

Prevalence of *netB* Gene among *Clostridium perfringens* Isolates Obtained from Healthy and Diseased Chickens

A. Toloee, B. Shojadoost, S.M. Peighambari and Y. Tamaddon
Department of Clinical Sciences, Faculty of Veterinary Medicine,
University of Tehran, P.O. Box 14155-6453, Tehran, Iran

Abstract: Necrotic Enteritis (NE) is the most clinically dramatic bacterial enteric disease of poultry induced by *C. perfringens* that affects industrial poultry worldwide. *C. perfringens* produces a large range of potent toxins that are responsible for severe diseases in humans and animals. The critical importance of *netB*, the recently identified pore-forming toxin for the development of NE is still under discussion. In this study *C. perfringens* isolates from healthy and diseased poultry flocks were analyzed by PCR in order to determine the presence of *netB* for the first time in Asia. The *netB* was detected in 52.8% isolates from diseased flocks but not in any isolates from healthy ones. The products of four positive *netB* PCR reactions were sequenced. Comparison of the isolates sequences revealed 100% identity to each other and to the *netB* sequences available in GenBank. *netB* can not be the only *C. perfringens* virulence factor involved in NE, since not all *C. perfringens* isolates from birds associated with NE contain the *netB* gene. However, further investigations are required to determine the role of *netB* in development of NE.

Key words: *Clostridium perfringens*, necrotic enteritis, toxin, *netB*, chicken, isolates sequences

INTRODUCTION

Among pathogenic bacteria, different species of the *Clostridia* genus produce the most variable type of toxins which are considered as the main virulence determinants of these bacteria (Popoff and Bouvet, 2009). *Clostridium perfringens*, an anaerobic gram-positive sporeforming bacterium is ubiquitous in the intestinal flora of human and animals and known to be the most widely distributed pathogen in nature. *C. perfringens* produce a large range of potent toxins and enzymes that are responsible for severe diseases in humans and other animals (Popoff and Bouvet, 2009). In the last decade, a great effort has been made to understand the mechanism of action, structure and function of these toxins.

Some toxins are known as potent virulence factors while the implication of other toxins in pathogenicity is questionable (Rood, 1998). In a commonly used classification scheme, *C. perfringens* is divided into five toxinotypes (A-E) based on the production of four major toxins (alpha, beta, epsilon and iota) where each type carries a different combination of the toxin genes however, this bacterium also produces at least 13 other toxins such as *C. perfringens* Enterotoxin (CPE), theta toxin, beta2 toxin and *netB* toxin (Keyburn *et al.*, 2008; Petit *et al.*, 1999). Specific *C. perfringens* toxin types are

associated with particular human and animal diseases. *C. perfringens* is often found in the intestinal tract of healthy birds but it can cause outbreaks of disease in many species of poultry and especially in broiler and turkey flocks (Songer, 1996). Clostridiosis occurs both as an acute clinical disease called as Necrotic Enteritis (NE) causing high mortality and as a subclinical disease with focal necrosis in the intestine (subclinical NE) or *C. perfringens*-associated hepatitis with cholangio-hepatitis or fibrinoid necrosis in the liver (Cooper and Songer, 2009). NE is the most clinically dramatic bacterial enteric disease of poultry induced by *C. perfringens* which affects industrial poultry worldwide and is a global problem (Cooper and Songer, 2009). Much of the current research on NE has focused on finding a definitive toxin that is responsible for causing disease. The majority of *C. perfringens* isolates from poultry belong to toxin type A but a few belong to type C (Cooper and Songer, 2009). The majority of the chicken strains are toxinotype A, meaning that they carry the *cpa* gene encoding alpha toxin (Petit *et al.*, 1999). For a long time, it was believed that this alpha toxin was the major virulence factor involved in NE (Fukata *et al.*, 1988). Recently, the role of *C. perfringens* alpha toxin in NE is disputed. A *cpa* knockout mutant from a virulent *C. perfringens* chicken strain was still capable of inducing necrotic lesions in the

gut of experimentally infected broilers (Keyburn *et al.*, 2006). In addition, almost 2 years ago, the netB toxin and its encoding gene (netB) were first identified in an Australian strain of *C. perfringens* type A that was isolated from a chicken suffering from NE (Keyburn *et al.*, 2008). Its importance in NE was shown when a netB mutant *C. perfringens* strain did not cause NE in an experimental chicken model and virulence was restored when a functional netB gene was introduced back into the mutant strain (Keyburn *et al.*, 2008).

