

Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing NetB toxin

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Abstract – A novel toxin, NetB, has recently been identified in virulent avian *Clostridium perfringens* isolates and shown to be an essential virulence factor in a clinical necrotic enteritis isolate. To assess whether NetB is more generally associated with avian necrotic enteritis isolates we have screened a range of *C. perfringens* strains from geographically diverse locations for both the presence and expression of the *netB* gene. Forty-four isolates were derived from necrotic enteritis disease cases from Australia, Belgium, Denmark and Canada and 55 isolates from healthy chickens from Australia and Belgium. The majority of strains isolated from necrotic enteritis-affected birds were *netB* positive (70%) and there was an absolute correlation between the presence of *netB* and in vitro expression of the NetB protein. Only two of the *C. perfringens* isolates from healthy chickens carried *netB*. Sequencing of the *netB* gene from 23 positive isolates showed that NetB is highly conserved, with only one predicted amino acid (A168T) difference, in six isolates, compared to the published sequence. This change did not alter the in vitro activity of the NetB toxin. The gene encoding the recently discovered TpeL toxin was also screened using PCR and only found in a small proportion of NetB-positive isolates from diseased birds. A selection of NetB-negative isolates, originating from diseased birds, was unable to cause disease in a necrotic enteritis induction model. This study provides further evidence that NetB is important in pathogenesis and advances our current understanding of *C. perfringens* virulence factors in avian necrotic enteritis.

Clostridium perfringens / necrotic enteritis / NetB toxin / virulence factor / TpeL

1. INTRODUCTION

Necrotic enteritis in chickens is a common bacterial disease in the poultry industry and is caused by an increase in the population of pathogenic *Clostridium perfringens* in the chicken's gastrointestinal tract [4, 5]. *C. perfringens* iso-

lates are categorized into five toxin types: A, B, C, D and E, depending on the production of four major toxins: α , β , ϵ and ι toxins [18]. Type A strains produce α -toxin, but do not produce any of the other three typing toxins (β , ϵ , ι). In addition, all strains may produce other toxins such as enterotoxin (CPE) and β 2-toxin. Type A strains of *C. perfringens* are the most common toxin type isolated from chickens

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suffering from necrotic enteritis [20, 22]. It was previously thought [1, 2, 9] that α -toxin was the major toxin involved in necrotic enteritis but we have shown that a α -toxin null mutant is still virulent [11].

Recently, we identified a new toxin, NetB, in avian *C. perfringens* type A strains. Initial studies indicated that NetB is only associated with strains isolated from chickens [12], although a recent study reported the recovery of a *netB*-positive *C. perfringens* isolate from a cow with liver abscesses [14]. NetB has limited sequence identity to the β -toxin from *C. perfringens*, which is associated with gastrointestinal syndromes in both humans and animals [23]. The importance of NetB in avian necrotic enteritis was demonstrated when a *netB* mutant did not cause necrotic enteritis in an experimental chicken model and virulence was restored when a functional *netB* gene was introduced back into the mutant strain [12]. This study demonstrated that NetB is an essential virulence factor, at least in one avian isolate.

Recent strain surveys in North America have found that the majority of *C. perfringens* isolates from chickens with clinical signs of necrotic enteritis carry *netB* whereas only a small percentage of isolates from healthy birds carry the gene [4, 14]. No surveys have yet investigated whether *netB* in each strain are functional and are expressed. This is an important issue since there is precedent for toxin genes being present but not expressed in *C. perfringens* [6].

Although we have clearly demonstrated that NetB is an essential virulence factor in strain EHE-NE18 [12], several studies have found that a minority of *C. perfringens* strains isolated from clear cases of necrotic enteritis do not carry *netB* [3, 12, 14]. The question therefore arises as to whether these *netB*-negative strains produce an alternative toxin that is responsible for necrotic enteritis. Another toxin, TpeL, has recently been identified in some chicken derived *C. perfringens* strains, although only in some *netB*-positive isolates [4].

