NetB, a New Toxin That Is Associated with Avian Necrotic Enteritis Caused by Clostridium perfringens

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For over 30 years a phospholipase C enzyme called alpha-toxin was thought to be the key virulence factor in necrotic enteritis caused by Clostridium perfringens. However, using a gene knockout mutant we have recently shown that alpha-toxin is not essential for pathogenesis. We have now discovered a key virulence determinant. A novel toxin (NetB) was identified in a C. perfringens strain isolated from a chicken suffering from necrotic enteritis (NE). The toxin displayed limited amino acid sequence similarity to several pore forming toxins including beta-toxin from C. perfringens (38% identity) and alpha-toxin from Staphylococcus aureus (31% identity). NetB was only identified in C. perfringens type A strains isolated from chickens suffering NE. Both purified native NetB and recombinant NetB displayed cytotoxic activity against the chicken leghorn male hepatoma cell line LMH; inducing cell rounding and lysis.

To determine the role of NetB in NE a netB mutant of a virulent C. perfringens chicken isolate was constructed by homologous recombination, and its virulence assessed in a chicken disease model. The netB mutant was unable to cause disease whereas the wild-type parent strain and the netB mutant complemented with a wild-type netB gene caused significant levels of NE. These data show unequivocally that in this isolate a functional NetB toxin is critical for the ability of C. perfringens to cause NE in chickens. This novel toxin is the first definitive virulence factor to be identified in avian C. perfringens strains capable of causing NE. Furthermore, the netB mutant is the first rationally attenuated strain obtained in an NE-causing isolate of C. perfringens; as such it has considerable vaccine potential.

Introduction

Clostridium perfringens is the main causative agent of avian necrotic enteritis (NE), an enteric disease of chickens that was first described in 1961 [1] and has since been found in all poultry producing countries. NE in chickens manifests as an acute or chronic enterotoxemia [2]. The acute disease results in significant levels of mortality whereas the chronic disease leads to loss of productivity and welfare concerns. It has been estimated that the disease costs the international poultry industry in excess of $US2 billion per year [3–5]. NE is primarily caused by C. perfringens type A and to a lesser extent type C strains. Clinical NE is thought to occur when C. perfringens proliferates to high numbers in the small intestine and produces extracellular toxins that damage the gastrointestinal tract.

Early studies on NE suggested that the major virulence factor involved in the disease was secreted by the bacteria [6], which led to the proposal that alpha-toxin was the major toxin involved in pathogenesis. This hypothesis was based on the observations that alpha-toxin is a major secreted protein and that cell-containing and cell-free cultures were able to cause necrotic lesions typical of NE in the gastrointestinal tract of chickens [6–8]. We have constructed an alpha-toxin (ple) null mutant of a virulent necrotic enteritis isolate, EHE-NE18, and have shown that it can still cause disease in chickens, proving that alpha-toxin is not an essential virulence factor and questioning the role of alpha-toxin in the disease process [9].

C. perfringens is a Gram-positive anaerobe and is ubiquitous in the environment, being found in the soil, in decaying organic matter and as a member of the normal intestinal flora of many humans and animals [10]. It has been implicated in numerous diseases [11]. C. perfringens strains produce many different secreted toxins including beta-toxin, a pore-forming toxin that is related to alpha-toxin from Staphylococcus aureus. The S. aureus alpha-toxin, which is not related to the C. perfringens alpha-toxin, forms functional oligomers in membranes and beta-toxin forms a heptameric oligomer on the surface of HL60 cells [12]. Beta-toxin has been implicated in diseases of both man and animals; these diseases are generally characterized by an acute onset with lethal hemorrhagic mucosal ulceration or severe mucosal necrosis of the small intestine [13].

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Author Summary

*Clostridium perfringens* can cause gas gangrene and food poisoning in humans and causes several enterotoxemic diseases in animals including avian necrotic enteritis. This disease affects all chicken producing countries worldwide and is a considerable burden on the commercial chicken production industry. Until recently alpha-toxin was thought to be the major virulence factor involved in necrotic enteritis. However, by using an alpha-toxin null mutant it has been demonstrated that this toxin is not essential for disease. This paper details the identification and characterisation of a novel toxin, NetB, and provides evidence that the protein is an essential factor in causing necrotic enteritis in chickens. NetB has limited protein sequence identity to the beta-toxin of *C. perfringens*, which causes mucosal necrosis of the small intestine in humans and animals. We demonstrate that NetB null mutants can no longer cause disease in chickens, whereas both the wild-type and mutant complemented with a wild-type netB gene caused significant levels of necrotic enteritis. The identification of this important toxin advances our understanding of the pathogenesis of the disease and opens significant opportunities for the development of novel vaccines against necrotic enteritis in poultry.

