APPLICATION OF MOLECULAR-BIOLOGY METHODS TO THE DIAGNOSIS OF BOTULISM IN MALLARD DUCKS

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Abstract

The application of molecular-biology methods based on PCR and Real–time PCR in the diagnosis of botulism in mallard ducks was described. The participation of Clostridium botulinum C1 toxin in the causing of the disease was demonstrated. The results were confirmed by the sequencing of PCR product. The application of molecular-biology methods provides fast and specific analysis of the occurrence and types of Clostridium botulinum toxins.

Key words: mallard ducks, Clostridium botulinum, botulism, diagnosis.

Botulism is highly fatal disease of human and animals. Aetiological factors of botulism are toxins, which are produced by the heterogeneous, anaerobic, Gram–positive, spore–forming rods from the species Clostridium botulinum. There are seven toxinotypes of Clostridium botulinum, which are marked by the letters from A to G (4, 13). In the aetiology of botulism in animals, C and D toxinotypes are of the great importance. The disease is seldom caused by the A, B, and E toxinotypes. Geographical scope of botulism is very wide. It has been noticed at all continents, besides Antarctica (15).

Toxinotype C plays an important role in the aetiology of botulism in birds. Botulism in birds is rarely caused by the toxins, which are produced by the A, B, and E Clostridium botulinum toxinotypes. The most affected with botulism are migratory birds, especially waterfowl and shorebirds. More than million deaths due to type C of avian botulism have been reported in localised outbreaks in some wetlands in a single year (1, 15).

The detection of Clostridium botulinum is difficult, because of lack of specific microbiological media for the isolation of the microorganism. The most popular method for the detection of Clostridium botulinum is based on biological test on laboratory mice, which confirms the ability to produce botulinum toxins by suspected strains (9, 10). Biochemical identification does not guaranteed distinguishing Clostridium botulinum strains from other bacteria, which have the same biochemical features (5, 8). Increasingly, in the laboratory practice, molecular-biology methods based on PCR and Real–time PCR are applied. The principle of these methods is based on the detection of genes, which determine the ability to produce botulinum toxins (3, 6, 7, 12, 14).

The aim of this article was describing the application of molecular-biology methods for the detection and determination of toxinotypes of Clostridium botulinum in the diagnosis of botulism in mallard ducks.

Material and Methods

Case description. In October 2008, specimens from the vitals of three mallard ducks (Anas platyrhynchos) from one of the public gardens in Mazowieckie province were collected: a fragment of the intestine from the duck marked 1, and kidney, liver, and fragment of the intestine from ducks marked 2 and 3. The specimens were sent to the Institute in order to conduct analysis for the detection and determination of toxinotypes of Clostridium botulinum, because the ducks were suspected of having been poisoned by botulinum toxin. The analysis was conducted by using culture methods and molecular-biology methods based on Real–time PCR and PCR. The results of the analysis were confirmed by the sequencing of PCR product. Sequencing was performed in the Department of Microbiology of the Institute.

Specificity. Specificity of PCR was proved by using reference strains of Clostridium botulinum: NCTC 887 (toxinotype A), NCTC 3815 (toxinotype B), NCTC 8266 (toxinotype E), NCTC 10281 (toxinotype F), toxinotype D (own isolate), and also other strains from the genus Clostridium: C. chauvieri, C. tetani, C.
septicum, C. sporogenes, C. septicum, C. oedematiens, C. sordelli, C. pasteurianum, C. novyi, C. fallax, C. histolyticum (own isolates), and C. perfringens (ATCC 13124). Because of the lack in our collection of reference strain of C. botulinum toxinotype C and in order to approve the specificity of PCR – characteristic product of PCR was submitted to the sequencing analysis.

**Culture of specimens and reference strains.**
About 1 g of specimens was inoculated into the tubes with TPGY broth. Each tube was submitted to the pasteurisation process at 70°C for 15 min and then incubated in an anaerobic jar at 37°C for 48 h, under anaerobic conditions. In order to obtain anaerobic conditions the AnaeroGen system (Oxoid) was used. Reference strains were incubated under the same conditions as the specimens.

**DNA isolation.** One millilitre of liquid culture was removed from the bottom of each tube. Isolation of DNA from the culture was performed by using a kit for DNA isolation - „Genomic Mini AX Bacteria“ (A&A Biotechnology). The isolation was conducted according to the instructions supplied with kit.

**Real – time PCR screening analysis for detection of C. botulinum.** For the detection of C. botulinum, the set of seven primers and TaqMan probe with LNA bases (locked nucleic acids) in 4, 6, 12, and 16 positions were used, according to Raphael et al. (12). This set facilitated the amplification of a highly-conserved region of gene, which determines the production of nonhaemagglutinin component (NTNH), common in all seven toxinotypes of C. botulinum (11, 12). Description of the primers and probe is in Table 1.

### Table 1
**Sequence of primers and probe for Real-time PCR based screening method**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5′GATTTAAGTGAAAAATTATTTAATAT′3</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>5′CCACCTAAGTATTAAATGAAA′3</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>5′TGATGAAATACCTAATAGTGTAAAT′3</td>
<td></td>
</tr>
<tr>
<td>CD2F</td>
<td>5′GACATATCAGATGTATTGAGGA′3</td>
<td>12</td>
</tr>
<tr>
<td>R1</td>
<td>5′TTTACCCATACAATTTAAT′3</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>5′ACTAGCCATACAAAATTAGATC′3</td>
<td></td>
</tr>
<tr>
<td>R3</td>
<td>5′TATTTAAACTTTCTTGCAT′3</td>
<td></td>
</tr>
<tr>
<td>Probe</td>
<td>NTNH410 5′FAM-ATCATTGGGACACATATTATAGTCA-BHQ′3</td>
<td></td>
</tr>
</tbody>
</table>

