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The Prevalence of *netB* Gene in Isolated *Clostridium perfringens* From Organic Broiler Farms Suspected to Necrotic Enteritis

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Abstract

Background: *Clostridium perfringens* causes necrotic enteritis (NE) and NetB is a critical pore-forming toxin in the development of this disease in chickens.

Objectives: The aim of this study was to evaluate the prevalence of *C. perfringens* in organic broiler farms and to assess the presence of *netB* gene among isolates and its occurrence with respect to NE disease.

Materials and Methods: A total of 103 intestinal samples (from eight farms clinically suspected to NE) were collected and evaluated by biochemical tests and polymerase chain reaction (PCR).

Results: Genotyping results showed the prevalence of 43.69% (n = 45) for *C. perfringens*. All isolates belonged to type A, and other toxinotypes of bacterium were not detected. Eight isolates (17.78%) from four farms were positive for *netB* gene. The present study represented the prevalence of the *netB* gene for the first time in organic broiler farms.

Conclusions: The results indicate that the role of *netB* in the induction of NE needs to be further investigated, to clarify the role of *C. perfringens* as commensal or pathogenic and to authorize a much better correlation between gene expression of *netB* toxin and the pathogenic capacity of *C. perfringens* strains from organic systems.

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Background

Clostridium perfringens (*C. perfringens*), a major enteric pathogen, can lead to both clinical and subclinical disease in broiler chickens.[1] This bacterium was divided to five types (A, B, C, D and E) based on the presence of major toxins (α, β, ε and ι). It produced some important minor toxins such as enterotoxin, beta$_2$, necrotic enteritis toxin B (NetB), TpeL and perfringolysin O (PFO).[2] *C. perfringens* is responsible for causing necrotic enteritis (NE) of poultry, especially by type A and rarely type C.[3] *C. perfringens* type A is the most frequently isolated clostridial type from NE cases.[4,5]

NE is an economically important disease with severe gastro-intestinal signs in commercial broiler farms and was reported for the first time by Parish.[6] Two forms of NE were described: clinical and subclinical.[7] Clinical NE, primarily in young chickens (between two to six weeks), is characterized by severe necrosis in the mucosa of proximal jejunum and associated with high mortality rates.[8] Subclinical NE is led to a decreased performance (reduced growth, reduced feed efficiency) without mortality, due to the extensive mucosal damage.[9]

Keyburn et al discovered a pore forming toxin of *C. perfringens* which they named NetB and the encoding gene, *netB* and recognized this gene in *C. perfringens* isolates recovered from chickens. They showed the relationship between presence of *netB* gene and NE outbreaks and reported that NetB is critical to the development of NE, in chickens.[10]

To our knowledge, there were not published data about NE outbreaks and responsible toxins for causing this disease in organic broiler farms.

Objectives

This study was firstly aimed to genotype the pathogenic *C. perfringens* isolates in organic broiler farms and secondly to assess the presence of *netB* gene among them and its occurrence with respect to the disease NE.

Materials and Methods

Sampling

A total of 103 intestinal samples of broiler chickens, clinically suspected to NE, were obtained from eight organic farms. Samples were collected aseptically in plastic bags in the post-mortem examination of chickens and quickly transported to the laboratory in ice-cooled containers.

The sampling farms were randomly selected. The analysis for bacteria isolation started as soon as samples arrived to the laboratory.

Isolation and Biochemical Identification

Intestinal contents were processed according to a routine protocol as previously described by authors.[11] The pure cultures of isolated *C. perfringens* were submitted to the following biochemical tests as described by MacFaddin[12]: lecithinase, lipase, gelatinase, motility and skim milk coagulation (stormy reaction). Furthermore, for confirmation of *C. perfringens* isolates, all strains were incubated in selective tryptose-sulfite cycloserine (TSC) agar (Merck, 1.11972), as shown black colonies. Isolates were stored in 25% glycerol at -80˚C for further analyses.

Reference Strains

Positive and negative controls were used for confirmation of *C. perfringens* isolates by multiplex polymerase chain reaction (PCR). The *C. perfringens* ATCC13124 and *C. perfringens* type B (CN228), type C (CN301) and type D (CN409) reference strains were used as positive controls as well as *C. septicum* (CN913) as negative control (Reference strains were obtained from the bacterial isolate archive of the Razi Institute of Iran). Also, distilled H_2O was applied as a negative control to confirm the absence of contamination of material and facilities and removal of experimental errors and to prove the exclusion of non-target DNA.