In the work reported here the carriage of *netB* and *tpeL* and the production of NetB toxin have been assessed in geographically diverse *C. perfringens* necrotic enteritis isolates from Belgium, Denmark, Australia, and Canada, as

well as isolates from healthy birds. In addition, we sequenced *netB* from many of these isolates to determine its sequence variation. Finally, we carried out virulence studies in chickens on *netB*-negative and *netB*-positive strains derived from diseased chickens. The results provide further evidence that NetB is important in the pathogenesis of avian necrotic enteritis.

2. MATERIALS AND METHODS

2.1. Bacterial isolates and growth conditions

The *C. perfringens* strains used in this study were isolated from chickens displaying clinical signs of necrotic enteritis (Tab. I) and from healthy chickens. *C. perfringens* were grown in tryptone-proteose peptone glucose (TPG) [13], fluid thioglycollate (FTG) (Becton, Dickinson and Company, NJ, USA) or TSC agar (Oxoid, Hampshire, UK). Agar cultures were grown at 37 °C in an atmosphere containing 10% H₂, 10% CO₂ and 80% N₂.

2.2. Detection of the *netB* and *tpeL* genes in various strains

The presence of *netB* and *tpeL* in *C. perfringens* strains was investigated by PCR. Clostridial genomic DNA was isolated as previously reported [17]. PCR amplification of the genomic DNA used Taq DNA polymerase (Roche) and 0.5 μ M concentration of each primer. Denaturation (94 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 1 min) steps were performed for 35 cycles. Internal primer pairs AKP78 (5'GCTGGTGCTGGAATAAATGC3') and AKP79 (5'TCGCCATTGAGTAGTTTCCC3') and AKP80 (5'ATATAGAGTCAAGCAGTGGAG3') and AKP81 (5'GGAATACCACTTGATATACCTG3') were used to screen the *C. perfringens* strains for the presence of *netB* and *tpeL*, respectively. For sequencing of *netB*, the PCR and sequencing primers were JRP3943 (5'TTTTCTTTAGACATGTCCATAGGC3'), which binds 268 bp upstream of the *netB* open reading frame (ORF), and JRP3944 (5'CCA TC CTTATTTTCATCAGCATTTA3'), which binds 348 bp downstream of the *netB* ORF. The primers CPNE3416F (5'CACCATGAGTGAATTAATGA CATAAAC3') and CPNE3416R (5'CAGATAATAT TCTATTTTATGATCTTG3') were also used to sequence the amplified PCR products. PCR products were purified using the QIAquick PCR purification

Table I. Necrotic enteritis *C. perfringens* strains and PCR, Western blotting and animal trial results.

Strain	Source ^a	<i>netB</i> ^b	NetB ^c	<i>netB</i> sequence ^d	% disease induction ^e	Reference
NAG-NE1	Aus	–	–		0	[19]
EHE-NE3	Aus	+	+	Con		[19]
EHE-NE4	Aus	+	+	Con		[19]
EHE-NE5	Aus	+	+	Con	47	[19]
EHE-NE7	Aus	+	+	G769A (A168T)		[19]
EHE-NE9	Aus	+	+	G769A (A168T)		[19]
EHE-NE13	Aus	+	+	Con	87	[19]
EHE-NE14	Aus	+	+	Con		[19]
EUR-NE15	Aus	+	+	Con	75	[19]
EHE-NE16	Aus	+	+	Con		[19]
EHE-NE17	Aus	+	+	Con		[19]
EHE-NE18	Aus	+	+	Con	100	[19]
EHE-NE20	Aus	+	+	Con		[11]
EHE-NE21	Aus	+	+	Con		[11]
EHE-NE22	Aus	+	+	Con		[11]
NAG-NE23	Aus	–	–			[19]
NAG-NE24	Aus	–	–		0	[19]
NAG-NE25	Aus	–	–		11	[19]
UNK-NE30	Aus	+		NA	154	This study
NAG-NE31 ^f	Aus	+		NA	68	This study
NAG-NE32 ^f	Aus	+		NA		This study
BER-NE33	Aus	–			0	This study
SOM-NE34	Aus	+		NA	46	This study
SOM-NE35	Aus	+		NA	77	This study
GrE ABAT	Can	+		Con	77	M. Boulianne, pers. com.
3 MB 2003	Can	+		G769A (A168T)		M. Boulianne, pers. com.
6 MB 2006	Can	+		Con	77	M. Boulianne, pers. com.
PF3	Can	–			0	M. Boulianne, pers. com.
SHY07 383	Can	–				M. Boulianne, pers. com.
ENV D4	Can	–				M. Boulianne, pers. com.
R04-134 ^T	Can	–				M. Boulianne, pers. com.
R04-104	Can	–				M. Boulianne, pers. com.
R03-382	Can	+		NA		M. Boulianne, pers. com.
48	Bel	–	–		0	[10]
67	Bel	+	+	Con		[10]
56	Bel	+	+	Con	80	[10]
37	Bel	+	+	G769A (A168T)		[10]
200302-1-1-Ba	Den	+	+	Con		L. Bjerrum, pers. com.
99.63206-34 ^f	Den	+	+	G769A (A168T)		L. Bjerrum, pers. com.
97.78247-2	Den	–	–		0	L. Bjerrum, pers. com.
75.65948-1	Den	–	+	Con		L. Bjerrum, pers. com.
00.82196-2	Den	–	–			L. Bjerrum, pers. com.
98.78718-2	Den	+	+	NA		L. Bjerrum, pers. com.
301001-1-B1 ^f	Den	+	+	G769A (A168T)		L. Bjerrum, pers. com.

