Short communication

In vitro production of necrotic enteritis toxin B, NetB, by *netB*-positive and *netB*-negative *Clostridium perfringens* originating from healthy and diseased broiler chickens

Lone Abildgaard^a, Teis E. Sondergaard^a, Ricarda M. Engberg^a, Andreas Schramm^b, Ole Højberg^{a,*}

^a Department of Animal Health and Bioscience, Faculty of Agricultural Sciences, Aarhus University, Blichers Alle 20, P.O. Box 50, DK-8830 Tjele, Denmark

^b Department of Biological Sciences, Microbiology, Aarhus University, Ny Munkegade, Building 1540, DK-8000 Aarhus C, Denmark

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ABSTRACT

The *Clostridium perfringens* necrotic enteritis toxin B, NetB, was recently proposed as a new key virulence factor for the development of necrotic enteritis (NE) in broilers. The aim of the present study was to investigate the presence of the *netB* gene and the *in vitro* production of the NetB toxin in a well characterized collection of 48 *C. perfringens* Type A isolates, obtained from Danish broiler flocks. The investigation revealed *netB* gene prevalences of approx. 50% and 60% among isolates from diseased (NE) and healthy flocks, respectively. Only minor nucleotide variations were observed between the isolates in the coding sequence (CDS) of the *netB* gene, and the promoter region was observed to be completely conserved. However, *in vitro* NetB production was only observed in 4 out of 14 *netB*-positive *C. perfringens* isolates recovered from healthy birds, whereas 12 out of 13 *netB*-positive isolates from NE birds were shown to produce the NetB toxin. It is therefore proposed that genotype, i.e. presence of the *netB* gene, in itself is inadequate for predicting virulence of *C. perfringens*, and future investigations should focus on the bacterial phenotypes; the regulatory mechanisms involved in the expression of NetB, and potentially also other toxins, and its implications for the virulence of individual *C. perfringens* strains.

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1. Introduction

Necrotic enteritis (NE) is a common and financially devastating bacterial disease in modern broiler flocks (Kaldhusdal and Løvland, 2000; Van der Sluis, 2000; Van Immerseel et al., 2004). NE in broilers is caused by the Gram-positive, spore-forming, anaerobe, *Clostridium perfringens*. However, the exact mechanism behind the pathogenesis of *C. perfringens* is poorly understood (Cooper and Songer, 2009a; Timbermont et al., 2009b; Van Immerseel et al., 2009). For many years, the α -toxin has

been regarded as the major *C. perfringens* virulence factor for the development of NE in chickens (Al-Sheikhly and Truscott, 1977a,b; Wages and Opengart, 2003b). This perception was recently questioned by studies demonstrating that an α -toxin knock-out mutant of *C. perfringens* induced NE lesions to the same degree as the wild-type isolate (Keyburn et al., 2006; Timbermont et al., 2009a). Moreover, *in vitro* α -toxin production level of *C. perfringens* isolates and health status of the host chickens have been reported not to correlate (Gholamiandekordi et al., 2006; Timbermont et al., 2009a; Abildgaard et al., 2009a). Necrotic enteritis toxin B, NetB, was recently proposed as a new key virulence factor of *C. perfringens* associated with NE (Keyburn et al., 2008). Loss of cytotoxicity was associated with a mutation in the *C. perfringens netB* gene,

* Corresponding author. Tel.: +45 89991183; fax: +45 89991166.
E-mail address: ole.hojberg@djf.au.dk (O. Højberg).

whereas the wild-type and complemented mutants caused NE. Moreover, [Keyburn et al. \(2008\)](#) reported that most (14 out of 18) *C. perfringens* isolates obtained from diseased (NE) Australian broiler flocks were *netB*-positive and produced the NetB toxin *in vitro*, whereas the *netB* gene was not detected in 32 non-NE-derived strains. The latter strains were, however, not derived from healthy chickens, but from other animal species and humans. Investigations of broiler flocks in the United States and Canada also reported most *C. perfringens* outbreak isolates to be *netB*-positive, but some isolates recovered from healthy birds were found to be *netB*-positive as well ([Chalmers et al., 2008a](#); [Martin and Smyth, 2009](#)). Moreover, a recent study has demonstrated NE induction by five *netB*-negative *C. perfringens* strains ([Cooper and Songer, 2009b](#)), questioning the clear cut correlation between NE status of the host birds and *netB* genotype of the *C. perfringens* isolates. The aim of the present study was, as a directly follow-up on these studies, to investigate the presence of the *netB* gene as well as *in vitro* NetB production in a well characterized collection of *C. perfringens* isolates recovered from Danish broiler flocks with or without NE.

