C. Fluorescence was measured after each annealing. Recombinant *cpa* in a linear range from 10^2 to 10^9 copies was included as a standard in each run. To mimic digesta samples, the standards also included the same amount of cDNAs generated from samples with no C. *perfringens* colony counts. Tests of samples and standards were repeated three times and arranged on different well positions of the plates. The standard curves were generated by plotting threshold cycles of the standards against $\log_{10}$ copy numbers of *cpa* using GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA). The amplification efficiency was calculated as $-1 + 10^{(-1/\text{slope})}$ (20).

Statistical analyses. Statistical analyses, including *t* test, correlation analysis, and polynomial regression modeling ($y = b_0 + b_1X + b_2X^2$, where y is the colony count or the copy number and X is days postchallenge) were performed using statistical software (SAS version 8.00; SAS Institute Inc., Cary, NC). Since NE score is a discrete variable, effects of the colony counts and the copy numbers on development of NE lesions were analyzed by a logistic proportional odds model according to the SAS logistic procedure.

Animal growth. Two birds in the non-bacitracin-treated group died following clostridial challenge. Significant differences ($P < 0.05$) were observed only in average daily gain, average daily feed intake, and feed conversion ratio of postchallenge birds between bacitracin-treated and untreated groups; in these categories, untreated birds demonstrated a reduction in performance (Table 1).

***C. perfringens* proliferation and NE lesions.** As summarized in Table 2, 1 and 2 out of 12 birds from bacitracin-treated and untreated groups of chickens, respectively, had barely detectable counts of hemolytic *C. perfringens* in the ileum on day 0 (i.e., before challenge). Bacitracin in the diet effectively controlled the cell proliferation of *C. perfringens* and development of NE. However, non-bacitracin-treated chickens demonstrated high incidence and lesion of NE with a high level of *C. perfringens* counts.

Alpha-toxin gene expression. Figure 1 shows a representative standard curve for quantification of *cpa* mRNA. A linear relationship was found between 10^2 and 10^9 copies of the mRNA ($r^2 = 0.99$). The average real-time PCR amplification efficiency summarized from 108 separate runs was 0.92 ± 0.14 ($n = 108$). The Q-RT-PCR assay demonstrated good reproducibility. The standard deviations from six standard curves ranged from 0.29 to 0.98 in six separate PCR assays (triplicates for each sample) for calibration of *cpa* mRNA in the digesta RNA samples. The detection limit of the assay with the digesta

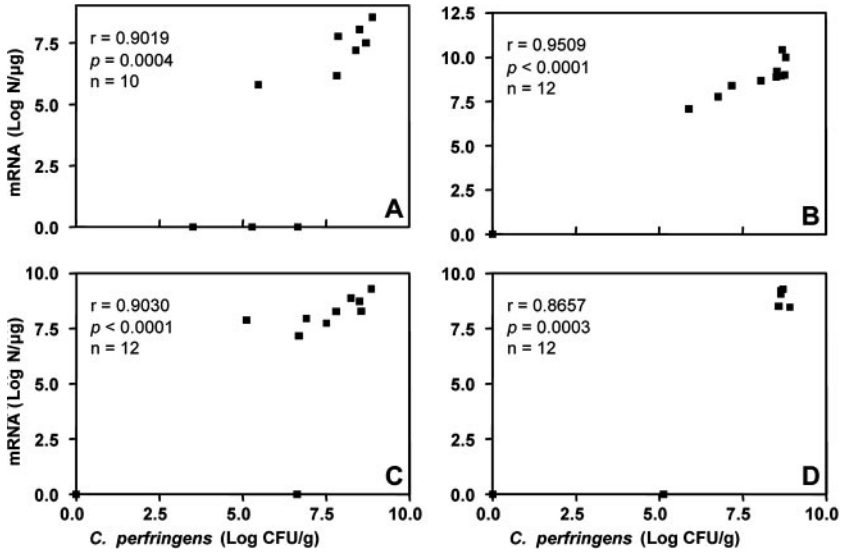

FIG. 3. Correlation between the cell proliferation and alpha-toxin gene expression of *C. perfringens* in the ileal digesta of non-bacitracin-treated postchallenge chickens. Panels A through D show data from days 1 through 4 postchallenge, respectively. Log CFU/g, $\log_{10}$ CFU per gram of ileal digesta; N, copy numbers of alpha-toxin gene mRNA; *r*, Spearman's rank correlation coefficient. Chickens were challenged with *C. perfringens* on day 18.

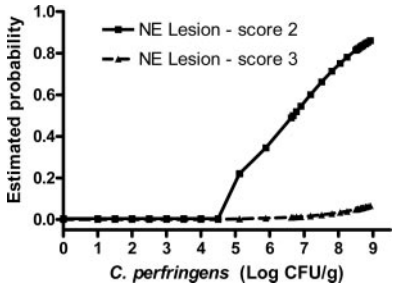

FIG. 4. Relationship between *C. perfringens* cell proliferation and the incidence of NE.

samples was 100 copies of *cpa* mRNA molecules per reaction, which is equal to 10^4 copies/μg total RNA.

Like *C. perfringens* proliferation and NE lesion, the expression of *cpa* was also effectively controlled by bacitracin treatment (Table 2). In non-bacitracin-treated chickens, however, *cpa* expression increased significantly after the challenge, reached a peak on day 2 postchallenge, and then declined at the end of the trial. Some birds in the same group had no detectable level of *cpa* mRNA regardless whether they had NE lesions, which led to relatively large standard deviations for gene expression.

Quantitative relationships. The progress of cell proliferation and *cpa* expression of *C. perfringens* in the ilea of non-bacitracin-treated chickens during postchallenge days can be described by a regression model: $y = b_0 + b_1X - b_2X^2$ (Fig. 2). Both cell proliferation and *cpa* expression demonstrated a progress trend in a parabolic curve. The expression of *cpa* was behind the cell proliferation during the first 2 days postchallenge but reached the same level at the last day of the trial. The level of *cpa* expression was positively correlated ($P \leq 0.0004$) with the cell proliferation of *C. perfringens* each day postchallenge ($r = 0.87$ to 0.95) (Fig. 3). The same positive correlation ($P < 0.0001$, $r = 0.91$) was also observed when the data from total 46 birds examined in the last 4 days postchallenge were used.

The relationship of the incidence of NE to the level of *cpa* expression and *C. perfringens* burden in the ilea of non-bacitracin-treated chickens was also analyzed. While the development of NE lesions was marginally explained by the level of *cpa* expression ($P = 0.10$), the cell proliferation of *C. perfringens* can be used to predict the incidence of NE lesion ($P = 0.0175$) (Fig. 4). The estimated probability shows that the average *C. perfringens* count of 5 $\log_{10}$ CFU/g in the digesta appears to be a threshold for developing NE with a lesion score of 2.

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