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Alpha-Toxin of *Clostridium perfringens* Is Not an Essential Virulence Factor in Necrotic Enteritis in Chickens[▽]

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The *Clostridium perfringens* alpha-toxin has previously been implicated as the major virulence factor in necrotic enteritis in chickens, although definitive proof has not been reported. In this study an alpha-toxin mutant was constructed in a virulent chicken isolate and shown to retain full virulence in a chicken disease model. These results demonstrated that alpha-toxin is not an essential virulence factor in the pathogenesis of necrotic enteritis in chickens.

Avian necrotic enteritis (NE) was first described by Parish in 1961 (33), and since then it has been reported to occur in almost all poultry-producing countries (8, 17, 24, 29). NE is an enteric disease that is caused predominantly by *Clostridium perfringens* type A and to a lesser extent by type C strains (41). Clinical NE is thought to occur when *C. perfringens* proliferates to high numbers in the small intestine and produces extracellular toxins that damage the intestine. The major toxin believed to be involved is the alpha-toxin, but its precise role in the disease process is not completely understood. The alpha-toxin is a secreted zinc-metalloenzyme which has both phospholipase C and sphingomyelinase activity and is the major toxin involved in the pathogenesis of human gas gangrene (5, 42). All five toxin types of *C. perfringens* (A to E) carry and express the alpha-toxin structural gene, *plc*.

Early studies on the reproduction of the disease involved intraduodenal infusion of large volumes of broth culture (2) or crude toxin (3) into chickens. Typical lesions of NE were seen as early as 5 h after infusion of *C. perfringens* cells. From these studies the authors concluded that because alpha-toxin was the major toxin secreted by *C. perfringens*, alpha-toxin must be the major virulence factor causing NE in chickens. Another study used oral inoculation with broth culture to cause characteristic necrotic lesions (20) and found that although many germfree birds died, no commercial birds died. A third of the birds inoculated with semipurified alpha-toxin died, but no bird died after receiving culture supernatant neutralized by antiserum raised against the semipurified alpha-toxin. Over the years, these studies have been the principle evidence given for the proposed major role of alpha-toxin in NE in chickens. The limitation of this interpretation is that it does not take into account other secreted toxins that the bacteria may have produced. While *C. perfringens* isolates are toxin typed by the presence of four major toxins, α, β, ε, and ι, various strains can also produce a range of other toxins (CPE, β2 toxin, perfringolysin O [θ-toxin], collagenase [κ-toxin], etc.).

Other studies call into doubt the causative role of alpha-toxin in NE. In one report, no difference in alpha-toxin levels was found when in vitro alpha-toxin levels were compared between isolates from diseased and healthy birds (22). Yet another study found that the intestinal level of alpha-toxin was not correlated with disease lesion scores (46). Another inconsistency in the assumptions regarding the role of alpha-toxin is the extent of heterophil, lymphocyte, and plasma cell infiltration in infected tissues (2, 21, 36). In clostridial myonecrosis (gas gangrene), a disease primarily mediated by the same alpha-toxin, there is marked leukostasis and lack of inflammatory infiltrate in tissues infected by *C. perfringens* cells (19). By contrast, in NE there is a typically extensive immune cell infiltration, indicating quite a different etiology compared to classical alpha-toxin-induced disease. The construction and virulence testing of *plc* mutants (5, 12, 31) have been important in determining the role of alpha-toxin in gas gangrene, but to date there have been no reports of the construction of a defined *plc* mutant in a *C. perfringens* chicken isolate.

In this study, alpha-toxin levels of chicken isolates were compared to virulence data obtained from the same isolates in commercial birds. We also report the construction of chromosomal *plc* mutants of a virulent poultry isolate. Virulence testing of the resultant defined chromosomal mutants showed that alpha-toxin was not an essential virulence factor in NE.