2. Materials and methods

2.1. Bacterial isolates, PCR and sequence analysis

From a previously described collection of 65 *C. perfringens* Type A isolates with different PFGE profiles ([Nauerby et al., 2003](#); [Abildgaard et al., 2009a](#)), 48 isolates recovered from broiler chickens with known health status regarding NE were selected for use in the present study. For detecting presence of the *netB* gene, samples of DNA were extracted from all *C. perfringens* isolates and subjected to *netB* gene amplification using the primers AKP78 and AKP79 ([Table 1](#)) as well as the PCR protocol from [Keyburn et al. \(2008\)](#), modified with a 10 min 94 °C preheating step. The *netB* gene was amplified in a total volume of 25 µL containing 1× PCR buffer, BSA (0.4 µg/µL final conc.), 1 pmol of each primer, 200 µM of each dNTP, 4 U DyNAzyme II Hotstart DNA polymerase (Finnzymes Oy, Espoo, Finland), and 3 µL (~25 ng/ml) of DNA extract. DNA extracts which did not reveal a PCR product of the expected size for *netB* (383 bp) were re-analysed for the presence of the *netB* gene by the above-mentioned PCR protocol as well as for the presence of the housekeeping gene, *gyrA*, using primers and PCR protocol from [Rooney et al. \(2006\)](#). For verification of *netB* gene identity, the obtained PCR products were purified using the QIAquick kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified PCR products were sequenced using the AKP78 and AKP79 primers, the BigDye® Terminator v1.1

Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and the following PCR conditions: 99 cycles of 95 °C for 10 s, 50 °C for 5 s, and 62 °C for 4 min. Sequenced PCR products were purified using a sodium acetate/ethanol precipitation step and analysed using an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). Furthermore, to obtain sequences covering the complete coding sequence, CDS, of the *netB* gene (969 bp) as well as the promoter region, the primers *netB*(–100)F, *netB*(210)R and *netB*(1278)R ([Table 1](#)) were constructed based on the twenty-three 1533 bp *netB* gene sequences submitted to GenBank by [Keybourn et al. \(2009\)](#) with accession numbers FJ189481–FJ189503. The numbers included in the primer names indicate primer position relative to the start codon (TTG) of the *netB* gene CDS. Two PCR amplicons of 977 and 783 bp were obtained by use of the primer sets *netB*(–100)F/AKP79 and AKP78/*netB*(1278)R, respectively. The *netB* gene was amplified in a total volume of 10 µL containing 1× PCR buffer, 0.5 µM of each primer, 200 µM of each dNTP, 0.2 µL Phire Hot Start DNA polymerase (Finnzymes, Espoo, Finland), and 1 µL (~200 ng/µL) of DNA extract and PCR conditions according to the recommendations of Finnzymes with an annealing temperature of 64.4 °C. The PCR products were purified by use of ExoSAP-IT (USB Corporation, Cleveland, Ohio). The two amplicons were sequenced (Eurofins MWG Operon, Ebersberg, Germany) by use of the primers *netB*(–100)F, *netB*(210)R and AKP79 for the first amplicon (977 bp) and primer AKP78 alone for the second amplicon (783 bp). The *netB* gene sequences were assembled using CLC DNA Workbench 5.5 (CLC Bio A/S, Aarhus, Denmark) and refined by manual inspection. The identity of the assembled sequences was verified via NCBI BLAST ([Altschul et al., 1990](#)) and they have been deposited in GenBank with accession numbers GU433314–GU433340.

