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Comparison of the Pathogenicity of Five *Clostridium perfringens* Isolates Using an *Eimeria maxima* Coinfection Necrotic Enteritis Disease Model in Commercial Broiler Chickens

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SUMMARY. *Clostridium perfringens* (CP) is the etiologic agent of necrotic enteritis (NE) in broiler chickens that is responsible for massive economic losses in the poultry industry in response to voluntary reduction and withdrawal of antibiotic growth promoters. Large variations exist in the CP isolates in inducing intestinal NE lesions. However, limited information is available on CP isolate genetics in inducing NE with other predisposing factors. This study investigated the ability of five CP isolates from different sources to influence NE pathogenesis by using an *Eimeria maxima* (EM) coinfection NE model: Str.13 (from soil), LLY_N11 (healthy chicken intestine), SM101 (food poisoning), Del1 ($netB^+tpeL^-$) and LLY_Tpel17 ($netB^+tpeL^+$) for NE-afflicted chickens. The 2-wk-old broiler chickens were preinfected with EM (5×10^3 oocysts) followed by CP infection (around 1×10^9 colony-forming units per chicken). The group of the LLY_Tpel17 isolate with EM coinfection had 25% mortality. No mortality was observed in the groups infected with EM alone, all CP alone, or dual infections of EM/other CP isolates. In this model of EM/CP coinfections, the relative percentages of body weight gain showed statistically significant decreases in all EM/CP groups except the EM/SM101 group when compared with the sham control group. Evident gut lesions were only observed in the three groups of EM/LLY_N11, EM/Del1, and EM/LLY_Tpel17, all of which possessed an essential NE pathogenesis locus in their genomes. Our studies indicate that LLY_Tpel17 is highly pathogenic to induce severe gut lesions and would be a good CP challenge strain for studies investigating pathogenesis and evaluating the protection efficacy for antibiotic alternative approaches.

RESUMEN. Comparación de la patogenicidad de varios aislados de *Clostridium perfringens* utilizando un modelo de enteritis necrótica por coinfección con *Eimeria maxima* en pollos de engorde comerciales.

La bacteria *Clostridium perfringens* (CP) es el agente etiológico de la enteritis necrótica (NE) en pollos de engorde que es responsable de pérdidas económicas masivas en la industria avícola como respuesta a la reducción o retiro voluntarios de antibióticos promotores de crecimiento. Existen grandes variaciones en los aislamientos de *C. perfringens* con relación a la inducción de lesiones intestinales por enteritis necrótica. Sin embargo, hay información limitada disponible sobre la genética de los aislamientos de *C. perfringens* para inducir enteritis necrótica con otros factores predisponentes. Este estudio investigó la capacidad de cinco aislados de *C. perfringens* de diferentes orígenes para influir en la patogénesis de la enteritis necrótica mediante el uso de un modelo de coinfección con *Eimeria maxima* (EM): Str.13 (con origen en suelo), LLY_N11 (origen de intestino de pollos sanos), SM101 (asociada con intoxicación alimentaria), Del1 ($netB^+$ $tpeL^-$) y LLY_Tpel17 ($netB^+$ $tpeL^+$) para pollos afectados por enteritis necrótica. Los pollos de engorde de dos semanas de edad fueron preinfectados con *E. maxima* (5×10^3 oocistos) seguido de la infección por *C. perfringens* (alrededor de 1×10^9 unidades formadoras de colonias por pollo). El grupo del aislado LLY_Tpel17 con coinfección con *E. maxima* tuvo una mortalidad del 25%. No se observó mortalidad en los grupos únicamente infectados con *E. maxima*, o con todos los grupos inoculados con *C. perfringens* únicamente o con infecciones duales con *E. maxima*/otros aislados de *C. perfringens*. En este modelo de coinfecciones *E. maxima*/*C. perfringens*, los porcentajes relativos de ganancia de peso corporal mostraron disminuciones estadísticamente significativas en todos los grupos inoculados con *E. maxima*/*C. perfringens* con excepción del grupo *E. maxima*/SM101 en comparación con el grupo de control no inoculado. Se observaron lesiones intestinales leves en pollos infectados con los grupos *E. maxima*/Str.13 o con *E. maxima*/SM101. Sin embargo, se observaron lesiones evidentes en los tres grupos de *E. maxima*/LLY_N11, *E. maxima*/Del1 y *E. maxima*/LLY_Tpel17, todos los cuales poseían un locus de patogénesis de enteritis necrótica esencial en sus genomas. Estos estudios indican que la cepa LLY_Tpel17 es altamente virulenta para inducir lesiones intestinales graves y sería una buena cepa de *C. perfringens* para los estudios que investigan la patogénesis y evalúan la eficacia de la protección para enfoques alternativos con antibióticos.