In this study we report the identification and purification of a novel toxin, NetB, from *C. perfringens*, and show that it has limited similarity to *C. perfringens* beta-toxin. A *C. perfringens* netB mutant was constructed and found to be avirulent in an NE induction animal model; a process that was reversed when the mutation was complemented by the wild-type netB gene. Therefore, the results show that NetB is a critical virulence factor in the pathogenesis of NE in chickens. The netB mutant represents the first example of a rationally attenuated strain obtained by direct targeting of a specific virulence factor of chicken-derived strains of *C. perfringens*.

Results

EHE-NE18 Culture Supernatant Is Cytotoxic to LMH Cells

We previously showed that a plc mutant of the chicken isolate EHE-NE18 was still virulent in chickens [9]. Consequently, to determine if NE strains of *C. perfringens* produced extracellular toxins other than alpha-toxin, sterile filtered culture supernatants of strain EHE-NE18 and its plc mutant NE18-M1 [9] were incubated with various cell lines including Vero, DF1, HD11 and the chicken leghorn male hepatoma (LMH) cell line for 16 h. Cytotoxic effects were observed with both EHE-NE18 and NE18-M1 supernatants on LMH cells at a dilution of up to 1/32 (Figure 1). By contrast, supernatant derived from EHE-NE18 but not NE18-M1 displayed cytopathic effects on Vero cells, indicating that this activity was caused by alpha-toxin. No cytopathic effects were seen after treatment of either DF1 or HD11 cells. Furthermore, no cytopathic effects were observed when LMH cells were incubated with supernatants from JIR325, a strain known to produce high levels of alpha-toxin but which does not cause NE in chickens [9]. These results indicated that the cytotoxicity for LMH cells was produced by a secreted component distinct from alpha-toxin. This cytopathic effect provided a biological assay that we used to identify the presence of toxin during its subsequent isolation and purification from EHE-NE18 supernatant fractions.

Purification of a Cytotoxic Protein from EHE-NE18

Ten ml of a 100× concentrated supernatant from a late log-phase culture of EHE-NE18 was loaded onto a Hi-Trap Mono-Q anion exchange column (GE Life Sciences) in 10 mM Tris-HCl buffer, pH 8.5. Proteins were eluted off the column stepwise, with increasing concentrations of NaCl (up to 1M). Analysis of the fractions in the LMH cytotoxicity assay revealed that only the flow-through fractions were positive (data not shown). These fractions were concentrated and analysed by SDS-PAGE and a major band was observed with a molecular mass of approximately 33 kDa (Figure 2A). N-terminal sequencing of this band identified the first 11 amino acids of the protein as S-E-L-N-D-I-N-K-I-E-L. Database searches failed to reveal any proteins with a similar sequence, indicating that a novel protein had been identified.

Identification of a Novel Toxin Gene in EHE-NE18 Genomic Sequence

In an alternative but complementary approach, genomic sequencing was used in an attempt to identify the cytotoxic protein produced by EHE-NE18. The incomplete EHE-NE18 sequence obtained from 454 Life Sciences contained 3,470,244 bp and was assembled into 381 contigs (av size = 9108 bp). The sequence was analysed for coding potential using the algorithm GeneMarkS [14], which led to the identification of 3771 putative ORFs, with 3081 being longer than 99 amino acids. All 3771 EHE-NE18 ORFs were compared against all CDS features from the *C. perfringens* strain 13 [15], *C. tetani* [16] and *C. acetobutylicum* [17] genomes using BLAST [18], with a match threshold set at 20% identity. These analyses identified 309 EHE-NE18 ORFs with no significant matches to the *C. perfringens* strain 13 ORFs, 229 of which showed no significant match to either *C. tetani* or *C. acetobutylicum* ORFs. These unique ORFs were further analysed by PSORTB [19] and Proteome Analyst [20] to identify predicted extracellular proteins. One of these predicted extracellular proteins was found to have similarity to beta-toxin from *C. perfringens* (38% amino acid sequence identity) and therefore was designated as NetB (Necrotic Enteritis Toxin B-like, EU143299). A potential ribosome binding sequence was located seven bases upstream of the putative TTG start codon of netB, which encoded a putative 323 amino acid protein, with a signal peptide region of 30 residues as predicted by SignalP [21]. The molecular size of the mature NetB toxin, as calculated from the deduced amino acid sequence, was 33 kDa, which correlated with the size of the unknown cytotoxic protein purified from the EHE-NE18 supernatant. Moreover, the predicted N-terminal amino acids of the mature form of the NetB-toxin were identical to the amino acids determined by N-terminal sequencing of the EHE-NE18 cytotoxic protein.