Isolation and characterization of *C. perfringens* from diseased flocks. Eighteen isolates (Table 1) were obtained from six broiler flocks from birds that suffered from NE. These strains were isolated from gut contents, intestinal samples, liver, and kidney. Multiplex PCR toxin typing (10, 28) carried out using reference strains (Table 1) as positive controls showed that all 18 isolates were *C. perfringens* toxin type A (Table 2), which agrees with surveys carried out in Europe (16, 22, 30), Korea (48), and North America (28). Two isolates were positive for the *cpb2* gene, which encodes the β2-toxin. While the β2-toxin has been implicated in NE in other animals,

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Plasmids		
pSM20	Kn^r *gfp*$^+$	27
pJIR750	Cm^r *oriCP oriEC*	9
pALK1	pSM20 Ω (BamHI-SpeI:pJIR750, 0.9 kb) Δ*gfp* (Cm^r Kn^r)	Recombinant
pALK2	pALK1 Ω (1.8-kb upstream *plc* fragment, *catP*, 2.1-kb downstream *plc* fragment) (Cm^r Kn^r)	Recombinant
Strains		
JIR325	Strain 13 Rif^r Nal^r	25
JIR4107	JIR325 *plc*::*erm*(B)	4
ATCC 13124	Type A (*plc*$^+$)	
CN 1884	Type B (*plc*$^+$ *cpb*$^+$ *etx*$^+$)	R. G. Wilkinson (Univ. Melbourne)
CN 2109	Type C (*plc*$^+$ *cpb*$^+$)	R. G. Wilkinson (Univ. Melbourne)
CN 462	Type D (*plc*$^+$ *etx*$^+$)	R. G. Wilkinson (Univ. Melbourne)
ATCC 27324	Type E (*plc*$^+$ *cpi*$^+$ *cpe*$^+$ *cpb2*$^+$)	
NE18-M1	EHE-NE18 Δ*plc*::*catP*	This work
NE18-M4	EHE-NE18 Δ*plc*::*catP*	This work
NAG-NE1	Isolated from gut contents of chicken with NE, flock 1	37
EHE-NE3	Isolated from liver of chicken with NE, flock 2	37
EHE-NE4	Isolated from liver of chicken with NE, flock 2	37
EHE-NE5	Isolated from kidney of chicken with NE, flock 2	37
EHE-NE7	Isolated from gut wall of chicken with NE, flock 2	37
EHE-NE9	Isolated from gut wall of chicken with NE, flock 2	37
EHE-NE13	Isolated from gut wall of chicken with NE, flock 2	37
EHE-NE14	Isolated from gut contents of chicken with NE, flock 2	37
EUR-NE15	Isolated from gut contents of chicken with NE, flock 3	37
EHE-NE16	Isolated from gut contents of chicken with NE, flock 4	37
EHE-NE17	Isolated from gut contents of chicken with NE, flock 4	37
EHE-NE18	Isolated from gut contents of chicken with NE, flock 4	37
EHE-NE20	Isolated from gut contents of chicken with NE, flock 5	This work
EHE-NE21	Isolated from gut contents of chicken with NE, flock 5	This work
EHE-NE22	Isolated from gut contents of chicken with NE, flock 5	This work
NAG-NE23	Isolated from gut wall of chicken with NE, flock 6	37
NAG-NE24	Isolated from gut wall of chicken with NE, flock 6	37
NAG-NE25	Isolated from gut wall of chicken with NE, flock 6	37

[a] Cm^r, chloramphenicol resistant; Kn^r, kanamycin resistant; Nal^r, nalidixic acid resistant; Rif^r, rifampin resistant.

TABLE 2. Alpha-toxin and θ-toxin production and PCR-based toxin typing of *C. perfringens* isolates derived from chickens diagnosed with NE