^a Aus, Australia; Can, Canada; Bel, Belgium; Den, Denmark.

^b PCR analysis of *C. perfringens* strains, + indicates *netB* positive product of 383 bp was amplified, – indicates no amplification product.

^c Western blot results probing with rNetB anti-serum, + indicates product of 33 kDa, – indicates no product.

^d *C. perfringens netB* sequence, NA indicates the *netB* sequence was not determined, Con indicates EHE-NE18 consensus sequence (EU143239), G769A (A168T) indicates nucleotide change resulting in amino acid change. GenBank accession numbers for *netB* sequences are FJ189481-FJ189503.

^e Results of strain use in disease induction model. The results are derived from a series of independent trials. To allow a simple comparison of all the results, the average lesion score for each group was compared to the lesion score for EHE-NE18 challenge in the same trial (included in all trials as our standard challenge strain).

^f Indicates *C. perfringens* strains that are *tpeL* positive with a product size of 474 bp.

^T Indicates *C. perfringens* isolate from diseased turkey.

kit before sequencing on an Applied Biosystems 3730S capillary sequencer.

2.3. Western blot analysis of *C. perfringens* isolates

Detection of NetB was performed as previously described [12]. Briefly, *C. perfringens* strains were grown in TPG broth to a turbidity at 600 nm of 0.6. Culture supernatants were obtained by centrifugation at 18 000 g for 10 min and separated by SDS-PAGE (NuPAGE® Novex 4–12% Bis-Tris gel, Invitrogen, CA, USA) in MES SDS running buffer (NuPAGE® MES SDS Running Buffer, Invitrogen). Proteins were transferred onto PDVF (PALL) membranes and probed with rabbit polyclonal anti-recombinant NetB (rNetB) anti-serum. Blots were developed with an ECL Western blotting kit (GE Healthcare Bio-sciences Pty. Ltd., Rydalmere, Australia) and the results recorded on autoradiographic film.

2.4. Cell line and cytotoxicity assay

Cytotoxicity assays were performed as previously described [12]. Volume normalized, dialyzed culture supernatant was added to the medium and incubated for 4 h at 37 °C. Lactate dehydrogenase (LDH) release into the supernatant was measured as an indicator of cytolysis using the Cyto-Tox (Promega, Alexandria, Australia) kit.

2.5. In vivo *C. perfringens* challenge model

The necrotic enteritis disease induction model was performed as previously described [11]. 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 20, feed was withdrawn and each bird was orally challenged with 1.5 mL of *C. perfringens* culture (10^9 to 10^{10} CFU). On day 21 birds were again orally challenged and feed contaminated with *C. perfringens* was administered. *C. perfringens* strains were grown in FTG broth with the addition of 1.5% soluble starch and 2.0% thiopeptone 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 euthanased with inhaled carbon dioxide gas and their small intestines (duodenum to ileum) examined for gross necrotic lesions. The results for each challenge strain are expressed as a percentage lesion score compared with

the standard challenge strain EHE-NE18. All animal experiments were assessed, approved and monitored by the Australian Animal Health Laboratory animal ethics committee.