The deduced amino acid sequence of NetB displayed limited sequence similarity to a range of pore forming toxins including *C. perfringens* beta-toxin (38% identity), *Bacillus cereus* haemolysin II (29% identity) and the *S. aureus* alpha-haemolysin precursor (Hla; 30% identity) (Figure 3). Comparison of NetB against the CDD conserved domain database [22] showed that it contained a leukocidin domain, which is also present in beta-toxin, HlyII and Hla, indicating that NetB belongs to the Leukocidin/Hemolysin family of toxins. To further explore the relationship between NetB and other members of the *S. aureus* pore-forming toxin family, we used...
ClustalX and treeview to visualize the phylogenetic relationships (Figure 4). These data showed that the closest orthologues of NetB were the beta-toxin proteins from *C. perfringens*. However, although the NetB-toxin is most similar to *C. perfringens* beta-toxin the level of identity is relatively low and NetB resides on its own deep branch (Figure 4) indicating that it should not be considered as a beta-toxin variant but as a distinct toxin.

Both the Native and Recombinant NetB Proteins Are Cytotoxic for LMH Cells

The netB gene was amplified from EHE-NE18 using PCR, cloned into the expression vector pDest41BA (Table 1) and recombinant NetB produced in *E. coli*. Expression from pDest41BA results in fusion of the recombinant protein with both an N-terminal NusA solubility tag and an N-terminal 6xHis tag. The expressed NetB fusion protein was purified on a nickel affinity column and the NusA and 6xHis fusion tags removed by TEV cleavage. SDS-PAGE indicated that the purified and cleaved protein (designated rNetB) ran at an identical molecular size to the native protein purified from *C. perfringens* (Figure 2A). Furthermore, antibodies raised in rabbits against the rNetB protein recognised both native and recombinant NetB (Figure 2B).

To determine if rNetB was active, LMH cells were treated with the purified protein (Figure 5A, panel 6). Cytopathic effects were observed at similar dilutions to purified native NetB. In addition, preincubation of both rNetB and NetB with the antiserum raised against the *E. coli*-derived rNetB protein neutralized both toxins in the cytotoxicity assay (Figure 5B and data not shown) while pre-treatment with pre-immune sera did not neutralize either toxin.

Osmotic Protection and Estimation of the NetB Pore Size in LMH Cells

Since bioinformatics analysis suggested that NetB may be a pore-forming toxin, cytotoxicity assays were carried out in the presence of polyethylene glycol (PEG) molecules of varying sizes to allow estimation of the NetB pore size in the LMH cell membrane. LMH cells were incubated with purified toxin and lactate dehydrogenase (LDH) release into the supernatant was measured as an indicator of cytolysis using the Cyto-Tox (Promega) kit (Figure 6A). The NetB-induced LMH cytotoxicity was not significantly inhibited by PEG 300 (hydrodynamic diameter of 1.16 nm), was partially inhibited by PEG 400 and PEG 600 (hydrodynamic diameters of 1.36 nm and 1.6 nm, respectively), and was strongly inhibited by PEG 1000 (hydrodynamic diameter of 1.8 nm) and PEG 1500 (hydrodynamic diameter of 2.4 nm) (Figure 6B). These results indicate that NetB forms a hydrophilic pore in the cell membrane with a functional diameter of approximately 1.6–1.8 nm.

Most NE-causing *C. perfringens* Strains Contain the netB Gene and Produce NetB-toxin

The presence of the netB gene in NE-causing and non-NE-derived strains of *C. perfringens* was investigated by PCR using netB-specific primers. Of the 18 poultry-derived NE *C. perfringens* strains tested, 14 were positive for netB (data not shown).
shown) although three of the four netB negative isolates were from the same NE outbreak and are most likely from the same clonal population. No netB-specific product was observed in any of the 32 non-NE-derived strains, which included C. perfringens type A, B, C and D strains isolated from cattle, sheep, pigs and humans. The production of NetB toxin in the netB-positive strains was confirmed by Western blotting of culture supernatants using rabbit antiserum against rNetB. There was an absolute correlation between the netB-PCR and the NetB-Western blots (data not shown) with a single band visualised for each positive strain at the appropriate size similar to native NetB. None of the strains that were negative in the PCR assay for netB produced an immunoreactive NetB band. Moreover, no cytopathic effect was observed when culture supernatants from the four netB-negative NE strains were incubated with LMH cells (data not shown).