Strain no.	Toxin type	Alpha-toxin[b] (U mg^{-1} protein)	θ-toxin[b] ($\log_2$ [titer])
NAG-NE1	A	$(2.3 \pm 1.0) \times 10^{-3}$	6.3 ± 0.2
EHE-NE3	A	$(1.9 \pm 0.6) \times 10^{-3}$	5.2 ± 1.1
EHE-NE4	A	$(2.0 \pm 0.7) \times 10^{-3}$	5.8 ± 0.1
EHE-NE5	A	$(13.3 \pm 3.4) \times 10^{-3}$	6.6 ± 0.1
EHE-NE7	A	$(4.3 \pm 3.8) \times 10^{-3}$	6.6 ± 0.3
EHE-NE9	A	$(8.3 \pm 1.1) \times 10^{-3}$	6.4 ± 0.1
EHE-NE13	A	$(4.0 \pm 0.8) \times 10^{-3}$	5.7 ± 0.1
EHE-NE14	A	$(4.0 \pm 0.9) \times 10^{-3}$	4.9 ± 0.1
EUR-NE15	A	$(2.1 \pm 0.2) \times 10^{-3}$	4.4 ± 0.4
EHE-NE16	A	$(5.2 \pm 2.0) \times 10^{-3}$	6.0 ± 0.2
EHE-NE17	A	$(4.8 \pm 0.8) \times 10^{-3}$	7.9 ± 0.4
EHE-NE18	A	$(1.9 \pm 0.3) \times 10^{-3}$	7.5 ± 0.1
EHE-NE20	A	$(1.1 \pm 0.3) \times 10^{-3}$	4.7 ± 0.1
EHE-NE21	A	$(2.7 \pm 0.1) \times 10^{-3}$	5.4 ± 0.3
EHE-NE22	A	$(2.3 \pm 0.6) \times 10^{-3}$	6.0 ± 0.2
NAG-NE23	A(*cpb2*$^+$)	$(2.6 \pm 1.4) \times 10^{-3}$	6.2 ± 0.4
NAG-NE24	A(*cpb2*$^+$)	$(2.0 \pm 0.5) \times 10^{-3}$	6.0 ± 0.2
NAG-NE25	A	$(3.6 \pm 1.2) \times 10^{-3}$	5.6 ± 0.5
JIR325[a]	A	$(16.6 \pm 3.3) \times 10^{-3}$	7.5 ± 0.3

[a] A derivative of strain 13, a human gas gangrene isolate, used as a control.
[b] Results represent averages of duplicate assays carried out on preparations from at least three separate cultures of each strain, ± standard deviations.

for example, in pigs (45), no correlation between NE disease isolates and β2-toxin has been established.

A selection of these isolates was evaluated in a chicken virulence model and found to cause lesions characteristic of NE, although the severity of disease varied among the isolates. Quantitative in vitro alpha-toxin (38) and θ-toxin (4) assays (Table 2) showed that there was no correlation between severity of disease and alpha-toxin or θ-toxin production. The amount of alpha-toxin produced by most of the NE disease isolates was considerably lower than that from the human gas gangrene strain JIR325. Among type A *C. perfringens* strains, JIR325 is regarded as producing a low level of alpha-toxin (11). Therefore, the alpha-toxin levels found in this collection of disease isolates must be regarded as very low. This result is similar to those of recent studies where no significant difference was found between the levels of alpha-toxin produced from disease isolates and healthy bird isolates (22), and there was no correlation between in vivo toxin levels and lesion scores (46). In one study, in vitro alpha-toxin levels of diseased birds were found to be higher than in uninfected birds (23), but the growing weight of evidence indicates that this is not a common finding.

Construction of *plc* mutants in the chicken *C. perfringens* isolate EHE-NE18. Isolate EHE-NE18 was chosen for subsequent studies because, unlike many of the isolates, it could be transformed (35) with plasmid DNA (pJIR750) and it caused significant disease in the NE induction model. The suicide plasmid pALK2 was constructed by cloning fragments of the *plc* gene region on either side of the *catP* cassette in pALK1 (Table 1), which led to an 890-bp deletion of the *plc* gene. This suicide plasmid was used to transform strain EHE-NE18 to thiamphenicol resistance (10 μg/ml). Two independently derived *plc* mutants, NE18-M1 and NE18-M4, were isolated from 20 independent transformation experiments, and PCR analysis was used to confirm that both mutants were derived from double reciprocal crossover events between pALK2 and the *plc* region on the EHE-NE18 chromosome. SmaI pulsed-field gel electrophoresis patterns were determined by using previously described methods (13, 40). EHE-NE18 and its mutants had identical profiles, which confirmed these strains were isogenic mutant isolates.