3. RESULTS

3.1. Prevalence of *netB* and *tpeL* in *C. perfringens* isolates from chickens

PCR analysis of *C. perfringens* isolates from both healthy and diseased chickens was used to determine whether these isolates carry *netB* or *tpeL*. Positive *C. perfringens* strains gave a 383 bp and 474 bp PCR product from internal *netB* and *tpeL* sequences, respectively. The *netB* gene was present in 70% (31/44) of the strains isolated from diseased birds: 3 out of 4 isolates from Belgium were *netB* positive, 5 out of 7 from Denmark, 19 out of 24 Australian isolates and 4 out of 9 Canadian strains (Tab. 1). The *tpeL* gene was only found in 9% (4/44) of isolates from diseased birds and was only present in *netB*-positive isolates. By contrast, only 2 of 55 isolates from healthy chickens from Australia and Belgium carried *netB* and none had *tpeL*.

Western blot analysis was carried out using rabbit anti-serum against rNetB; for those strains positive for NetB toxin, a single immunoreactive band was present at approximately 33 kDa (Fig. 1). The results (Tab. 1) showed that there was an absolute correlation between for the presence of the gene, as detected by PCR, and the ability to produce the NetB toxin.

3.2. The *netB* sequence is highly conserved

A PCR product encompassing the complete *netB* gene was amplified from 23 *netB*-positive strains and sequenced to determine the deduced amino acid sequence of the encoded 322 amino acid full-length NetB proteins. The toxins were all highly conserved in both nucleotide and amino acid sequence; only one different NetB sequence type was identified amongst these isolates, even though they represented several outbreaks of necrotic enteritis from different geographical locations (Tab. 1). The *netB* nucleotide sequences of 15 of these strains were

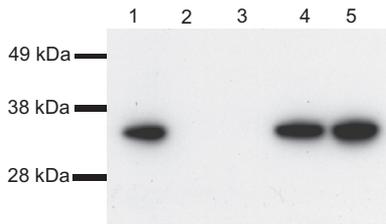


Figure 1. Western blot of NetB toxin from *C. perfringens* isolates from diseased chickens. Proteins were separated by 4–12% SDS-PAGE. SeeBlue Plus2 prestained marker (Invitrogen) was used as a size marker. Bands were transferred onto PVDF membranes and probed with rabbit polyclonal anti-rNetB antibody. Blots were developed with an ECL Western blotting kit and the results recorded on autoradiographic film. Strains: (1) EHE-NE18; (2) NAG-NE1; (3) 48; (4) 56; (5) 301001-1-B1.

identical to the previously determined EHE-NE18 sequence [12], across the entire region analysed. The remaining 8 strains had very few nucleotide changes, which were represented in several strains from different countries. Only one change resulted in a NetB amino acid sequence change, altering the alanine residue at position 168 to threonine (A168T). The 6 strains that had this change included isolates from Australia, Canada, Denmark and Belgium (Tab. I). Finally, analysis of the non-coding regions surrounding the *netB* gene showed that they were very well conserved. Only three changes were observed, in a total of 5 strains. One of these changes involved an insertion of 10 additional nucleotides when compared to EHE-NE18. However, all changes are downstream of the *netB* ORF and are unlikely to alter gene expression.

3.3. NetB (A168T) retains cytotoxic activity

To determine if the single amino acid change at residue 168 of NetB affects its cytotoxic activity, culture supernatants from several strains producing the variant toxin were tested in the LMH cell cytotoxicity assay (Fig. 2). LDH release was used as a measure of cytotoxicity. The results showed that all five of the