The critical importance of netB for the development of necrotic enteritis is still under discussion as occasionally isolates that lack the netB gene can be found in birds suffering from NE and NE has been reproduced with netB negative isolates (Abildgaard *et al.*, 2010; Cooper and Songer, 2010). However, the exact role of this toxin in pathogenicity of NE still needs to be elucidated. Various epidemiological studies of *C. perfringens* strains from varied geographical locations have been published but very few studies from Asian countries have been published on *C. perfringens*-induced NE in poultry and very little knowledge of the *C. perfringens* genetic profile in Asia is available. In the present study, for the first time in Asia, we analyzed the *C. perfringens* isolates from poultry flocks in a single PCR assay in order to determine the presence of netB gene and examined its occurrence with respect to NE in chickens.

MATERIALS AND METHODS

Bacterial isolates and bacteriological procedures: About 79 isolates of *C. perfringens* type A collected during the period of 2005-2008 and kept in the laboratory in 50% glycerol at -70°C were used for this study. The collection consisted of 36 isolates obtained from six NE-positive flocks (broiler) and 43 strains obtained from four NE-negative flocks (two broiler, one layer and one broiler breeder). Toxinotypes of *C. perfringens* isolates were determined by Multiplex PCR in the previous study. The frozen *C. perfringens* isolates were cultivated in Brain Heart Infusion (BHI) and incubated anaerobically at 37°C for 24-36 h.

Samples were sub-cultured anaerobically in blood agar plates containing 7% defibrinated sheep blood, Tryptose Sulfite Cycloserine agar (TSC) and Tryptose Sulfite Neomycin agar (TSN). The identity of the isolates was confirmed by characteristic colony morphology, hemolytic pattern, gram staining and biochemical tests as previously described (Quinn *et al.*, 1994). All culture media and additives used in this study were provided from Merck (Germany). Due to recent discovery of netB gene, there was no netB-positive standard strain

of *C. perfringens* for using in PCR. However, the first identified netB-positive isolate was kept and used in all PCR reaction sets.

Single PCR for netB gene: To extract bacterial DNA, a single colony of each *C. perfringens* isolate grown on blood agar plate for overnight at 37°C suspended into 100 µL distilled water in a clean 1.5 mL microtube, boiled for 10 min and centrifuged for 10 min at 10000×g. The supernatants were carefully removed and used as template DNA. The concentration of DNA was determined by Biophotometer (Eppendorff, Germany) and adjusted to approximately 50 ng for each PCR reaction. To detect netB gene previously developed forward (5'-GCTGGTGCTGGAATAAATGC-3') and reverse (5'-TCGCCATTGAGTAGTTTCCC-3') primers were used (Keyburn *et al.*, 2008). Amplification reactions were carried out in a 25 µL reaction volume containing: 2 µL 10×PCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs mixture, 2.5 units of Taq DNA polymerase, 0.1 µM of each primers, dH₂O and 5 µL of template DNA solution. Negative and positive controls were included in all PCR reaction sets. Amplification was programmed in a thermocycler (Gradient Mastercycler, Eppendorff, Germany) as follows: 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec and a final extension at 72°C for 12 min (Keyburn *et al.*, 2008). The amplification products were detected by gel electrophoresis (Apelex, France) in 1.5% agarose gel in 1×TAE buffer, stained with 0.5 µg mL⁻¹ EtBr. Amplified bands were visualized and photographed under UV transillumination (UVP Visi-Doc-It System, UK). The primers and other materials used in PCR reaction were provided by Cinnagen (Tehran, Iran).

DNA sequencing of netB PCR products: Four *C. perfringens* isolates (ATBS61IR, ATBS65IR, ATBS94IR and ATBS120IR) obtained from separate diseased flocks were selected and their relevant PCR amplified products for netB were purified using the GeneJET™ Gel Extraction Kit (Fermentas Life Science, Germany) according to the manufacturer's instructions and submitted for automated sequencing in both directions at the Geneservice™, Source BioScience (Cambridge, England) using PCR primers as sequencing primers. The sequence data were submitted to GenBank under the accession numbers GU453172 and GU581173-5.