2.2. Western blot analysis of NetB toxin

The *C. perfringens* isolates were grown overnight in Anaerobe Basal Broth (CM0957; Oxoid Ltd., Basingstoke, Hampshire, UK) at 37 °C. Culture supernatants (10 ml) were obtained by centrifugation (5500 × g, 10 min). Proteins in the supernatant were precipitated using 10% trichloroacetic acid and pelleted by centrifugation (14,000 × g, 15 min). The protein concentrations of the samples were determined on a NanoDrop® ND-1000 spectrophotometer (NanoDrop technologies, Inc., Wilmington, DE) and equal amounts (~20 µg) were separated by SDS-PAGE (NuPAGE® Novex 4–12% Bis-Tris gel, Invitrogen) in MES SDS running buffer (NuPAGE® MES SDS Running Buffer, Invitrogen). Proteins were transferred to Hybond™-C Extra Nitrocellulose membranes (Amer-

Table 1
Primers used for targeting the *netB* gene.

Primer	Sequence 5'–3'	Origin
<i>netB</i> (–100)F	CCA GTT ATG TAT AAA TTT TGA CCA GTT	This study
<i>netB</i> (210)R	TCC AAC CTT TAT GTG AAG CGG	This study
<i>netB</i> (1278)R	AAA CTT TAG TAT TCC TCT CAT TTT TTA TCC C	This study
AKP78	GCT GGT GCT GGA ATA AAT GC	Keyburn et al. (2008)
AKP79	TCG CCA TTG AGT AGT TTC CC	Keyburn et al. (2008)

sham Bioscience). The membranes were blocked with 5% skim milk before probing with rabbit polyclonal anti-rNetB antiserum (Keyburn et al., 2008) overnight in 5% bovine albumin (1:1000). Blots were developed using peroxidase-labelled anti-rabbit antibodies (1:10,000) with ECL Western Blotting Kit (GE Healthcare) and the results were recorded on autoradiographic films.

3. Results

3.1. Presence of the *netB* gene

The 48 *C. perfringens* isolates included in the present study were all previously found to be of Type A (Nauerby et al., 2003) and harbouring the α -toxin encoding *plc* gene. Using the *netB*-specific primers of Keyburn et al. (2008), a *netB* PCR product of the expected 383 bp in length was obtained in 27 out of the 48 isolates (Table 2). The 21 *netB*-negative isolates were re-analysed and further tested for the presence of *gyrA*, defined as a *C. perfringens* house-keeping gene (Rooney et al., 2006), using the primers and the PCR protocol provided by Rooney et al. (2006). The 21 isolates were confirmed to be *netB*-negative and were all positive for the *gyrA* gene (data not shown), indicating that lack of *netB* PCR product was not due to insufficient DNA extraction or general PCR inhibition. The health status regarding NE of the chickens from which the isolates were isolated was known for all 48 isolates investigated; among the *C. perfringens* isolates recovered from NE (25) and healthy (23) chickens, 13 and 14 were observed to be *netB*-positive in the two groups, representing a *netB* gene prevalence of 52% and 61%, respectively (Table 2 and 3).

3.2. Analysis of *netB* sequences

The nucleotide sequences of the *netB* amplicons cover both the 969 bp complete CDS (Keybourn et al., 2009)

Table 2

Host chicken health status, *netB* genotype, and *in vitro* NetB production of the investigated *Clostridium perfringens* isolates.

Host bird health status	Genotype	Number of isolates	<i>In vitro</i> NetB production ^a
Healthy	<i>netB</i> -negative	9	0
	<i>netB</i> -positive	14	4
Necrotic enteritis	<i>netB</i> -negative	12	0
	<i>netB</i> -positive	13	12

Please see [supplementary material](#), for further information.

^a Number of NetB-producers for each group of isolates.