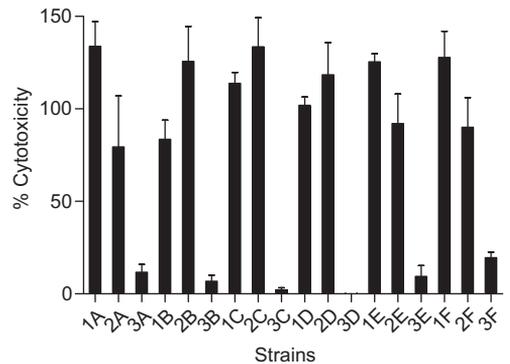


Figure 2. Cytotoxic activity of NetB (A168T) supernatants on LMH cells. The LMH cells were grown to 70% confluence in a 96 well tissue culture plate and culture supernatant added to the medium for 4 h at 37 °C. Cytotoxicity was measured by release of LDH from the LMH cells using the CytoTox kit (Promega). (A) EHE-NE18; (B) EHE-NE7; (C) EHE-NE9; (D) 3 MB-2003; (E) 37; (F) 301001-1-B1. (1) Dialyzed culture supernatant; (2) culture supernatant pre-incubated with pre-immune serum for 1 h at 37 °C; (3) culture supernatant pre-incubated with rNetB anti-serum for 1 h at 37 °C. The values are averages of triplicate assays with error bars representing SD.

NetB_{A168T} variants tested had similar toxicity for LMH cells as NetB from EHE-NE18. In addition, when supernatants from either NetB or NetB_{A168T} derivatives were pre-incubated with anti-serum raised against the *Escherichia coli*-derived rNetB protein, a reduction in LDH release was observed. Incubation with pre-immune serum did not affect the cytotoxic activity of any of the NetB variants.

3.4. NetB negative strains from diseased chickens do not cause necrotic enteritis in an experimental model

To investigate the role of NetB in necrotic enteritis a selection of *netB* positive and negative strains was tested in a necrotic enteritis induction model. All 11 strains tested that contained *netB* caused disease in the disease induction model (Tab. I). However, the 7 *netB* negative *C. perfringens* strains, all of which were derived from birds suffering from necrotic

enteritis, induced little or no signs of disease when used to challenge these chickens. In the NAG-NE25 (*netB*-negative) challenge, the *C. perfringens* cells recovered from the few small lesions (approximately 1 mm in diameter) were clearly from a different source (presumably environmental) as PCR analysis showed that they carried *netB*.

4. DISCUSSION

In this study we found that 70% of *C. perfringens* isolates from chickens with necrotic enteritis carry *netB*. Importantly, all of the isolates that carried this gene also expressed the NetB protein in vitro. This result is in contrast to studies on $\beta 2$ toxin, which recently showed that only 54.5% of the strains that were positive for its structural gene, *cbp2*, produced $\beta 2$ toxin in vitro [6]. The *C. perfringens* isolates analysed in this study were sourced from a range of geographical locations, with NetB-positive strains detected from 4 countries, on 3 separate continents. There were insufficient numbers to determine if there was any statistically significant difference in *netB* carriage in these countries. Previous studies reported *netB* incidences of 7 out of 12 for isolates from the USA and a very high carriage rate of 92% for Canadian isolates [3, 14]. In agreement with our results, these strain surveys found a low incidence of *netB* in *C. perfringens* strains isolated from healthy birds.

These results, in conjunction with our previous finding that a *netB* mutant of a virulent isolate no longer causes disease [12], support the conclusion that NetB plays an important role in the pathogenesis of necrotic enteritis in chickens. However, recent studies have also found that there are some isolates from diseased birds that do not carry *netB*. There are several possible explanations for this finding, ranging from alternative virulence factors to the need for complex associations of other microflora required for disease production. In this study, 7 *netB*-negative strains isolated from diseased birds in all 4 countries were tested for their ability to induce disease in a standard disease induction model. No confirmed *netB*-negative

isolates were able to induce disease whereas a range of *netB*-positive strains, from different geographical locations, were able to induce lesions typical of necrotic enteritis. These results strongly suggest that these isolates are not capable of causing disease without NetB. Of course, we cannot categorically rule out the possibility that these *netB*-negative isolates can cause disease in the field either by themselves or as part of a wider microbial consortium. The results reported here are consistent with our previous conclusion that NetB is important for virulence.