Key words: *Clostridium perfringens*, *Eimeria maxima*, necrotic enteritis, pathogenicity

Abbreviations: BHI = brain-heart infusion broth; CP = *Clostridium perfringens*; CPU = colony-forming units; EM = *Eimeria maxima*; NE = necrotic enteritis; *netB* = necrotic enteritis toxin B-like; PBS = phosphate-buffered saline; RBWG% = relative body weight gain%

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Table 1. *Clostridium perfringens* sources and their genomic information used in this study.

Isolates	Genotype	Source	Chromosome	Plasmid	Reference
Str. 13	A ($plc^+ netB^- tpeL^-$)	Soil	NC_003366.1	NC_003042.1	23, 24
SM101	F ($plc^+ netB^- tpeL^-$)	Food poisoning	NC_008262.1	NC_008263.1	5
LLY_N11	A ($plc^+ netB^- tpeL^-$)	Healthy chicken	NZ_CP023410.1	NZ_CP023411.1 NZ_CP023412.1 NZ_CP023413.1	25, 26
Del1	G ($plc^+ netB^+ tpeL^-$)	Field NE	NZ_CP019576.1	NZ_CP019577.1 NZ_CP019578.1 NZ_CP019579.1 NZ_CP019580.1	4
LLY_TpeL17	G ($plc^+ netB^+ tpeL^+$)	Field NE	NZ_VFFA00000000.1	NZ_VFFA00000000.1	27

Necrotic enteritis (NE), a disease caused by *Clostridium perfringens* (CP), is one of the top two important enteric infectious diseases in commercial broiler chicken flocks (1). NE is estimated to cause an annual loss of around US$6 billion in the poultry industry worldwide (2). CP is a ubiquitous bacterium in soil, dust, animal production environments, and the gastrointestinal tracts of healthy and sick animals, and it may cause food poisoning and various enterotoxemias in humans and animal species (1,3,4,5,6). CP isolates are traditionally classified into five toxinotypes, A to E, according to the major toxins produced (alpha, beta, epsilon, iota, enterotoxin) (7,8). The amount of alpha-toxin (PLC) detected in the gut content and mucosa in birds challenged with either the *plc* mutant or CP wild type A strain is found to be directly correlated with gut lesion severity (9). The presence of the *netB* gene in the newly created type G ($plc^+ netB^+$) strains is critical for NE pathogenicity (10,11).

The etiology of NE is very complex, and many predisposing factors, such as coccidiosis coinfection and immune suppression as well as high levels of animal protein in the diet, have been identified (12,13). Coccidiosis is one of the most evident factors caused by several distinct species of *Eimeria* parasites that are ubiquitous in chicken farms and that induce intestinal epithelium damage which may facilitate CP infection (14,15). *Eimeria maxima* (EM) is one of several *Eimeria* species causing chicken coccidiosis. As a common pathogen, EM invades the small intestines and damages the epithelium, thereby increasing gut permeability, providing nutrients for CP replication and causing the NE lesion (15,16). Therefore, *Eimeria* spp. are often coinfected with CP to induce experimental NE disease (17,18,19).

The availability of reliable NE disease models is critical for host-pathogen interaction studies and for development of NE prevention and control strategies. Many key factors important in successful experimental reproduction of NE in chickens need to be identified and optimized, such as selection of critical CP strains, timing of bacterial cultures, inoculation times, and dietary manipulations (13). Preexposure to coccidia infection followed by coinfection with a $netB^+$ CP strain, especially a virulent strain of EM, produces a more consistent NE disease with typical gut lesions (6,19). This dual infection experimental model has been used to examine the protection efficacy of many vaccine candidates and feed additive alternatives to antibiotics against NE (20,21,22).

Limited information on the pathogenicity comparison among the CP isolates from different genetic backgrounds hinders development of a good dual infection model of NE. To better understand the nature of CP interactions with *Eimeria* spp., five isolates of CP from different sources and genetic backgrounds: LLY_N11 (isolated from healthy chicken intestine), Str.13 (from soil), SM101 (from food poisoning case), Del1 ($netB^+ tpeL^-$), and LLY_Tpel 17 ($netB^+ tpeL^+$) were analyzed in pathogenicity studies in commercial broiler chickens using the EM/CP model that was previously described (6).