and the upstream promoter region, including sequences potentially representing the RNA polymerase/sigma factor recognition sites (–35: TTGAAA; –10: TATAAT) as well as the ribosomal binding site (AGGAGG). The sequences obtained from the 27 *netB*-positive isolates, all revealed ~100% identity to each other as well as to the 23 full length *netB* gene sequences presently available in GenBank (accession numbers FJ189481–FJ189503). Thus, according to the sequence data, a more systematic nucleotide variation (A/G) only seems to occur in CDS position 502 of the *netB* gene (A replaced by G in 6 isolates [GU433316, GU433317, GU433319, GU433330, GU433332 and GU433338]), leading to a shift from Thr (encoded by ACT) to Ala (encoded by GCT) in amino acid position 168 of the NetB protein. However, the observed variation could be related neither to health status of the host birds nor *in vitro* NetB production status of the *C. perfringens* strains. Further to this, single nucleotide variations were observed in the *netB* gene of a few isolates at CDS position 10 (T replaced by C in 4 isolates [GU433314, GU433318, GU433331 and GU433340]; no AA shift) and CDS position 497 (C replaced by T in 2 isolates [GU433327 and GU433334]; shift from Ala to Val in AA position 166). No nucleotide variations, whatsoever, were observed in the 100 bp upstream region covering the *netB* gene promoter.

3.3. Production of NetB toxin

All *C. perfringens* isolates were tested for *in vitro* production of NetB in batch cultures and the Western blot analysis rendered a highly unambiguous result; we either observed a clear NetB antibody-reactive band or no band at all as illustrated in Fig. 1. Thus, none of the *netB*-negative isolates were observed to accumulate NetB toxin in the culture supernatant (Table 2). Among the *netB*-positives, 12 out of the 13 isolates obtained from broilers suffering from NE were shown to accumulate NetB toxin in the supernatant following overnight incubation (Table 2). For *netB*-positive isolates obtained from healthy birds, however, accumulation of NetB toxin was only detected in 4 out of 14; no NetB production was observed for the other 10 isolates (Table 2). The total amount of protein analysed in the Western blots was comparable for all investigated isolates as verified by spectrophotometric measurements and inspection of the corresponding comassie blue stained SDS-PAGE gels (data not shown).

4. Discussion

The present investigation revealed a prevalence of *netB*-positive *C. perfringens* isolates of 52% and 61% in diseased

Fig. 1. Western blot analysis of *C. perfringens* batch culture supernatants using the NetB antibody. The expected size of NetB toxin is 36.8 kDa. Lanes 1–3 and 11–13: *netB*-positive isolates from birds suffering from NE. Lanes 18 and 19: *netB*-negative isolates from birds suffering from NE. Lanes 4–9 and 14–17: *netB*-positive isolates from healthy birds. Lane 10: pre-stained marker (units in kDa).

Table 3Prevalence of *netB* gene-harboured *Clostridium perfringens* isolated from healthy and diseased broilers in four regional studies.

Region	Host broiler health status				Data source
	NE ^a		Healthy		
	Prevalence	Isolates ^b	Prevalence	Isolates	
Denmark	52%	25	61%	23	This study
Canada	95%	41	35%	20	Chalmers et al. (2008a)
United States	58%	12	9%	80	Martin and Smyth (2009)
Australia	78%	18	ND ^c	0	Keyburn et al. (2008)

^a Necrotic enteritis.^b Number of isolates.^c Not determined.

and healthy broilers, respectively (Table 3). This result differs slightly from the investigations performed in Australia, Canada and the United States (Chalmers et al., 2008a; Keyburn et al., 2008; Martin and Smyth, 2009), where the prevalence of the *netB* gene in *C. perfringens* isolates obtained from broilers suffering from NE were generally higher (58.3–95.1%). The Canadian and US investigations (Chalmers et al., 2008a; Martin and Smyth, 2009) also revealed *netB*-positive *C. perfringens* in healthy broilers, though with a somewhat lower prevalence (35% and 8.8%, respectively) than in our case. The Australian investigation (Keyburn et al., 2008) did not include *C. perfringens* strains isolated from healthy chickens. Overall, the prevalence data should of course be interpreted with caution due to the relatively low number of isolates included in all studies. However, collectively, these observations illustrate that despite the potential importance of the NetB toxin in the development of NE, presence or absence of *netB*-positive *C. perfringens* in itself is insufficient for predicting broiler flock health status.