Construction of a netB Mutant of EHE-NE18

To determine the role of NetB in the pathogenesis of NE in chickens, a netB mutant was isolated. The ΔnetB suicide plasmid pALK16 (Table 1) was constructed and used to transform strain EHE-NE18 to thiamphenicol resistance. Two independently derived netB mutants, NE18ΔnetB1 and NE18ΔnetB2, were isolated from five separate transformation experiments. Their genotypes were confirmed by PCR (data not shown) and Southern blot analysis (Figure 7). Genomic DNA from EHE-NE18 and NE18ΔnetB1 was digested with HindIII and analysed separately with DIG-labelled catP and netB specific probes. As expected, the netB probe bound to DNA from both the wild-type and mutant strains, hybridising to the expected 3.8-kb genomic DNA fragment in the wild-type strain and to a 4.4-kb fragment in the NE18ΔnetB1 mutant. The hybridising NE18ΔnetB1 fragment was less intense than the hybridising wild-type fragment as most of the sequence present in the probe had been deleted in the mutant strain. In NE18ΔnetB1 the catP probe hybridized to a band of the same size as the netB reactive band, but did not...
bind to EHE-NE18 DNA. These data confirmed that both mutants were derived from double reciprocal crossover events between pALK16 and the netB region in EHE-NE18. Similar results were obtained with the second independently derived netB mutant (NE18DnetB2). Culture supernatants from the netB mutant strains showed no cytotoxicity in the LMH assay (Figure 5A) but were capable of alpha-toxin and perfringolysin O expression as demonstrated by the presence of zones of precipitation or hemolysis on egg yolk agar and sheep blood agar, respectively (data not shown).

To confirm that the loss of cytotoxicity was associated with the mutation in netB, both mutants were complemented in trans with the multicopy plasmid, pALK20 (Table 1), which contained a wild-type copy of the netB gene cloned into the shuttle vector, pJIR1457 (Table 1). These plasmids were introduced separately into the mutant strains and the resultant strains assayed for their cytotoxicity. Cytotoxic activity was restored when the netB mutant strains were complemented with pALK20 but not with pJIR1457 (Figure 5A). Therefore, NetB is responsible for the cytotoxic activity of EHE-NE18 supernatants on LMH cells.

The netB Mutants Do Not Produce NE in a Chicken Disease Model

To determine if the netB mutant could cause NE, groups of 10 Ross 308 broiler chickens were challenged with wild-type strain, the isogenic NE18DnetB1 mutant, or the complemented NE18DnetB1 strains in a NE disease induction model. No NE lesions were detected in birds infected with the netB mutant or the mutant complemented with the vector plasmid pJIR1457. By contrast, significant levels of disease were detected in birds infected with the wild-type parent strain (p < 0.01) or the strain complemented with the netB+ plasmid pALK20 (p < 0.05) (Figure 8). Similar results were obtained in independent virulence trials. Bacteria were cultured from the intestines of birds from all groups and with one exception only the infecting strain was isolated from the lesions. In one of the trials, two birds in the netB mutant challenge group showed a small number of lesions typical of NE. However, no thiamphenicol resistant C. perfringens were isolated from these birds and it was concluded that these lesions were likely to have been caused by environmental or endemic C. perfringens isolates and therefore are not included in our analysis.
In this paper we report the identification and initial characterization of NetB, a novel C. perfringens toxin. This toxin appears to be carried only by NE-causing C. perfringens type A strains as none of the 32 non-NE-derived strains tested either produced the toxin or carried the netB gene. The in vivo analysis of an isogenic netB mutant and its complemented derivative provided unequivocal evidence that NetB is an essential virulence factor in the pathogenesis of NE in chickens. When the netB gene was insertionally inactivated by a double crossover event the resultant mutant was avirulent in the NE disease model. Complementation with the wild-type netB gene restored the ability to cause NE in chickens, fulfilling the requirements of molecular Koch’s postulates. Importantly, the gross lesions observed in the animal model were consistent with NE lesions previously reported from experimental and field cases of the disease.

| Table 1. Bacterial Strains and Plasmids |
|----------------------------------------|
| **Strain or Plasmid** | **Name** | **Relevant Characteristics** | **Source/Reference** |
|-----------------------|----------|-----------------------------|---------------------|
| **Strains**           |          |                             |                     |
| JIR325                | Strain 13 RifR NalR                  | [50]               |
| NE18ΔnetB1            | EHE-NE18 ΔnetB::catP                 | This study        |
| NE18ΔnetB2            | EHE-NE18 ΔnetB::catP                 | This study        |
| NE18-M1               | EHE-NE18 Δpsi::catP                  |                     |
| JM109                 | F' traD36 proA::B- lacQ ΔlacZlM15/ Δ(lac-proAB) glmV44 e14- gyrA96 relA1 endA1 thi hsdR17 | New England Biolabs |
| **Plasmids**          |          |                             |                     |
| pJIR1457              | EmR oriCP oriEC oriT                 | [51]               |
| pJIR20                | CmR oriCP oriEC                      | [52]               |
| pALK1                 | pSM20 (BamHI-SpeI:pJIR750, 0.9 kb) Δgfp (CmR KnR) | [9]                |
| pALK15                | pALK1 (1.9 kb upstream netB fragment,catP,1.4 kb downstream netB fragment) (CmR KnR) | This study        |
| pALK16                | pALK15 (erm(B) oriT::pJIR1457, 1.7 kb) (CmR KnR) | This study        |
| pALK20                | pJIR1457 (EcoRI:netB+, 1.7 kb) (EmR) | This study        |
| pDEST41BA             | E. coli expression vector. Expresses recombinant fusion proteins with an N-terminal NusA and 6x-His tag | This study        |