MATERIALS AND METHODS

***C. perfringens* and *E. maxima* strains.** The following five isolates were used in this study: Str.13 (simplified as S13), Del1, LLN_N11 (simplified as N11), SM101, and LLY_Tpel17 (simplified as Tpel17; Table 1). The CP strains were first cultured in chopped meat glucose medium at 37 C for 24 hr in anaerobic chambers that utilized gas packets to generate anaerobic conditions ($O_2 < 2\%$, $CO_2 = 9\%–13\%$, Mitsubishi Gas Chemical Company, New York, NY), then 1.5 ml of cultured medium was added into 150 ml of Bacto™ brain-heart infusion broth (BHI, Becton Dickinson and Company, Sparks, MD) supplemented with 5% yeast extract and 0.5% L-cysteine (Sigma-Aldrich, St. Louis, MO; BYC medium) cultured at 37 C for 18 hr under the same anaerobic conditions. EM sporulated oocysts were prepared as described elsewhere (6).

Phylogenetic analysis and data mining of *C. perfringens* strains. Complete genome ID information of CP S13 and SM101 is listed in Table 1 (23,24), and CP Del1, N11, and Tpel17 were characterized and sequenced in our laboratory (4,5,6,27). Five annotated genomes used in the study are available in GenBank. The alignment for phylogenetic analysis was run with Mauve to align the 59 CP genomes (28) and the

Table 2. Experimental design for necrotic enteritis testing in groups infected with *Eimeria maxima* (EM) and/or *Clostridium perfringens* (CP) isolates.

Group No	Detailed group	Day14	Day 18
1	Sham control (S)	PBS	BYC
2	EM	EM	BYC
3	Str.13 CP alone	PBS	Str.13 CP
4	LLY-N11 CP alone	PBS	LLY-N11 CP
5	SM101 CP alone	PBS	SM101 CP
6	Del1 CP alone	PBS	Del1 CP
7	LLY-Tpel17 CP alone	PBS	LLY-Tpel17 CP
8	EM/Str.13 CP	EM	Str.13 CP
9	EM/LLY-N11 CP	EM	LLY-N11 CP
10	EM/SM101 CP	EM	SM101 CP
11	EM/Del1 CP	EM	Del1 CP
12	EM/LLY-Tpel17 CP	EM	LLY-Tpel17 CP

Note: Chickens were orally infected with 5×10^3 oocytes/bird of EM at day 14 and/or with 1×10^9 CFU/bird of CP at day 18. The birds were euthanized on day 20. BYC = BHI + yeast extraction + L-cysteine medium. A single dose of CP was given (1×; 3–7) by oral gavage in CP-alone and EM/CP infections (8–12).

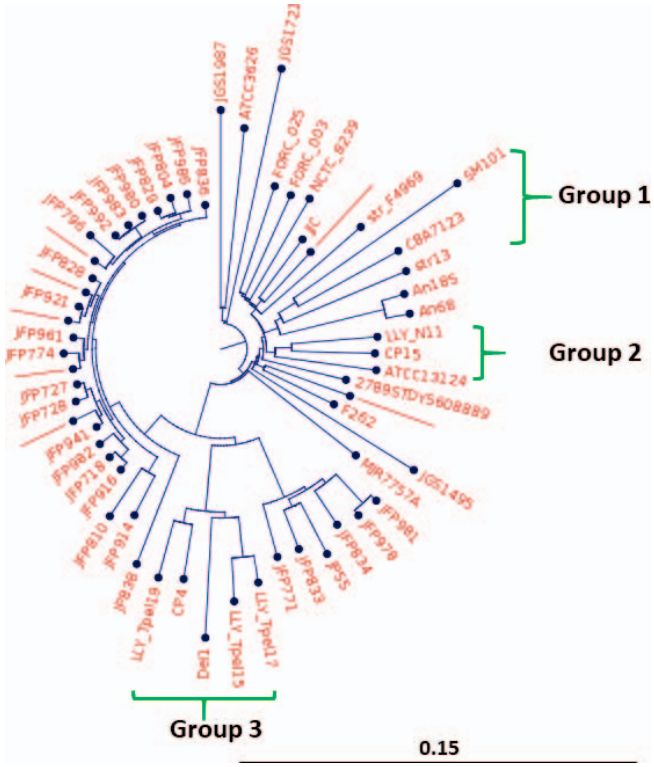

Fig. 1. Phylogenetic tree highlighting genetic relatedness of isolates LLY_N11, LLY_Tpel17, Del1, Str.13, and SM101 of *C. perfringens* in comparison with other 54 CP isolates. The alignment for phylogenetic analysis was run with Mauve (28) and the resulting phylogenetic tree was visualized using the CLC Genomics Workbench v.11.1 (29).