The fact that *netB*-positive *C. perfringens* could be isolated from healthy birds does not necessarily imply that these strains are non-virulent. It could as well be explained by a relatively high diversity of the *C. perfringens* population in healthy broilers (Engström et al., 2003), though this is not always observed (Chalmers et al., 2008b). Therefore, virulent *netB*-positive strains may be present in healthy birds but constituting an insignificant proportion of the total *C. perfringens* population. Moreover, being healthy at the time of sampling does not exclude that birds hosting the *netB*-positive strains could potentially have developed NE later on. In contrast to healthy birds, NE birds usually harbour a much less diverse population of *C. perfringens* that can be dominated by one or two virulent clones solely (Engström et al., 2003; Nauerby et al., 2003; Gholamiandekordi et al., 2006). Thus, recovering *netB*-negative strains from NE birds seems more unlikely, if the NetB toxin is actually a major provoking factor for NE development.

The technical validity of the negative *netB* PCR reactions obtained with the AKP78/AKP79 primer set was verified by running a parallel PCR targeting a bacterial housekeeping gene (*gyrA*). The *gyrA* PCR was positive for all isolates, but this does not rule out the possibility that *netB*-negatives in general could be a result of mismatches between the specific primers used and the target DNA. Though the *netB* gene sequence is highly conserved according to our

observations as well as the available GenBank sequences (accession numbers FJ189481–FJ189503), the variable CDS position 502 of the *netB* gene is located in the target site of the forward primer (AKP78) and could potentially result in PCR failure. However, positive *netB* PCR signals were obtained with the AKP78/AKP79 primer set irrespective of the nucleotide (A or G) at this position, indicating that the mismatching primer position (position 7 from the 5' end) was not crucial for the binding of primer AKP78. Moreover, in support of the negative *netB* PCR data, no accumulation of NetB was observed in any of the *netB*-negative isolates tested in the present study.

It has been reported that the β 2-toxin encoding *cpb2* gene in *C. perfringens* may contain a deletion that leads to a premature stop codon after only nine amino acid codons and thereby no production of β 2-toxin; antibiotic (gentamicin or streptomycin) treatment apparently induced a frameshift and restored toxin production (Vilei et al., 2005). The highly conserved CDS of the *netB* gene seems to exclude mechanisms like this to account for the observed lack of NetB production. In general, the highly conserved nature of the *netB* gene compared to e.g. the α -toxin encoding *plc* gene (Abildgaard et al., 2009a) may, however, seem somewhat peculiar and it could be speculated if it somehow indicates a more crucial function of the NetB protein. Further investigations of the NetB protein structure and function are needed, however, to make any decisive conclusions.

The sequence data presented here reveal no variations either in the proximal *netB* promoter region (100 nucleotides upstream of the CDS start codon) that could account for the observed differences in the NetB production patterns of the isolates. This is in accordance with the set of *netB* gene sequences in GenBank submitted by Keybourn et al. (2009); the latter moreover indicates that the conserved nature of the *netB* promoter region continues up to 250 nucleotides upstream of the start codon. Explanations for the apparent differences in NetB production among the isolates of the present study should therefore be found at other levels, like involved signal peptides and sigma factors, or at the post-transcriptional level.

Analyzing the *in vitro* production of the NetB toxin or any other protein may be considered critical, since it may be influenced by factors like choice of culture media and other growth conditions (pH, temperature, etc.), and *in vitro* activity cannot directly be extrapolated to *in situ*

activity of the strains in question. Still, *in vitro* toxin production is regularly used as a measure for evaluating virulence of pathogens, including *C. perfringens* strains (Gholamiandekhordi et al., 2006; Fernandez-Miyakawa et al., 2007; Timbermont et al., 2009a; Abildgaard et al., 2009b). We observed that nearly all (12 out of 13) *netB*-positive isolates, recovered from birds suffering from NE, produced the NetB toxin *in vitro*, in accordance with the observations of Keyburn et al. (2008). Remarkably, however, only 4 out of 14 *netB*-positive isolates obtained from healthy birds produced NetB toxin *in vitro*; the other 10 did not.

The data presented from this study neither confirms nor excludes NetB as being the key virulence factor for NE development in broiler chickens *per se*. However, this is the first study to report that the *netB*-gene can be present in *C. perfringens* without the NetB toxin being produced. Since this trait moreover seems to prevail in isolates recovered from healthy chickens, the observations certainly imply that investigations on NetB, and potentially other *C. perfringens* toxins, in relation to development of NE in broilers should take genotypic as well as phenotypic characteristics of the bacteria into consideration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2009.12.036.

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