CmR, chloramphenicol and thiamphenicol resistant; EmR, erythromycin resistant; KnR, kanamycin resistant; NalR, nalidixic acid resistant; RifR, rifampicin resistant.
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**Discussion**

In this paper we report the identification and initial characterization of NetB, a novel C. perfringens toxin. This toxin appears to be carried only by NE-causing C. perfringens type A strains as none of the 32 non-NE-derived strains tested either produced the toxin or carried the netB gene. The in vivo analysis of an isogenic netB mutant and its complemented derivative provided unequivocal evidence that NetB is an essential virulence factor in the pathogenesis of NE in chickens. When the netB gene was insertionally inactivated by a double crossover event the resultant mutant was avirulent in the NE disease model. Complementation with the wild-type netB gene restored the ability to cause NE in chickens, fulfilling the requirements of molecular Koch’s postulates. Importantly, the gross lesions observed in the animal model were consistent with NE lesions previously reported from experimental and field cases of the disease.

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**Figure 5. Cytotoxic Activity of Supernatants from EHE-NE18, EHE-NE18ΔnetB1, and Complemented Mutants**

The LMH cells were cultured until 70% confluence in 24 well plates coated in 0.2% gelatine and grown in EMEM medium at 37 °C. (A) Cytotoxicity assays. Culture supernatant was added to the medium and incubated for up to 16 h at 37 °C. (1) EHE-NE18 (1:16 dilution); (2) NE18ΔnetB1 (1:2 dilution); (3) NE18ΔnetB1 (pJIR1457) (shuttle plasmid) (1:2 dilution); (4) NE18ΔnetB1 (pALK20) (netB+ complementation plasmid) (1:16 dilution); (5) TPG culture medium (undiluted); (6) Purified rNetB (0.5 µg). (B) Neutralisation of NetB cytotoxicity. Pre-immune sera and antisera raised against rNetB were incubated with 0.5 µg/ml of purified native NetB-toxin (1:20) for 1 h at room temperature. Treated and non-treated samples where added to LMH cells and incubated for 16 h at 37 °C. Cytopathic effects were observed under the light microscope at 100X magnification. (1) NetB positive control; (2) NetB pretreated with pre-immune rabbit sera; (3) NetB pretreated with rabbit anti-rNetB antiserum.
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While, in this study, we only tested strains from chickens suffering NE and NetB was not present in non-NE strains, further more extensive epidemiological studies need to be carried out to determine the international distribution of this new toxin and its correlation with disease.

For many years alpha-toxin was believed to be the main virulence factor involved in NE pathogenesis in chickens. However, this hypothesis was based on studies that used culture supernatants to reproduce the disease [6] even though the supernatants could potentially contain many secreted proteins. Furthermore, subsequent studies [8,25] that used antibodies prepared against culture supernatants or partially purified toxin preparations did not explore the possibility that toxins other than alpha-toxin might be produced by the *C. perfringens* strains. The identification of NetB, together with our recent observation that alpha-toxin negative *C. perfringens* mutants remain virulent [9], adds further weight to the proposal that alpha-toxin plays only a minor role in NE disease pathogenesis.

In other studies the presence of beta2-toxin in *C. perfringens* type A strains has been associated with enteric diseases in human and animals [26–28]. The detailed analysis of these isolates has revealed that a high proportion of strains from avian sources contain an atypical beta2-toxin [29]. While this gene is present in a high proportion of non-porcine *C. perfringens* isolates it remains unclear if it is involved in enteric diseases of other animals including chickens. Importantly, *cpb2* positive strains can be found in both healthy and diseased chickens; its presence does not correlate with disease [30].