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Broiler chick husbandry. One-day-old Ross 708 broiler chickens (Longenecker's Hatchery, Elizabethtown, PA) were housed in CP- and coccidia-free Petersime starter brooder units and provided with feed and water *ad libitum*. The 14-day-old chickens were randomly assigned to 12 groups (Table 2) and transferred into large hanging cages (8 birds/group). All experiments were approved by the Institutional Animal Care and Use Committee at the Beltsville Agricultural Research Center (protocol number: 17-027).

***In vivo* evaluation of *C. perfringens* pathogenicity.** *Experimental design.* Chickens at day 14 were infected with 5×10^3 sporulated oocysts of EM Beltsville strain 41A per bird by oral gavage at 2 wk old, followed with an oral dosing of 1×10^9 colony-forming units (CFU) of CP per bird at day 18, as shown in Table 2. The birds dosed with phosphate-buffered saline (PBS), followed by receiving BYC medium, were used as sham control. Birds administrated with EM alone or CP alone were used as single infection controls. The doses of EM and CP were preoptimized. Birds were fed a starter diet (United States Department of Agriculture [USDA] Feed Mill) containing 16% crude protein and 61% carbohydrate before CP infection and a standard grower diet containing 24% crude protein and 54% carbohydrate after CP infection as described elsewhere (6,22).

Body weight gain. Body weights were measured at day 14 (chickens preinfected with EM), day 18 (chickens preinfected with CP), and day 20 (euthanatized). The relative percentage of body weight gain (RBWG%) was calculated with the following equation: (BW at day 20 − BW at day 18) / (BW at day 18) × 100%. The RBWG% in general effectively represents the growth rate by using the same birds as their own controls and greatly decreases effects of the large variations in body weight observed in broiler birds.

Lesion score evaluation. At day 20 (day 2 postinfection with CP), equal-length sections of mid gut intestines (flanking Meckel's Diverticulum, 20 cm) were removed and NE lesions were scored in a blinded fashion on scales from 0 (none) to 4 (high) as follows: 0 = no gross lesions; 1 = thin-walled or friable; 2 = focal necrosis or ulceration; 3 = larger patches of necrosis; 4 = severe and extensive necrosis (30).

Statistical analysis. RBWG% and lesion scores were analyzed by ANOVA with Dunnett's multiple comparison test for statistical analysis using GraphPad Prism software (31). Data were expressed as mean ± standard error of means (SEM). Differences were considered statistically significant when P values were <0.05.

RESULTS

Genomic comparative analysis. Fundamentally, sequence similarity by Basic Local Alignment Search Tool (BLAST) (32) analyses could reflect phylogenetic distance. However, heterogeneity, saturation, and specific compositional bias could exacerbate the ratios of similarity in these BLAST results. Basic genomic information for the five CP strains is shown in Table 1. Three isolates were in Genotype A (N11 and S13) and F (SM101) whereas two isolates traced to the newly created Genotype G (Del1 and Tpel17). In this study, the phylogenetic tree of CP strains is shown in Figure 1. Compared to the standard isolate ATCC13124, isolate N11 shared around 99% similarity to SM101, S13, Del1, and Tpel17 in genomic sequences, although N11 showed a higher sequence similarity to Del1 and Tpel17 compared to SM101 and S13. SM101 and S13 were more closely related to each other and were designated within Group 1. N11 was more genetically close to a standard isolate, ATCC13124, and clustered within Group 2 whereas Del1 and Tpel17 were more closely related to Group 3.

Table 3 shows the comparison of genes of toxins and other virulence factors among the five isolates. All genomes harbored the alpha-toxin gene (phospholipase C, *plc*), but none of them had epsilon- and iota-toxins (data not shown). As to major toxin genes, both Del1 and Tpel17 had *netB* and *netE*, but only Tpel17 contained the *tpeL* toxin gene. All the genomes except SM101 harbored the *beta2* toxin gene. CP SM101 lacked *nagI*, *nagK*, *and nagL* genes (encoding neuraminidase), *nanI* and *nanJ* genes (encoding sialidase), or the *pfoA* gene (encoding perfringolysin O) in the genome. In addition, SM101 and S13 lacked some important virulence genes in the plasmids, such as the *dgc* gene encoding diguanylate cyclase and the *pde* gene encoding phosphodiesterase, both of which are involved in cyclic-di-GMP system, or lacked genes encoding fimbrial adhesion proteins located in NElotus-1 site (cell wall surface protein, surface anchor protein, LPXTG-specific sortase A).