It is important when testing any isolates (e.g. *netB*-negative strains) in a disease model to test the genotype of isolates recovered from the lesions. The nature of the disease and the model systems are such that occasionally environmentally derived *C. perfringens* can initiate lesion formation and it is important to differentiate such lesions from lesions caused by the test organism. Other researchers have reported that some strains isolated from necrotic enteritis-affected chickens [4, 5] and strains from healthy birds [21], are not virulent in disease induction models, but the *netB* status of these strains was not reported.

Previous work has suggested that although different strains of *C. perfringens* can coexist in the cecum of healthy birds usually only one strain is involved in disease lesions in any particular bird [8, 16]. It would therefore seem unlikely that many of the apparently non-pathogenic isolates from diseased birds could result from mixed cultures of pathogenic and non-pathogenic strains coming from the sampled lesions. One possible explanation to consider is that the isolates have lost virulence during the in vitro culturing process that is necessary to derive a pure, defined culture from a lesion sample.

Other workers [3] surveyed *C. perfringens* strains from chickens and found that 11 out of 61 strains examined carried the *tpeL* gene, which encodes a potential glucosylating toxin. All of these *tpeL* positive strains also carried *netB*. Accordingly, we also screened for the *tpeL* gene, and obtained similar results. Only 4 out of 44 strains were *tpeL* positive, all of which also carried *netB*. Several strains that

were *tpel* negative caused typical disease in the disease induction model. It is clear from these studies that the putative TpeL toxin is not essential for necrotic enteritis in chickens.

Comparative sequence analysis of the *netB* genes revealed a very high degree of conservation with only one NetB variant detected (A168T) in 6 of the 23 genes sequenced. This difference was the result of the same single base substitution and the single amino acid change did not significantly alter the predicted physical properties of the encoded proteins (33.2 kDa, pI 6.14). All other *netB*-positive strains carried genes encoding the same protein as the prototype gene characterized in strain EHE-NE18 [12]. Alignment and structural modelling of NetB, in comparison to the related proteins, α -hemolysin from *Staphylococcus aureus* and β -toxin from *C. perfringens*, predicted that the A168T substitution occurs in a region that is expected to be within a membrane-spanning region [15]. The relatively conservative nature of the amino acid substitution would not be expected to significantly affect the tertiary structure of the protein and indeed the cytotoxicity assays showed that the variant protein was functional and had similar activity to the original NetB toxin.

The generally accepted model [22] for the development of necrotic enteritis in chickens proposes that *C. perfringens* normally resides in the gastrointestinal of birds, essentially behaving as a benign commensal organism. Some type of environmental or nutritional change or stress, results in overgrowth of the resident *C. perfringens* population, resulting in lesion formation. This study and other reports in the literature [3, 4, 7, 14] are giving a different picture of the disease process. It is now clear that the overall gastrointestinal population of *C. perfringens* in the normal healthy chicken is different to that of the organisms that cause disease. Whereas a mixed population of *C. perfringens*, containing a low percentage of NetB expressing clones, is present in healthy birds it has been found that a single clone tends to dominate the *C. perfringens* population in diseased birds. It is now clear that the clonal populations seen in diseased birds usually carry and express the *netB* gene. It now remains to be determined whether

the increase in overall *C. perfringens* numbers that occurs during the disease process is due to a specific increase in the number of *netB*-positive organisms or whether there is a general overgrowth of all *C. perfringens* cells but only the *netB*-positive strains colonise, proliferate and cause cell and tissue damage.

In summary, we have shown that *C. perfringens* strains from chickens suffering necrotic enteritis produce highly conserved NetB toxins that closely resemble the NetB found in chicken isolate EHE-NE18. A high proportion of the *C. perfringens* strains from diseased birds were *netB*-positive, all of which were able to express NetB in vitro. Virulence testing of numerous strains in a standard disease induction model showed that only strains producing NetB were capable of producing disease. These results provide further evidence that NetB is an essential virulence factor in the pathogenesis of necrotic enteritis. Other toxin-encoding genes are present in some virulent strains but their role in virulence remains unclear. These results open up further studies aimed at the development of diagnostic tests and vaccines for the control and treatment of *C. perfringens* infections of commercial chickens; this work is currently being pursued in our laboratories.

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