RESULTS AND DISCUSSION

Single PCR for netB gene: The presence of the netB gene was examined in all isolates by single PCR (Fig. 1). The netB gene was detected in 19/36 (52.8%) isolates from diseased flocks but not in any isolates from healthy

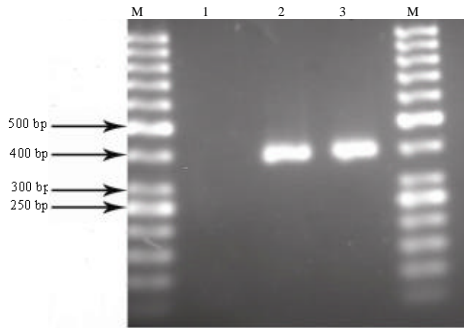


Fig. 1: Agarose gel (1.5%) electrophoresis results of the PCR assay for the detection of a 384 bp fragment of the *netB* gene in *C. perfringens*. Lanes M: Size marker (GeneRuler 50 bp DNA Ladder, Fermentas); Lane 1: Negative control; Lanes 2-3: *netB*-positive *C. perfringens* isolates from birds suffering from NE

flocks. All isolates from a single flock showed an identical pattern except in flock no. 4 in which one isolate was negative for *netB* gene but the rest were positive (Table 1).

Sequence analysis of netB PCR products: Comparison of four Iranian *C. perfringens* isolates sequences by Blastn (<http://www.ncbi.nlm.nih.gov/Blast>) at the nucleotide level revealed 100% identity to each other and to the *netB* sequences of *C. perfringens* strains available in GenBank.

The recently discovered *netB* toxin gene was exclusively found in NE-associated flocks in the study although, all *C. perfringens* isolates from three NE-positive flocks were *netB* negative (Table 1).

Since the discovery of *netB* in 2008, the presence of the *netB* gene have been screened among a wide variety of *C. perfringens* isolates from Australia, Canada, United States and some European countries (Chalmers *et al.*, 2008; Johansson *et al.*, 2010; Keyburn *et al.*, 2009; Martin and Smyth, 2009). However, this study for the first time reports the presence of *netB* gene among *C. perfringens* isolates from Asia. Similar to the findings, the *netB* gene has not been detected in all isolates from definitive cases of NE (Abildgaard *et al.*, 2010; Chalmers *et al.*, 2008; Johansson *et al.*, 2010; Keyburn *et al.*, 2009; Martin and Smyth, 2009).

Despite the first report on the detection of *netB* gene among isolates from diseased chickens, the presence of this gene among *C. perfringens* isolates from healthy chickens was also confirmed in later studies (Abildgaard *et al.*, 2010; Chalmers *et al.*, 2008; Johansson *et al.*, 2010; Keyburn *et al.*, 2008, 2009;

Table 1: Characteristics of *Clostridium perfringens* isolates of this study

Flock no.	Flock status	Flock type	No. of isolates	Toxin types	<i>netB</i>
1	Diseased	Broiler	4	Type A	-
2	Diseased	Broiler	8	Type A	+
3	Diseased	Broiler	4	Type A	-
4	Diseased	Broiler	7	Type A	6+/1-
5	Diseased	Broiler	8	Type A	-
6	Diseased	Broiler	5	Type A	+
7	Healthy	Broiler	25	Type A	-
8	Healthy	Broiler	4	Type A	-
9	Healthy	Layer	10	Type A	-
10	Healthy	Breeder	4	Type A	-