Mortality associated with EM/CP dual infection. In the birds infected with EM followed by CP infection (EM/CP), a mortality of 25% ($n = 8$ birds) was observed in the EM/Tpel17 group. No mortality was observed in the EM-alone or the CP-alone groups, or any of the EM/other CPs groups.

Relative percentage of body weight gain. Fig. 2a shows the RBWG% data to measure the growth rates among all the groups. Compared with the uninfected sham control, there were no significant differences between sham control and all the CP-alone groups, or between sham control and the EM-alone group ($P > 0.05$). However, there were significantly lower RBWG% in all the EM/CP dual infection groups ($P \leq 0.05$) except EM/SM101 ($P < 0.01$, $P < 0.05$, $P < 0.01$ and $P < 0.001$ for EM/S13, EM/N11,

Table 3. Toxins and other virulence factors of *C. perfringens* strains.

Virulence factor	Gene symbol	GenBank accession no.	Bacterial strains[A]				
			Del1	T17	N11	SM101	S13
Adherence							
Fibronectin-binding protein	*fbp*	WP_011590967.1	+	+	+	+	+
GroEL	*groEL*	WP_011967678.1	+	+	+	+	+
Regulation							
VirR/VirS two-component system	*virR*	WP_003449818.1	+	+	+	+	+
VirR/VirS two-component system	*virS*	WP_011592434.1	+	+	+	+	+
Toxin							
Alpha-clostripain	*cloSI*	WP_011010069.1	+	+	+	+	+
Bacteriocin	*bcn5*	M14481.1	−	−	+	+	+
Kappa-toxin (collagenase)	*colA*	WP_011009653.1	+	+	+	+	+
Collagen adhesin	*cna*	BAO58422.1	+	+	+	−	+
Enterotoxin	*cpe*	M98037	−	−	−	+	−
Enterotoxin	*entA*	WP_011592255.1	+	+	+	+	+
Enterotoxin	*entB*	WP_011590766.1	+	+	+	+	+
Enterotoxin	*entC*	BAB80158.1	−	−	+	−	+
Enterotoxin	*entD*	WP_011009927.1	+	+	+	+	+
Beta2-toxin	*cpb2*	CP025503.1	+	+	+	−	+
Hemolysin	*Hemolysin*	WP_003455347.1	+	+	+	+	+
Hemolysin	*Hemolysin_0*	WP_011592412.1	+	+	+	+	+
Hemolysin	*Hemolysin_1*	WP_003458690.1	+	+	+	+	+
Hemolysin	*Hemolysin_2*	WP_011592763.1	+	+	+	+	+
Mu-toxin (hyaluronidases)	*nagH*	WP_011009663.1	+	+	+	+	+
Mu-toxin (hyaluronidases)	*nagI*	WP_011010095.1	+	+	+	−	+
Mu-toxin (hyaluronidases)	*nagJ*	WP_003467250.1	+	+	+	+	+
Mu-toxin (hyaluronidases)	*nagK*	WP_011590733.1	+	+	+	−	+
Mu-toxin (hyaluronidases)	*nagL*	WP_003468301.1	+	+	+	−	+
Sialidase	*nanH*	WP_011590492.1	+	+	+	+	−
Sialidase	*nanI*	WP_011009995.1	+	+	+	−	+
Sialidase	*nanJ*	WP_011009886.1	+	+	+	−	+
Necrotic enteritis toxin B-like (TX428)	*netB*	ACN73257.1	+	+	−	−	−
Cytolysis (NetE)	*netE*	KJ606985.1	+	+	−	−	−
Perfringolysin O (theta-toxin/PFO)	*pfoA*	WP_003462918.1	+	+	+	−	+
Alpha-toxin	*plc*	WP_011009584.1	+	+	+	+	+
Glycosyl transferase	*tpeL*	AB262081.1	−	+	−	−	−
Other functions							
Type IV pili (AI109)	*CPE2277*	WP_011010855.1	+	+	+	+	+
Type IV pili (AI109)	*CPE2278*	WP_003454175.1	+	+	+	+	+
Type IV pili (AI109)	*CPE2280*	WP_011010856.1	+	+	+	+	+
Type IV pili (AI109)	*CPE2281*	WP_011010857.1	+	+	+	+	+
Fibronectin-binding protein (AI156)	*fbpA*	WP_011010006.1	+	+	+	+	+
Type IV pili (AI109)	*pilA1*	WP_011010863.1	+	+	+	+	+
Type IV pili (AI109)	*pilB*	WP_011010636.1	+	+	+	+	+
Type IV pili (AI109)	*pilB2*	WP_011010862.1	+	+	+	+	+
Type IV pili (AI109)	*pilC*	WP_011010635.1	+	+	+	+	+
Type IV pili (AI109)	*pilC2*	WP_011010861.1	+	+	+	+	+
Type IV pili (AI109)	*pilD*	WP_003462279.1	+	+	+	+	+
Type IV pili (AI109)	*pilM*	WP_011010859.1	+	+	+	+	+
Type IV pili (AI109)	*pilN*	WP_011010858.1	−	−	+	−	−
Type IV pili (AI109)	*pilT*	WP_003451114.1	+	+	+	+	+
Major pilins	*tcp cluster*	DQ338473.1	+	+	+	−	−
Antibiotic resistance	*tetA*	WP_003479690.1	+	+	+	−	−
Antibiotic resistance	*tetB*	AQW28570.1	+	+	+	−	−
Bacteriocin	uviB	AAA98258.1	−	−	+	+	−
Fimbrial adhesion							
Cell wall protein	NELoc-1 locus	AFV15055.1	+	+	+	−	−
Surface protein	NELoc-1 locus	AFV15058.1	+	+	+	−	−
Sortase A, LPXTG specific	NELoc-1 locus	AFV15059.1	+	+	+	−	−
c-di-GMP system							
Diguanylate cyclase	*dgc*	WP_003476383.1	+	+	+	−	−
Bifunctional diguanylate Cyclase/phosphodiesterase	*pde*	WP_057230428.1	+	+	+	−	−

