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

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**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 the 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 cannot 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 toxintypes (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 (Sorger, 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 Sorger, 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 Sorger, 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 Sorger, 2009). The majority of the chicken strains are toxintype 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 (Eppendorf, Germany) and adjusted to approximately 50 ng for each PCR reaction. To detect netB gene previously developed forward (5'-GTGTTGCTGGAATTAAATGC-3') and reverse (5'-TGCCTATTGTAGTTTCC-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, 1.4 μL of template DNA solution. Negative and positive controls were included in all PCR reaction sets. Amplification was programmed in a thermocycler (Gradient Mastercycler, Eppendorf, 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 transilumination (UVP Visi-Document 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, ATBS63IR, ATBS94IR and ATBS120IR) obtained from separate diseased flocks were sequenced and their relevant PCR amplified products for netB were purified using the Gene JET™ 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 GU881173-5.

**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; 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 (38.3%) whereas only a small percentage (6.8%) 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 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

<table>
<thead>
<tr>
<th>Flock no.</th>
<th>Flock status</th>
<th>Flock type</th>
<th>No. of isolates</th>
<th>Toxin types</th>
<th>netB</th>
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<tbody>
<tr>
<td>1</td>
<td>Diseased</td>
<td>Broiler</td>
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<td>Type A</td>
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<td>Diseased</td>
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<td>3</td>
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<td>4</td>
<td>Diseased</td>
<td>Broiler</td>
<td>7</td>
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<td>Layer</td>
<td>10</td>
<td>Type A</td>
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<tr>
<td>10</td>
<td>Healthy</td>
<td>Breeder</td>
<td>4</td>
<td>Type A</td>
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Table 1: Characteristics of Clostridium perfringens isolates of this study.
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 toxicogenic *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 results 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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