Martin and Smyth, 2009). Chalmers *et al.* (2008) found the presence of *netB* gene only in isolates associated with NE outbreaks in Canada but not in any isolates from healthy birds which correspond with the findings in this study. In another North American survey, it was found that the majority of *C. perfringens* isolates from chickens with clinical signs of NE carried the *netB* gene (58.3%) whereas only a small percentage (8.6%) of isolates from healthy birds carried this gene (Martin and Smyth, 2009). The highest percentage (>90%) of *netB* gene positive among *C. perfringens* isolates from diseased chickens was reported in a single broiler flock in Sweden. However, in the same flock, 25% of the isolates from apparently healthy birds were also *netB* positive (Johansson *et al.*, 2010). Possibly, the reason for the detection of a high percentage of *netB* positive cases was that all isolates were from a single flock which represents the dominant *C. perfringens* population in the chicken organ lesions. Recently, Keyburn *et al.* (2009) studied various *C. perfringens* isolates from NE-positive and NE-negative flocks from three continents and found 70% (31/44) and 3.6% (2/55) *netB* positive isolates, respectively. In an Italian study, 107 *C. perfringens* isolates were tested for the presence of *netB* gene and it was found that 27% (29/107) of isolates were *netB* positive in which 93% (27/29) of isolates had been were originated from birds affected by NE and other intestinal disorders (Drigo *et al.*, 2009). A recent Danish study showed a prevalence of approximately 50 and 60% of *netB* gene among isolates from NE-associated and healthy flocks, respectively (Abildgaard *et al.*, 2010). The Danish study was the first and the only one that reported the presence of *netB* gene in isolates from healthy chickens more than that of in isolates from NE-positive chickens.

The presence of *netB* gene has also been shown in one non-poultry related *C. perfringens* isolate. Martin and Smyth (2009) reported isolation of the first *netB*-positive *C. perfringens* from the liver abscesses in a cow died with gastrointestinal disease. This non-poultry isolate was shown to cause lesions characteristic of avian NE in a chicken disease model (Smyth and Martin, 2010). This

finding was in contrast to another recent study which demonstrated the failure of a non-avian netB-positive *C. perfringens* isolate to effectively colonize birds following challenge with a high titre of infective inoculum. It was suggested that the overall deficiency in poultry colonization factors may be the reason for this failure (Cooper *et al.*, 2010).

The presence of toxin genes in *C. perfringens* isolates does not solely determine the clinical importance of the isolates and there are some predisposing factors that have been associated with the selection of toxigenic *C. perfringens* and consequently, the development of disease. Not all *C. perfringens* strains isolated from birds that clearly displayed signs of necrotic enteritis were netB positive and in one particular study two *C. perfringens* isolates that were negative for netB gene produced NE in 6.7 and 16.7% of inoculated birds (Cooper and Songer, 2010). These results imply that although there is a clear association between the presence of netB toxin gene and development of NE, there may be other (yet to be determined) virulence factors that are produced by these netB-negative disease-producing isolates. While it is possible that NE may result from the interaction of several toxic molecules, further investigation on this issue appears to be necessary. It is noteworthy that all netB-positive isolates identified in the study were cpb2-positive too. A recent study has revealed that in several NE-positive isolates the netB gene is part of a large potential pathogenicity locus (Lepp *et al.*, 2010). The identification of pathogenicity loci may lead to the discovery of additional virulence factors that are involved in the pathogenesis of disease (Lepp *et al.*, 2010).

CONCLUSION

This study demonstrated the presence of netB gene in more than half of the isolates from diseased flocks but not in any isolates from healthy flocks. However, netB can not be the only *C. perfringens* virulence factor involved in avian NE, since not all *C. perfringens* isolates from birds with NE contain the netB gene. According to the information obtained from the study and other investigations, netB toxin may not be an obligate requirement for poultry *C. perfringens* virulence and at least the presence of netB may not be essential for the disease process in all *C. perfringens* isolates. Although, the role of netB in the induction of NE needs further investigation. In addition, comparison of four Iranian *C. perfringens* isolates sequences revealed 100% identity to each other and to the netB sequences of *C. perfringens* strains available in GenBank.

Future investigations should focus on the ability of toxin production by this gene, experimental studies in a disease model to investigate the disease producing capabilities of the netB positive and negative strains

recovered from cases of NE and also the netB negative strains recovered from healthy chickens, vaccination with various toxoids and subsequent challenge for opening significant opportunities for the development of novel vaccines against NE in poultry, study on the regulatory mechanisms involved in the expression of netB toxin, identification the netB in *C. perfringens* isolates from species other than chicken, considering the roles of external and predisposing factors in pathogenesis of NE and identification new genes for toxins or related enzymes in future as was as the case for *C. perfringens* netB.