[A]Strains: T17 = LLY_Tpel17; N11 = LLY_N11; S13 = Str.13.

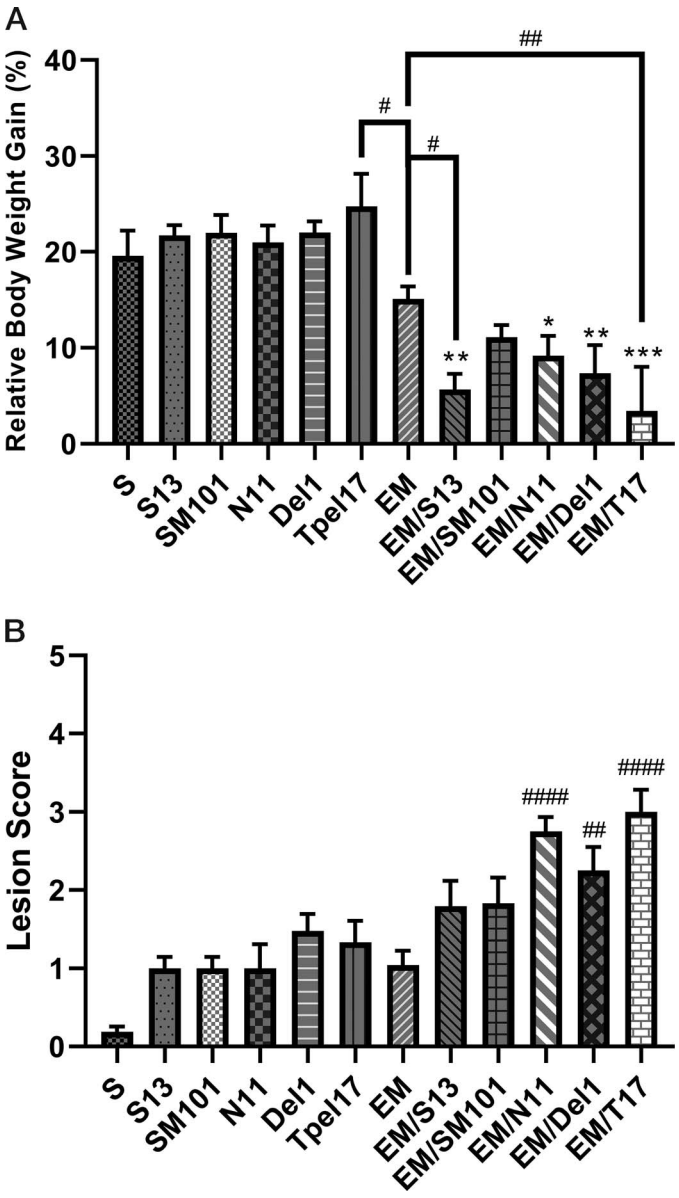

Fig. 2. (a) Relative body weight gain% (RBWG%, and (b) jejunum lesion scores in chickens with a single dosage of CP infection in chickens and/or *E. maxima* (EM)/*C. perfringens*. The 14-day-old broiler chickens were preinfected with EM (5×10^3 oocysts) followed by single CP infection (around 1×10^9 CPU per chicken) at day 18. At 2 days post CP infection (day 20), birds were euthanatized for analysis of RBWG% and the lesion score estimation. The RBWG% was calculated with the following equation: (BW at day 20 − BW at day 18) / (BW at day 18) × 100%. S = sham control, S13 = Str.13, N11 = LLY_N11, Tpel17 = LLY_Tpel17. The # marks mean statistically significant differences (#$P < 0.05$; ##$P < 0.01$, ####$P < 0.0001$) when compared to the value in EM group while * marks indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$) when compared to the value of sham control group (S). Chicken number: $n = 8$ for all groups.