Quantification of the levels of alpha-toxin and θ-toxin produced by the *plc* mutants showed that neither mutant synthesized active alpha-toxin (the mutant levels were below the detection limit of the assay; EHE-NE18 produced $[1.9 \pm 0.3] \times$

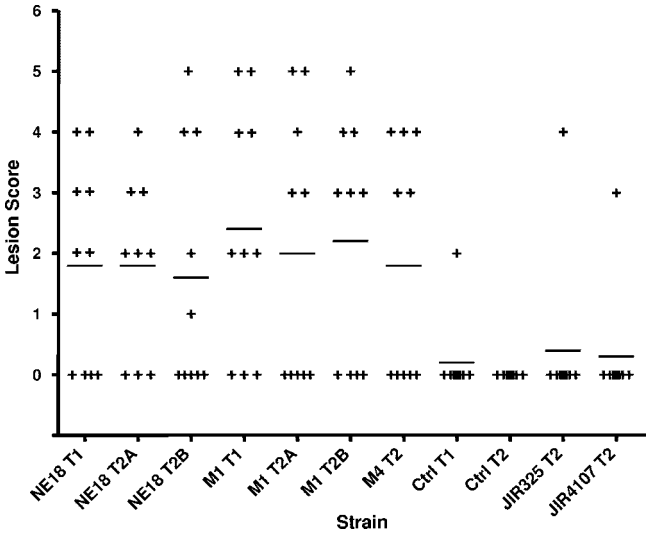

FIG. 1. NE challenge model. Lesion scores of individual 24-day-old broiler chickens challenged with *C. perfringens* are shown. Each group consisted of 10 birds. The solid bars represent the average lesion score in each group. Intestinal lesions in the small intestine (duodenum to ileum) were scored as follows: 0, no gross lesions; 1, thin or friable walls; 2, focal necrosis or ulceration (1 to 5 foci); 3, focal necrosis or ulceration (6 to 15 foci); 4, focal necrosis or ulceration (16 or more foci); 5, patches of necrosis 2 to 3 cm long; 6, diffuse necrosis typical of field cases. The results are from two trials (T1 and T2), with some strains tested in duplicate in the second trial (T2A and T2B). Birds not challenged with *C. perfringens* acted as negative controls (Ctrl). The *C. perfringens* isolates tested and their abbreviations are as follows: NE18, EHE-NE18; M1, NE18-M1; M4, NE18-M4. One-tailed, nonparametric *t* test analyses of the challenge (EHE-NE18) and mutant derivatives against their respective controls all showed a statistical difference ($P < 0.05$), but no statistical significance was seen between the mutant and the wild-type strains.

10^{-3} U · mg^{-1}). Both mutants produced similar levels of perfringolysin O (NE18-M1, 7.5 ± 0.1; NE18-M4, 7.6 ± 0.8; EHE-NE18, 7.5 ± 0.2; units are $\log_2$[titer]).

The *plc* mutants still produce NE in a chicken disease model. Chickens were challenged with the two *plc* mutants and the isogenic wild-type strain. Other strains tested included the strain 13 derivative JIR325 and its *plc* mutant, JIR4107 (4). Commercial Ross 308 broiler chickens were fed an antibiotic-free chicken starter diet containing 20% protein for 13 days. On day 14 feed was changed to a wheat-based feed containing 50% fish meal. On day 20, feed was withdrawn and each bird was orally challenged with 1.5 ml of *C. perfringens* stationary-phase culture. On day 21, birds were again orally challenged and feed was returned infected with *C. perfringens* (20 ml culture in first 100 g of feed per group). On day 24, chickens were euthanized with inhaled carbon dioxide gas and their small intestines (duodenum to ileum) were examined for gross necrotic lesions. In each group the survival rate was 100%. The results (Fig. 1) showed that the lesion scores in birds infected by the two independent *plc* mutants were not significantly different from those in birds infected by the wild-type parent strain. These results were reproducible, since the same results were obtained in independent virulence trials. The unchallenged negative control group from trial 1 had a single bird with a single 1-mm necrotic lesion, presumably resulting from infection by a fortuitous environmentally derived strain. The negative control group in the second trial had no lesions. *C. perfringens* strains were reisolated directly from the lesions of affected birds. Twenty colonies from each bird were subcultured onto sheep blood agar with or without thiamphenicol. As expected, the lesions sampled from birds challenged with the isolates from the wild-type strain were susceptible to thiamphenicol and produced both alpha-toxin and θ-toxin, whereas birds infected with the mutants yielded only thiamphenicol-resistant *C. perfringens* colonies that produced θ-toxin but not alpha-toxin. PCR analysis confirmed that the *plc* gene in the latter isolates remained insertionally inactivated. These results clearly demonstrate that the alpha-toxin-negative mutants produce lesions of equal severity to those in the wild-type strain from which they were derived.