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REFERENCES

- Abildgaard, L., T.E. Sondergaard, R.M. Engberg, A. Schramm and O. Hojberg, 2010. *In vitro* production of necrotic enteritis toxin B, NetB, by netB-positive and netB-negative *Clostridium perfringens* originating from healthy and diseased broiler chickens. Vet. Microbiol., 144: 231-235.
- Chalmers, G., H.L. Bruce, D.B. Hunter, V.R. Parreira and R.R. Kulkarni *et al.*, 2008. Multilocus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. J. Clin. Microbiol., 46: 3957-3964.
- Cooper, K.K. and J.G. Songer, 2009. Necrotic enteritis in chickens: A paradigm of enteric infection by *Clostridium perfringens* type A. Anaerobe, 15: 55-60.
- Cooper, K.K. and J.G. Songer, 2010. Virulence of *Clostridium perfringens* in an experimental model of poultry necrotic enteritis. Vet. Microbiol., 142: 323-328.
- Cooper, K.K., J.R. Theoret, B.A. Stewart, H.T. Trinh, R.D. Glock and J.G. Songer, 2010. Virulence for chickens of *Clostridium perfringens* isolated from poultry and other sources. Anaerobe, 16: 289-292.
- Drigo, I., F. Agnoletti, C. Bacchin, A. Guolo, M. Cocchi, M. Bonci and L. Bano, 2009. Diffusion of *Clostridium perfringens* NetB positive strains in healthy and diseased chickens. Ital. J. Anim. Sci., 8: 761-764.
- Fukata, T., Y. Hadate, E. Baba, T. Uemura and A. Arakawa, 1988. Influence of *Clostridium perfringens* and its toxin in germ-free chickens. Res. Vet. Sci., 44: 68-70.

- Johansson, A., A. Aspan, M. Kaldhusdal and B.E. Engstrom, 2010. Genetic diversity and prevalence of netB in *Clostridium perfringens* isolated from a broiler flock affected by mild necrotic enteritis. *Vet. Microbiol.*, 144: 87-92.
- Keyburn, A.L., J.D. Boyce, P. Vaz, T.L. Bannam and M.E. Ford *et al.*, 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. *PLOS Pathog.*, 4: e26-e26.
- Keyburn, A.L., S.A. Sheedy, M.E. Ford, M.M. Williamson, M.M. Awad, J.I. Rood and R.J. Moore, 2006. Alpha-toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens. *Infect. Immun.*, 74: 6496-6500.
- Keyburn, A.L., X.X. Yan, T.L. Bannam, F. Van Immerseel, J.I. Rood and R.J. Moore, 2009. Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing NetB toxin. *Vet. Res.*, 41: 21-21.
- Lepp, D., B. Roxas, V.R. Parreira, P.R. Marri and E.L. Rosey *et al.*, 2010. Identification of novel pathogenicity loci in *Clostridium perfringens* strains that cause avian necrotic enteritis. *PLOS One*, 5: e10795-e10795.
- Martin, T.G. and J.A. Smyth, 2009. Prevalence of netB among some clinical isolates of *Clostridium perfringens* from animals in the United States. *Vet. Microbiol.*, 136: 202-205.
- Petit, L., M. Gibert and M.R. Popoff, 1999. *Clostridium perfringens*: Toxinotype and genotype. *Trends Microbiol.*, 7: 104-110.
- Popoff, M.R. and P. Bouvet, 2009. Clostridial toxins. *Future Microbiol.*, 4: 1021-1064.
- Quinn, P.J., M.E. Carter, B.M. Maarkey and G.R. Carter, 1994. *Clinical Veterinary Microbiology*. Wolfe Publication Company, UK.
- Rood, J.I., 1998. Virulence genes of *Clostridium perfringens*. *Annu. Rev. Microbiol.*, 52: 333-360.
- Smyth, J.A. and T.G. Martin, 2010. Disease producing capability of netB positive isolates of *C. perfringens* recovered from normal chickens and a cow and netB positive and negative isolates from chickens with necrotic enteritis. *Vet. Microbiol.*
- Songer, J.G., 1996. Clostridial enteric diseases of domestic animals. *Clin. Microbiol. Rev.*, 9: 216-234.