EM/Del1, and EM/Tpel17, respectively). When compared with the EM-alone group, only the CP Tpel17-alone group showed significantly higher RBWG% ($P < 0.05$) while EM/S13 and EM/Tpel17 dual infection groups had significantly lower RBWG% ($P < 0.05$, and $P < 0.01$ for EM/S13 and EM/Tpel17, respectively).

Mid gut lesion scores. Intestinal lesion scores were determined at day 2 post CP infection. Lesions of varied degrees were observed for all EM/CP groups with severe lesions in the EM/Tpel17 group (Fig. 2b). Compared with the lesion score of the EM-alone group, three EM/CP groups (EM/N11, EM/Del1, and EM/Tpel17) showed generally higher scores with significant differences ($P \leq 0.001$ for EM/N11, $P < 0.01$ for EM/Del1, and $P < 0.001$ for EM/Tpel17) while there were no significant differences between EM-alone and EM/S13 or between EM-alone and EM/SM101. Some birds in the EM/Tpel17 group showed typical Turkish towel-like lesions in the small intestinal mucosa (figure not shown). No difference was found between the EM-alone and CP-alone groups ($P > 0.05$).

DISCUSSION

In this study, five isolates of CP from different genetic backgrounds were analyzed for potential correlation between the presence of virulence factors and their pathogenicity in our NE disease model. CP S13, a natural isolate from soil, causes gas gangrene in humans and has been used to establish experimental gas gangrene in a murine model (24). Isolate N11 was initially obtained from the intestine of a healthy chicken to show the potential to induce severe NE lesions in an EM/CP NE experimental model (5,6), but CP isolate N10, which was also isolated from a healthy chicken, did not cause NE in the same EM/CP model. Smyth and Martin (33) also found that some CP isolates from normal chickens could cause NE in chicken.

CP isolate SM101, a Type F isolate originated from a food poisoning case, produces enterotoxin (CPE) upon sporulation (7,26). This CPE-positive isolate induces epithelial cell death and diarrhea in the intestinal tract of the infected host by specifically binding to certain claudin-receptors on the intestinal epithelial membrane surface and by inducing histologic damage accompanied by intestinal fluid and electrolyte transport changes in CPE-treated rabbit small intestinal loops (34,35,36). The CP SM101 strain has neither *nanI* and *nanJ* nor the perfringolysin O (*PfoA*) or collagen adhesion (*cna*) gene (25). In this study, the EM/SM101 coinfected group did not show a significantly lower RBWG% than the sham control, or significantly higher NE lesion scores than the EM-alone group.

Del1, an isolate belonging to a newly classified Genotype G, was originally isolated from the intestine of an NE-afflicted chicken and showed virulence in an NE disease model with prior EM exposure in broiler chickens (6). The Del1 genome contains many important genes encoding alpha-toxin, NetB, beta2-toxin (CPB2), five hyaluronidases, sialidase (NanH-J), collagenase, adhesin, and theta-toxin (4), which may contribute to toxin generation and bacterial colonization and growth. The Tpel17 CP strain, isolated from an NE field disease outbreak in a commercial poultry farm, possesses *netB* and *tpeL* genes, and demonstrated high pathogenicity post multiple CP infections without a prior EM exposure in combination with a higher protein diet of fishmeal (27). In our study, chickens inoculated with the Tpel17 CP isolate ($netB^+tpeL^+$) showed the most severe intestinal lesions, lowest RBWG%, and higher mortality rates of 25% in our EM/CP dual infection NE model. As there are large variations in the absolute body weight and in relative growth rate post EM/CP infections, a larger bird sample set should be used in the future to avoid variation.