The mature NetB toxin was similar in molecular size to mature beta-toxin (33.2 kDa vs 34.8 kDa). However, the two toxins have limited sequence identity (38%) and phylogenetic analysis indicated that NetB was clearly a distinct toxin that is not a member of the beta-toxin clade (Figure 5B). However, the presence of conserved amino acid residues in NetB and beta-toxin suggested that NetB may also act as a pore forming toxin [13]. NetB displays many of the conserved residues found in other pore-forming toxins of the *S. aureus* alpha-toxin family [31]. Site-directed mutagenesis has been used to characterize many amino acids critical for function in *C. perfringens* beta toxin and *S. aureus* alpha toxin [32,33] and NetB contains many of these key residues including R200 (R212 from beta-toxin). The corresponding residue in *S. aureus* alpha-toxin (R200) is important for binding and oligomerization of the protein as well as for hemolysis [34]. Mutation of the beta-toxin residue Y203 (Y191 in NetB) to phenylalanine resulted in a 2.5-fold increase in the LD50.
corresponding residue in \textit{S. aureus} alpha-toxin (Y191) is located at the predicted membrane binding surface of the protein [35]. Mutation of the beta-toxin residue D167 (D156 in NetB) resulted in a complete loss of functional protein expression, suggesting that this region participates in crucial protomer-protomer interactions as well as being important for conformational rearrangements involved in multimer formation in the membrane bound form [35]. Since NetB aligns at many of these specific residues essential for beta-toxin and \textit{S. aureus} alpha-toxin function we postulate that NetB is likely to be a pore-forming toxin.

We have shown that NetB induced morphological changes in LMH cells, resulting in significant rounding and cell lysis. Since these morphological changes and LDH release were blocked by PEG1000 and PEG1500, it was likely that these changes were caused by toxin-dependent formation of pores in the plasma membrane. Osmotic stabilizers such as PEG can inhibit the lysis of target cells if they are unable to pass through the pore generated by a pore-forming toxin [36]. This observation has been used to estimate the size of the pore formed by various other pore-forming toxins, including the \textit{Xenorhabdus nematophila} fimbrial shaft protein (MrxA) [37], the \textit{E. coli} hemolysin [38], the \textit{C. septicaen} alpha-toxin [36] and the \textit{Vibrio metchnikovii} cytolyisin [39]. Based on the estimated Stokes radii for various PEG molecules [40] the results obtained here suggest that NetB forms a hydrophilic pore with a functional diameter of 1.6–1.8 nm in the cell membrane [41]. This NetB pore diameter is slightly larger to the pore size predicted for beta-toxin (1.36–1.6 nm) in HL 60 cells [12].

Although a high proportion (14/18) of NE strains contained \textit{netB} and expressed the NetB toxin we identified four NE strains that were \textit{netB}-negative. These strains were isolated from chickens suffering NE and were able to cause NE in our experimental model (data not shown) however, three of these strains were isolated from the same NE outbreak and may represent the same clonal population. These data indicate that the presence of NetB may not be essential for the disease process in all \textit{C. perfringens} isolates. In EHE-NE18 it is clear that production of NetB toxin is required for disease. However, in the virulent \textit{netB}-negative strains other, as yet unidentified factors, may be sufficient for disease production. Indeed, there may be distinct classes of NE-causing \textit{C. perfringens} strains and we are currently analysing these other strains for novel toxins.

In conclusion, we have identified a novel \textit{C. perfringens} pore-forming toxin which we designated NetB. While it is possible that NE may result from the interaction of several toxic molecules it is clear from the evidence provided here that NetB is a critical \textit{C. perfringens} EHE-NE18 virulence factor and to our knowledge is the first virulence factor unequivocally shown to be associated with NE in chickens. The identification of this important toxin opens significant opportunities for the development of novel vaccines against NE in poultry and these studies are currently underway in our laboratories.

\textbf{Materials and Methods}

\textbf{Bacterial strains and growth conditions.} Bacterial strains and plasmids are described in Table 1. \textit{C. perfringens} was grown in tryptone-proteose peptone glucose (TPG) [42], fluid thioglycollate broth (FTG, Beckson, Dickinson and Company), TSC agar (Oxoid), egg-yolk agar (EYA) [43] and Brain Heart Infusion agar with 5% sheep blood (SBA). Agar cultures were grown at 37°C in an atmosphere containing 10% H2, 10% CO2 and 80% N2. \textit{C. perfringens} media were supplemented with thiamphenicol (5 μg/ml) or erythromycin (50 μg/ml) as required. \textit{E. coli} strains were derivatives of JM109 and were grown in LB broth or agar under aerobic conditions at 37°C. \textit{E. coli} media were supplemented with ampicillin (100 μg/ml), erythromycin (150 μg/ml) or chloramphenicol (30 μg/ml) as required.

\textbf{Genomic sequencing and bioinformatics of EHE-NE18.} The genomic DNA sequence of \textit{C. perfringens} EHE-NE18 was determined by 454 Life Science Corp. GeneMarkS was used to predict possible open reading frames [14]. Signal peptide prediction was performed using the SignalP v 3.0 program [21]. Sequences homologous to the deduced amino acid sequence were searched using the gapped BLAST program [18]. A multiple sequence alignment was executed using the CLUSTAL W program [44]. ClustalX [45] with Neighbour-Joining bootstrap (1000 iterations) analysis was used to construct a phylogenetic tree of NetB and related toxins. The tree was visualized using TreeView [46].