DNA Extraction

Both, the isolated and reference strains were cultured in tubes with 10 mL thioglycolate broth and incubated anaerobically overnight at 37˚C. Then, bacterial cultures were centrifuged for 10 minutes at 7500 g and collected 10-20 mg of pellets in 1.5 mL microtubes. DNA was extracted using the protocol provided in the DNA extraction kit (DN8115C, Cinnagen, Iran).

Polymerase Chain Reaction Amplification and Assay

Molecular typing of *C. perfringens* isolates were performed by multiplex PCR, as described by authors.[11] Specific primers (Sinaclon, Iran) were used for amplification of genes (Table 1). Also, all isolates were examined for the presence of the *netB* gene by a duplex PCR reaction as previously described.[10] The PCR assay was performed using a thermal cycler (Bio-Rad, California, USA) with a total reaction volume of 50 µL with the following reagents: 5 µL of 10X PCR buffer, 2 µL of 50mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate, 5 U of recombinant Taq DNA polymerase (TA7506C, Sinaclon, Iran), 0.25µM of each of the primers, 5 µL of template DNA and distilled water till 50 µL.

Ten microliters of PCR products were evaluated for expected amplicons by electrophoresis on 1.5% agarose gel. The 100 bp DNA ladder (NL1402, Vivantis, Malaysia) was used as molecular marker to indicate the size of amplicons. DNA safe stain (PR881603, Sinaclon, Iran) was used for detecting nucleic acid in agarose gels. It is as sensitive as ethidium bromide and can be used exactly the same way in agarose gel electrophoresis. The amplified bands were visualized and photographed under UV illumination.

Results

C. perfringens was isolated in 43.69% (n = 45) of 103 intestinal samples from all organic broiler farms and the rates of isolation ranged from 18.18% to 64.29% between different farms (Table 2). Multiplex PCR results showed that all isolates belonged to type A and non-enterotoxin producers, harbouring the alpha toxin gene (*cpa*). Other types of *C. perfringens* (B, C, D and E) were not detected (Figure 1). Duplex PCR for detection of *netB* gene was performed and eight isolates (17.78%) from four farms were positive for this gene (Figure 2).

Discussion

Herein, *C. perfringens* was recovered from all organic farms involvement NE. Forty-five isolates were genotyped by PCR and revealed that all isolates were positive for *cpa* gene and negative for *cpb*, *etx*, *iap* and *cpe* genes. This means that all *C. perfringens* isolates from organic broiler

Table 1. Primers Used in Molecular Identification of Isolated *Clostridium perfringens* in This Study

Gene (Toxin)	Primers	Primers Sequence (5'–3')	Amplicon Size (bp)	Reference
cpa (α)	CPA5L CPA5R	AGTCTACGCTTGGGATGGAA TTTCCTGGGTTGTCCATTTC	900	[13]
cpb (β)	CPBL CPBR	TCCTTTCTTGAGGGAGGATAAA TGAACCTCCTATTTTGTATCCCA	611	[13]
etx (ε)	CPETXL CPETXR	TGGGAACTTCGATACAAGCA TTAACTCATCTCCCATAACTGCAC	396	[13]
iap (ι)	CPIL CPIR	AAACGCATTAAAGCTCACACC CTGCATAACCTGGAATGGCT	293	[13]
cpe (enterotoxin)	CPEL CPER	GGGGAACCCTCAGTAGTTTCA ACCAGCTGGATTTGAGTTTAATG	506	[13]
netB (NetB)	AKP78-F AKP79-R	GCTGGTGCTGGAATAAATGC TCGCCATTGAGTAGTTTCCC	384	[10]
Clostridium cluster I	CI-F1 CI-R2	TACCHRAGGAGGAAGCCAC GTTCTTCCTAATCTCTACGCAT	231	[14]

Table 2. Genotyping Results of *Clostridium perfringens* in Different Organic Broiler Farms

Farms	No. of Samples	No. (%) of Samples Positive for *cpa* Gene	No. (%) of Samples Positive for *netB* Gene
F1	11	2 (18.18)	0 (00.00)
F2	12	4 (33.33)	1 (08.33)
F3	13	7 (53.85)	2 (15.38)
F4	14	9 (64.29)	3 (21.43)
F5	13	6 (46.15)	0 (00.00)
F6	14	5 (35.71)	0 (00.00)
F7	13	5 (38.46)	0 (00.00)
F8	13	7 (53.85)	2 (15.38)
Total	103	45 (43.69)	8 (07.77)