The human *C. perfringens* isolate JIR325, which produced significantly higher levels of alpha-toxin than EHE-NE18, did not produce an appreciable level of disease; just a single bird in the treatment group had lesions. There was no difference in disease incidence between the wild-type JIR325 strain and its alpha-toxin mutant, JIR4107 (4).

At necropsy, segments of ileum or jejunum measuring approximately 2 to 4 cm were collected into 10% sodium phosphate-buffered formalin. The small intestine samples were cross-sectioned at 4-mm intervals, and segments were processed to paraffin-embedded blocks for routine histology, cut at 4 to 5 μm, and stained with hematoxylin and eosin (H&E). A comparison of the gross pathology of the lesions resulting from infection with EHE-NE18 and NE18-M1 (Fig. 2a and b) showed that there were no observable macroscopic differences between the infected groups. The macroscopic lesions had the typical signs of NE in that the lesions were sharply demarcated from the surrounding mucosa. The histopathology of unchallenged, wild-type, and mutant gut tissue sections was compared. Microscopic examination of the infected tissue (Fig. 2d and e) revealed a small intestine with a thick lumenal lining of acellular coagulative necrotic debris covered by a thick layer of rod-shaped bacteria, with a layer of degenerate and necrotic debris separating the underling viable lamina propria and a heavy invasion of immune cells in the underlying tissue. This typical innate inflammatory response is in contrast with the alpha-toxin-mediated inhibition of neutrophil invasion that is the hallmark of gas gangrene (15). While there was no histologically observable difference between the microscopic lesions from the wild-type and mutant infections, there were significant differences between the challenged and unchallenged birds (Fig. 2c).

The in vivo analysis of the isogenic *plc* mutants has provided clear evidence that the alpha-toxin is not an essential virulence factor in the pathogenesis of NE in chickens. The inactivation of the chromosomal *plc* gene had no effect on the virulence of the resultant strains in chickens. As the wild-type isolate was obtained from a clinical case of chicken NE and produced a disease pathology similar to that reported in many other disease induction models (6, 14, 32, 34, 44, 47), we conclude that our results can be readily extrapolated to other virulent strains.

The observation that the NE18-M1 and NE18-M4 mutants were still completely virulent contrasts with a recent study in which isolated spontaneous alpha-toxin mutants were isolated by repeated passaging and found to be avirulent in their model