CP is the major pathogen leading to NE in chickens. Alpha-toxin was previously assumed to be a major toxin in causing NE during

the early years (37,38). However, an alpha-toxin CP mutant was still able to retain full virulence in an NE disease model (39). Another group later discovered that a necrotic enteritis B-like toxin gene (*netB*) encoding pore-forming toxin is critical for CP to cause NE in chickens (13,40,41). Existence of other toxins involved in NE pathogenesis has been shown in a recent study where *netB*$^+$ CP strains were found in the healthy birds but did not cause NE (42,43,44). Another important virulence factor, TpeL toxin, is a large CP toxin family member that is associated with adhesion but not with the invasion process (45) and which has been shown to be a potential virulence factor for NE (13,46). Besides *netB*, other NE virulence factors have been also recognized including the recognition of three pathogenicity loci (NELoc-1 to -3) and intracellular bacterial second messenger cyclic diguanosine monophosphate (c-di-GMP), which plays critical roles in regulating multiple bacterial processes including motility and surface adhesiveness (40,47,48). Two genes are involved in the c-di-GMP signaling system on the NELoc-1 locus: *dgc* encoding diguanylate cyclase for its synthesis and *pde* encoding a phosphodiesterase for its degradation (47,48). Mutations of these two genes on NELoc-1 locus significantly attenuate virulence in an avian NE CP isolate (47). By genomic analysis, Del1, Tpel17, and N11 harbored *dgc* and *pde* genes while S13 or SM101 did not. On the other hand, a cluster of seven genes encoding proteins involved in generation, secretion, and anchoring of an adhesin, such as cell wall proteins AFV15055.1, surface anchoring protein AFV15058.1, and sortase A AFV15059.1, exist between *dgc* and *pde* on NELoc-1 (48). These genes were also absent in CP isolates S13 or SM101 but present in Tpel17, Del1, and N11 (Table 3). In summary, the presence of two genes (*dgc* and *pde*) for c-di-GMP signaling system and other adhesion-related genes may account for the enhanced pathogenicity capabilities of isolates Del1 and Tpel17, and especially N11.

In our studies, two *netB*$^+$ CP isolates, Del1 and Tpel17, showed higher gross lesion scores in the small intestine in the EM/CP model. However, N11, SM101, and S13, although *netB* negative, produced low levels of NE lesions in the EM/CP model. These results suggest that there are other virulence-promoting factors beyond NetB that can give rise to NE lesions, especially when an *Eimeria* sp. infection is present. As indicated above, N11 also contained the essential virulence factors such as the c-di-GMP signaling system and adhesion-related ones on a large pathogenesis locus (NetLoc-1). Both natural and experimental NE disease inductions are complex and may involve many host- and parasite-related predisposing factors. The results of our study confirmed the critical role of coccidiosis as the most frequent predisposing factor inducing field NE infection (20). In our study with EM/CP coinfection in the NE model, all the groups except the EM/SM101 group had a lower RBWG% in broiler chickens as compared with those in the uninfected sham control. These results strongly suggested that these two infections together promote the NE disease process. The ubiquitous presence of *Eimeria* spp. and CP in broiler farms where litter is reused may create bigger challenges in developing alternatives to antibiotics.

The results obtained in this study showed that the non-*netB* harboring CPs such as S13 could reproduce mild gut lesions in the EM/CP dual infection model, but Cooper *et al.* (49) found that CP S13 and SM101 and other non-*netB* carrying strains failed to reproduce NE disease in the CP-alone infection model. This discrepancy may be due to the different NE model and the variations in CP strain pathogenicity. The interaction between EM and S13 caused significant decreases of RBWG% when compared to the sham control, even though neither EM nor S13 alone had a significant impact on RBWG%. In addition, a much higher dose of fresh CP bacterial culture with fresh toxins in the supernatant was directly administrated to the chickens via oral gavage in our model instead of the mixed feed administration with much lower doses which was used in other study (49).

In summary, five CP isolates from different genetic backgrounds were characterized for toxin/virulence factors throughout the genome, and their pathogenicity *in vivo* was analyzed. Essential NE virulence factors were found to exist in the NELoc-1 locus of Del1, N11, and Tpel17 isolates. Coinfection with EM and CP resulted in varied gross NE lesions in the small intestines of broiler chickens in this study. The NE lesions were evident in chickens infected with EM/N11, EM/Del1, and EM/Tpel17 related to the EM-alone group. The presence of *netB*$^+$*tpeL*$^+$ genes in the CP Tpel17 isolate significantly enhanced the NE severity in the intestine. One major conclusion of this study is that the Tpel17 strain is a good CP isolate for a dual-infection NE disease model because consistent gut lesions and NE clinical signs can be obtained. The availability of pathogenic CP strains and a reproducible NE disease model should facilitate the efficacy testing of antibiotic alternatives, including vaccines and novel feed additives, aimed to reduce the use of antibiotics in poultry production.

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