\textbf{Native NetB production and purification.} EHE-NE18 was grown in TPG broth to a turbidity of 0.6 at 600nm. Culture supernatant (3 L) was obtained by centrifugation at 18 000 g for 15 min at 4°C. The supernatant was concentrated 5x using ultrafiltration (Amicon 8400) through a 10kDa membrane (DIAFLO YM10–76 mm, Amicon) and supernatant proteins precipitated by overnight incubation in 40% (w/v) (NH4)2SO4 at 4°C overnight followed by centrifugation at 18 000 g for 2 h at 4°C. The precipitate, containing the toxin, was resuspended in 30 ml of PBS and dialysed against 10mM Tris-HCl buffer (pH 8.5) for 48 h at 4°C with 5 buffer changes. Proteins were separated by Sepharose Q FF (GE) anion exchange chromatography in 10 mM Tris-HCl buffer (pH 8.5) and the flow through collected. The NetB protein (detected at approximately 33 KDa) was
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Purification of recombinant NetB and generation of rabbit anti-NetB serum. The netB gene was PCR cloned into the Gateway vector pENTR/D-TOPO (Invitrogen), inserted into the modified Gateway™ expression vector pDestHIBA (Table 1). The recombinant fusion protein was purified on a nickel affinity column followed by gel filtration on Superdex 200. Peak fractions were analyzed by SDS-PAGE and the full-length fusion protein was validated using Western blotting and reloaded onto a nickel column to remove unclaved protein and the TEV protease. Recombinant protein (~1.3 mg) was sent to Chemicon (Chemicon-Millipore) for antibody production in rabbits. The rabbit-antibody raised against NetB toxin was used in Western blot analysis of C. perfringens strains and for neutralization studies. In the latter studies anti-NetB sera was incubated with semi-purified toxin and proteins were transferred onto PDVF membranes and probed with rabbit polyclonal anti-rNetB antisera. Blots were developed with an ECL Western Blotting kit (Amersham Biosciences) and the results recorded on autoradiographic film.

Construction of defined netB mutants of EHE-N18. DNA manipulations were carried out according to standard techniques [48]. Products were cloned into the pCP26 shuttle vector system (Promega) and subsequently sub-cloned as required. The marked, partial deletion, suicide plasmid, pALK17, was constructed by cloning fragments of the netB gene and surrounding region into either side of the attP cassette in pALK1 [9], which resulted in an 541 bp internal deletion within the netB gene. First, a 1490 bp MfeI-Spel fragment amplified using oligonucleotides AKP60 (5'-GAAATTCCTTTATTTGTGTGAT-3') and AKP61 (5'-GGCTTAGTTTACCAATTGCTATTG-3') was directionally cloned into the EcoRI-SpeI sites of pALK1, followed by cloning of a 1957 bp BamHI-Nhel fragment amplified using oligonucleotides AKP58 (5'-GGGATCCATTGGAACATCCTGATGAT-3') and AKP59 (5'-CAATTGGCAAGATCATAAAATAGAA-3') into the BamHI-Nhel sites of the resultant plasmid. Finally, the nmrB gene and the ort region were amplified from pJR185 and blunt-end cloned into the Smal site. The final suicide plasmid pALK16 was introduced into C. perfringens strain EHE-N18 as described previously [49]. After growth at 37 °C on TSC supplemented with thiampenicol, colonies were patched onto TSC supplemented with erythromycin to confirm that a double crossover event had occurred. The colonies were confirmed as thiampenicillin and erythromycin sensitive were selected for further analysis. Genomic DNA was prepared and PCR and Southern blot analysis was used to confirm that the mutants were derived from double crossover events within the netB gene region. The primer pairs of AKP33 (5'-AAGTGCCTG-CAGCTCAGTTAG-3') and AKP38 (5'-TGATCCCTTATTTAC-TAGT-G-3'), AKP48 (5'-TTGGCTCTAGGACGCGATCC-3') and AKP49 (5'-GGCTTAGTTTACCAATTGCTATTG-3') were used for confirmation of insertion within the netB region, and the netB region was then probed with labelled netB and ortP gene fragments. The complementation plasmid, pALK20, was constructed by cloning the wild-type netB gene into the C. perfringens shuttle vector pJR1457. The complementation plasmid pALK20 and the vector pJR1457 were introduced into confirmed mutants by electroporation [49] and erythromycin resistant transformants selected.