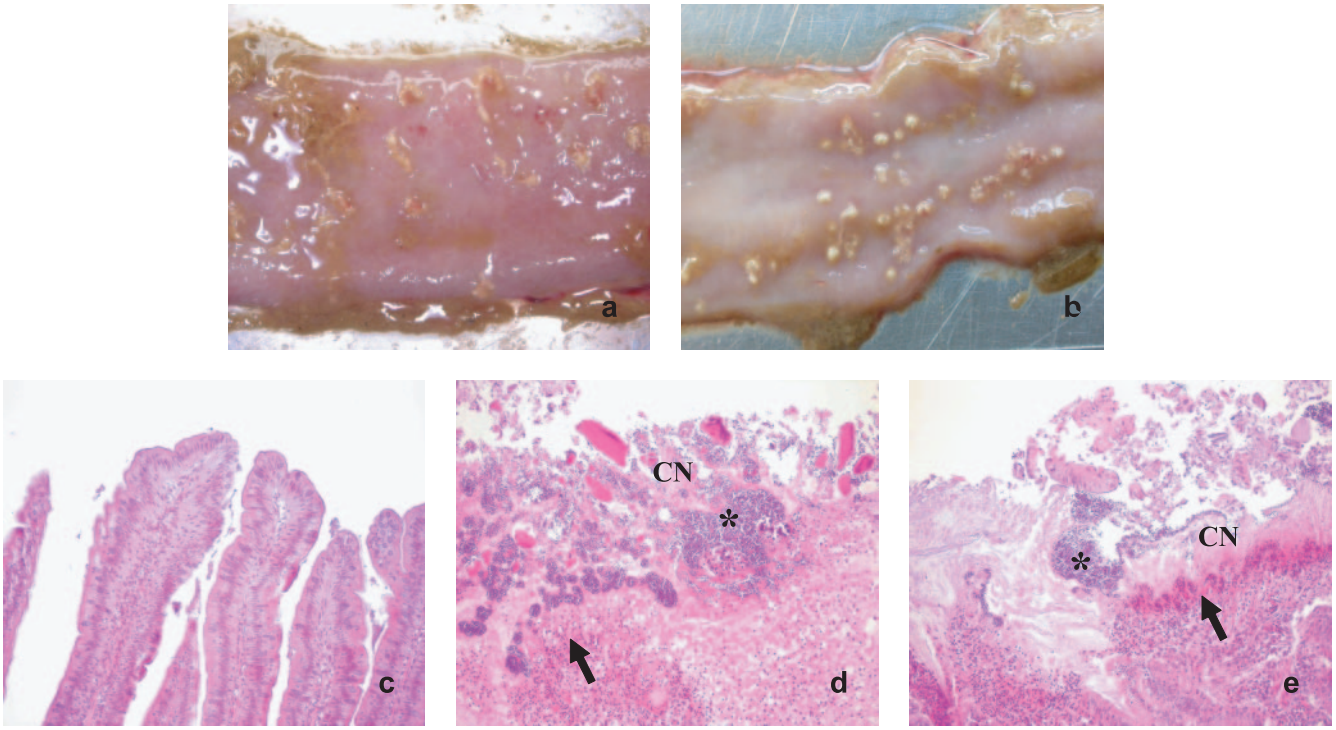

FIG. 2. Gross pathology and histopathology of infected birds. The small intestines of broiler chickens challenged with either EHE-NE18 (a and d) or NE18-M1 (b and e) are shown. (a) Gross pathology of the small intestine of a 24-day broiler chicken challenged with EHE-NE18 (lesion score, 4). (b) Gross pathology of the small intestine of a 24-day broiler chicken challenged with NE18-M1 (lesion score, 4). (c) Histopathology of the small intestine from a control chicken. (d) Histopathology of the small intestine with a thick diffuse layer of necrotic cellular debris mixed with large numbers of bacteria of an H&E-stained section. Magnification, ×100. (e) Histopathology of the small intestine with diffuse coagulative necrosis of the mucosa covered by a thick layer of bacteria/bacilli. The underlying viable tissue is separated from the necrotic mucosa by degenerate heterophils and cellular debris of an H&E-stained section. Magnification, ×100. Arrow, leukocyte or heterophils; *, bacterial cells; CN, coagulative necrosis.

(43). However, the alpha-toxin mutants were not complemented, failing to rule out the possibility that mutations in other regions of the genome resulted in the strain becoming avirulent.

All toxin types of *C. perfringens* produce alpha-toxin, yet only some type A and C strains cause disease in chickens. Clearly, alpha-toxin cannot be sufficient to cause disease, as otherwise all *C. perfringens* strains would be capable of inducing disease, which is clearly not so, as shown by the results obtained with JIR325 in this study. The NE-causing isolates must have other attributes that confer a virulence phenotype. The long-held belief that alpha-toxin is the main virulence factor for NE in chickens has been based on conclusions that extrapolate too much from the data provided. Early studies used culture supernatants to reproduce the disease (3), but these supernatants potentially contained many secreted proteins. Subsequent studies (1, 20) using antibodies prepared against culture supernatants or partially purified toxin preparations did not explore the possibility that toxins other than alpha-toxin may be produced by these bacteria. Since that time, there have been many studies investigating the many other toxins that *C. perfringens* can produce and their involvement in causing animal disease (7, 18, 26, 39, 45). However, no other candidate toxin that has been found in a majority of isolates that cause NE in chickens has been identified.

In conclusion, this study presents definitive evidence that alpha-toxin is not an essential causative agent of NE in chickens and provides the basis for further work to identify virulence factors that do play a crucial role in the development of this disease. Such studies are currently under way in our laboratories.

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