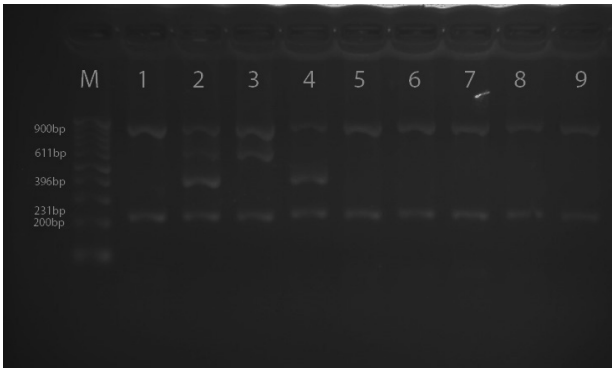

Figure 1. Multiplex PCR Typing of *C. perfringens* Isolates With α (900 bp), β (611 bp), ε (396 bp), ι (293 bp), Enterotoxin (506 bp) and *Clostridium* Cluster (231bp) Primers.
Lane M: VC 100bp DNA ladder (Vivantis, product No: NL1402); lane 1: *Clostridium perfringens* type A reference strain (ATCC 13124); lane 2: *C. perfringens* type B reference strain (CN228); lane 3: *C. perfringens* type C reference strain (CN301); lane 4: *C. perfringens* type D reference strain (CN409); lanes 5-9: *C. perfringens* type A field isolates.

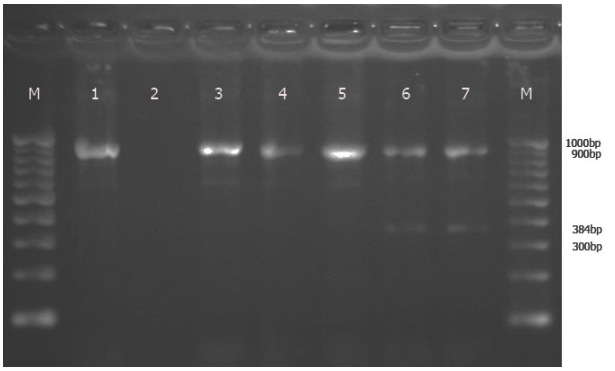

Figure 2. Duplex PCR of *C. perfringens* Type A Isolates for Detection of *netB* Gene (384 bp).
Lane M: VC 100bp DNA ladder (Vivantis, product No: NL1402); lanes 1 and 3-5: *C. perfringens* type A field isolates (negative for *netB* gene); lane 2: negative control (dH2O); lanes 6 and 7: *C. perfringens* type A field isolates (positive for *netB* gene).

farms are type A. These results were similar as previous studies in nonorganic poultry farms.[15-18]

The presence of *cpa* gene is not always correlated with clinical NE and other toxins are effective in NE outbreaks. Enterotoxin producer strains of *C. perfringens* may be associated with food poisoning.[19] In this study, none of the isolates were positive for the *cpe* gene. The absence of this gene is not surprising because this gene is rarely found in avian *C. perfringens* strains.[20,21] This result in organic broiler farms was similar to previous studies in broiler chickens.[17,18,22]

NetB, is a crucial toxin in NE outbreaks and it has been suggested that this potent toxin could contribute to disease effects in field isolates.[10,23] In current study, the *netB* gene was detected in eight out of 45 *C. perfringens* isolates (17.78%) by PCR. Previously studies showed that NetB is not essential to the development of NE in all cases.[15,24] But, our results showed that NetB has significant effect on the incidence of NE and negates those studies. Further studies are suggested to evaluate the relationship between the presence of the *netB* gene that can be produced NetB toxin and the occurrence of NE in organic broiler farms.

In conclusion, *C. perfringens* isolates from organic broiler farms with NE outbreak were found to be similar to those found in conventional systems based on typing characterization. The *netB* gene was detected for the first time at a low incidence (7.77%) in chickens with NE in organic broiler farms. In addition, the present study highlights the need for more studies to clarify the role of *C. perfringens* as commensal or pathogenic and to authorize a much better correlation between gene expression of NetB toxin and the pathogenic capacity of *C. perfringens* strains from organic systems.

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Authors Contributions
Mojtaba Alimolaei developed the original idea and the protocol, abstracted and analyzed data and wrote the manuscript. Majid Ezatkhah cooperated in the experiment and interpretation of data analysis. Neda Shahdadnejad performed sampling and preliminary experiments.

Conflict of Interest Disclosures
None.

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