Southern hybridization analysis. Genomic DNA of wild-type and netB mutant strains was digested with HindIII, separated by gel electrophoresis on a 0.8% agarose gel and transferred to a nylon membrane (Amersham). The blots were hybridized with DIG-labeled PCR amplified probes as specified by the manufacturer (Roche). The probes were either a 410-bp ortP-specific product amplified from pJR790 or a 563-bp netB product amplified from EHE-N18 genomic DNA. Hybridization was detected using a chemiluminescent detection system (Roche).

Detection of the netB gene in various strains. The presence of the netB gene in various strains of C. perfringens was investigated by PCR. A single colony of each strain was suspended in 100 μl distilled water, boiled for 10 min and then centrifuged at 10 000 g for 10 min. The supernatants were collected and used as the template for PCR. PCR was performed in a 25 μl reaction mixture containing 1x PCR buffer (Mg²⁺ free, Promega); 2.5 mM MgCl₂; 0.2 mM dNTP mixture; 2.5 units of Go Taq DNA polymerase (Promega); 50 μM of primers (5'-GGCTTAGTTTACCAATTGCTATTG-3' and 5'-TGGCATGATTGATTGTTCC-3'); and 5 μl of template solution. The following conditions were used: denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s; annealing at 55 °C for 30 s; and extension at 72 °C for 2 min; with the final extension step at 72 °C for 12 min. PCR products were analysed by electrophoresis on 1.5% agarose gels.

Cell lines and cytotoxicity assay. The chicken hepatoma cell line LMH (ATCC CRL-2117) and the Vero African green monkey kidney cell line (Vero, ATCC CCL-81) were maintained in Earl's minimum essential medium (EMEM) supplemented with L-glutamine, 10% fetal calf serum (FCS), 100 U/ml of penicillin and 100 μg/ml streptomycin and 100 μg/ml fungizone (LMH cells also require 0.2% gelatine for adherence to surfaces). Chicken embryo fibroblast cells (DF-1, ATCC CRL-12253) were used to measure LDH release and the results expressed as percentage cytotoxicity.

Pore size estimates. The size of the pore generated by NetB on LMH cells was determined by osmotic protection of toxin-treated cells with PEG preparations of various molecular sizes (PEG 300 - PEG 1500). LMH cells were cultured in 96-well tissue culture plates (Nunc) in 200 μl of EMEM culture medium. Osmotic protection of the cells by PEG (25 mM) was performed in the presence of 0.5 μg of NetB per well, which caused 50% lysis of the cells in 4 h. The experiment was repeated three times, and the values reported represent the mean ± the standard deviation of triplicate samples from one representative experiment. Percent protection was calculated based on the amount of LDH released where the LDH release in the absence of PEG was taken as 100% and in the absence of NetB was taken as 0%. Pore-size estimation was determined using the calculated Einstein-Stokes radius for each of the PEG molecules used [40,41].

In vivo C. perfringens challenge model. The disease induction model was performed as previously described [9]. Briefly, commercial 1-day-old 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% fishmeal. On day 21 birds were challenged with C. perfringens culture (10⁹ to 10⁶ CFU). On day 21 birds were again orally challenged and fed contaminated with C. perfringens was administered. C. perfringens strains were grown in FTG broth with the addition of 2% soluble starch and 1.5% thiopepton and incubated at 37 °C for 14 h. Groups of 10 chickens were kept in adjacent separate pens in an animal isolation facility. On day 24, chickens were euthanized with inhaled carbon dioxide gas and their small intestines (duodenum to ileum) examined for gross necrotic lesions. Intestinal lesions in the small intestine (duodenum to ileum) were scored as follows: 0 = no gross lesions; 1 = frond-like necrosis or ulceration (1–5 foci); 2 = focal necrosis or ulceration (6–15 foci); 3 = focal necrosis or ulceration (16 or more foci) 5 = patches of necrosis 2–3 cm long; 6 = diffuse necrosis typical of field cases. C. perfringens was recovered from the intestines of killed chickens by culturing on TSC agar. All animal experiments were approved and monitored by the Australian Animal Health Laboratories animal ethics committee.

Supporting Information

Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Entrez) accession numbers in this study are: NetB from C. perfringens: EU143239; Beta-toxin C. perfringens: CAA58246.1, AAA23584.1, CAB75343.1; Hla from S. aureus: NP_371687.1, YP_180636.1, 7AHLA, P09616.0, NP_658661.1, HlyII from B. cereus: AAB51156.1, YP_083859.1, NP_653326.1.
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Genomics Protein Production Unit for recombinant protein purification.

Author contributions. ALK, JIR, and RJM conceived and designed the experiments. ALK, JDB, MEF, ADR, and RJM performed the experiments. ALK, JDB, TLB, DJP, JIR, and RJM analyzed the data. ALK, JDB, PV, and RJM contributed reagents/materials/analysis tools. ALK, JIR, and RJM wrote the paper.

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