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

Majid Eztakhah¹, Mojtaba Alamolaei¹∗, Neda Shahdadnejad²

¹Department of Molecular Microbiology, Kerman Branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Kerman, Iran
²Department of Animal Science, Shahid Bahonar University of Kerman, Kerman, Iran

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.

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 i). It produced some important minor toxins such as enterotoxin, beta, 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. The pure cultures of isolated *Clostridium perfringens* were submitted to the following biochemical tests as described by MacFaddin: lecithinase, lipase, gelatinase, motility and skim milk coagulation (stormy reaction). Furthermore, for confirmation of *Clostridium perfringens* isolates, all strains were incubated in selective tryptose-sulfite cycloserine (TSC) agar (Merck, Darmstadt, Germany) for bacteria isolation started as soon as samples arrived to the laboratory.

Reference Strains

Positive and negative controls were used for confirmation of *Clostridium perfringens* by multiplex polymerase chain reaction (PCR). The *Clostridium 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 *Clostridium septicum* (CN913) as negative control (Reference strains were obtained from the bacterial isolate archive of the Razi Institute of Iran). Also, distilled H$_2$O 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 *Clostridium perfringens* isolates were performed by multiplex PCR, as described by authors. 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. 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 dexonucleotide triphosphate, 5 μM 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 *Clostridium 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, *Clostridium 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

<table>
<thead>
<tr>
<th>Gene (Toxin)</th>
<th>Primers</th>
<th>Primers Sequence (5'-3')</th>
<th>Amplicon Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpa (α)</td>
<td>CPA5L</td>
<td>AGTCTACCGCTTGGGATGGAA</td>
<td>900</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>CPA5R</td>
<td>TTTCTGGGTTGTCCATTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpb (β)</td>
<td>CPBL</td>
<td>TCTTTTCTTGGAGGGAGGATAAA</td>
<td>611</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>CPBR</td>
<td>TGAACCTCTATTGTTGATACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>etx (ε)</td>
<td>CPTEXL</td>
<td>TGGGAACTCTGGATACAAA</td>
<td>396</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>CPTEXR</td>
<td>TAACTCTATCTCCATAACTGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iap (ι)</td>
<td>CPII</td>
<td>AAACGCCATTTAAGCTCACACC</td>
<td>293</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>CPRR</td>
<td>CTGCTAACCCCTGGAAATGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpe (enterotoxin)</td>
<td>CPEL</td>
<td>GGGGAAACCCCTAGTGTCTCA</td>
<td>506</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>CPER</td>
<td>ACCAGCTGAGTATCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>netB (NetB)</td>
<td>AKP78-F</td>
<td>GCTGCTGCTGGATAATGCT</td>
<td>384</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>AKP79-R</td>
<td>TGGCCATTGAGTATTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium cluster I</td>
<td>CI-F1</td>
<td>TACCHRAGAGGAGGAGCCAC</td>
<td>231</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>CI-R2</td>
<td>GTCTCTCTAAATCTCACGCAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Genotyping Results of Clostridium perfringens in Different Organic Broiler Farms

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

Enterotoxin producer strains of C. perfringens may be associated with food poisoning. 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. This result in organic broiler farms was similar to previous studies in broiler chickens. NetB, a crucial toxin in NE outbreaks and it has been suggested that this potent toxin could contribute to disease effects in field isolates. 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. 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.

Acknowledgments

We are grateful Mr. Sadegh Aftzali and Mr. Moslem Aftzali for their helpful comments.

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.

Funding/Support

This research was supported by project 0-85-18-90010/5, and funded by the Razi Vaccine and Serum Research Institute (RVSRI), Iran.

References

4. Ołkowski A, Wojnarowicz C, Chirino-Trejo M, Laarveld B, Sawicki G. Sub-clinical necrotic enteritis in broiler chickens: novel etiological consideration based on ultra-structural...