A – indicates location of LNA (locked nucleic acid bases)
FAM - 6-carboxylfluorescein
BHQ - Black Hole Quencher

### Table 2
**Sequence of primers for multiplex PCR for the determination of A, B, E, and F C. botulinum toxinotypes**

<table>
<thead>
<tr>
<th>Toxinotype</th>
<th>Primer</th>
<th>Sequence</th>
<th>Length of PCR product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>IA_03_fw</td>
<td>5′GGGCGCTAGAGGTAGCCTTGT′3</td>
<td>101 bp</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>IA_03_rev</td>
<td>5′TCTTYATTTCCAGAAGCTATATT′3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>CBMLB1</td>
<td>5′CAGGAGAAGTTGGACGCCTAA′3</td>
<td>205 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBMLB2</td>
<td>5′CTTGCAGCTTGTTCCTTCTTG′3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CBMLE1</td>
<td>5′CCAAGATTTTTTACAGCCCTTA′3</td>
<td>389 bp</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>CBMLE2</td>
<td>5′GCTATTGATCCAAAACGCTGGA′3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>CBMLF1</td>
<td>5′CGGCTTTCTATTAGGAACGGG′3</td>
<td>543 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBMLF2</td>
<td>5′TAACTCCCTAGCCCGCAT′3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R= A, G
Y= C, T

### Table 3
**Sequence of primers used for the determination of C and D C. botulinum toxinotypes**

<table>
<thead>
<tr>
<th>Toxinotype</th>
<th>Primer</th>
<th>Sequence</th>
<th>Length of PCR product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>CS–11</td>
<td>5′ATAACTATGCTATATGAGCCTTG′3</td>
<td>290 bp</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>CS–22</td>
<td>5′TGGAGGATTTGGATTTCCAGGG′3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>DS–11</td>
<td>5′GTGATCTCTTTAATGACATG′3</td>
<td>497 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DS–22</td>
<td>5′TCCTGGCAATGTGAGGATGC′3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The final volume of reaction mixture was 20 µl and contained: 5 µl of DNA template, 4 µl of LightCycler TaqMan Master (prod. Roche), 0.7 µM of each primer, and 0.24 µM of NTNH410 probe. Thermocycling was performed on the LightCycler 2.0 instrument (Roche). Following a 10-min activation step at 95°C, reactions were subjected to 45 cycles at 95°C for 15 s, at 42°C for 15 s, and at 55°C for 60 s. Fluorescence data was acquired following the third step of each cycle (55°C).

**Multiplex PCR analysis for determination of A, B, E, and F toxinotypes of C. botulinum.** For determination of A, B, E, and F toxinotypes of C. botulinum, the set of eight primers was used, according to the CEN (2). This set allowed the detection of a gene, which creates botulinum neurotoxin production (BoNT) in particular toxinotypes. The sequences of primers are summarised in Table 2.

The final volume of reaction mixture was 25 µl and contained: 5 µl of DNA template, 2.5 µl of 10xTaq buffer with KC1 (Fermentas), 0.3 µM of each primer, 4 mM of MgCl2 (Fermentas), 200 µM of dNTP mixture (Fermentas), and 1.25U of Taq DNA polymerase (Fermentas).

Thermocycling was performed on the T1 thermocycler instrument (Biometria). Following initial denaturation step at 95°C for 60 s, reaction was subjected to 27 cycles at 95°C for 30 s, at 53°C for 30 s, and at 72°C for 3 min. Final extension was carried out at 72°C for 3 min. Detection of PCR products was performed on agarose gel.

**PCR–based analysis for determination of C and D toxinotypes of C. botulinum.** For determination of C and D toxinotypes of C. botulinum, the set of two primers was used, according to the Takeshi et al. (14). This set enabled the detection of a gene, which determines the production of light chain (Lc) in BoNT. The sequences of primers are summarised in Table 3.

The final volume of reaction mixture was 25 µl and contained: 5 µl of DNA template, 2.5 µl of 10xTaq buffer (Fermentas), 0.3 µM of each primer, 4 mM of MgCl2 (Fermentas), 200 µM of dNTP mixture (Fermentas), and 1.25U of Taq DNA polymerase (Fermentas).

Thermocycling was performed on the T1 thermocycler instrument (Biometria). Following an initial denaturation step at 95°C for 60 s, reaction was subjected to 30 cycles at 95°C for 30 s, at 55°C for 30 s, and at 72°C for 3 min. Final extension was carried out at 72°C for 3 min. Detection of PCR products was performed on agarose gel.

**Electrophoresis.** The PCR products were separated in 2% agarose gel stained with ethidium bromide, located in 1xTBE buffer (Fermentas) for 1.5 h under 100 V. Into each well, 10 µl of reaction mixture and 2 µl of loading buffer 6xDNA Loading Dye (Fermentas) were loaded. The molecular weight of the obtained products was determined on the basis of a molecular weight marker, which was GeneRuler™ 100 bp DNA Ladder Mix (Fermentas). After separation, PCR products were analysed under an UV light transiluminator (Vilber-Lourmat).

**Sequencing.** Positive result of PCR for the determination of C. botulinum C toxinotype was proved by a sequencing analysis. The analysis was performed with the same set of primers, which was used in PCR. Obtained result of sequencing was analysed by using the BLAST algorithm (Basic Local Alignment Search Tool) in order to detect the homology of PCR product sequence with those from GenBank (http://blast.ncbi.nlm.nih.gov/blast.cgi).

**Results and Discussion**

The screening analysis conducted by using the Real–time PCR-based method for detection of the gene, which determines the production of non-haemagglutinin component (NTNH) of botulinum protoxin indicated the occurrence of this gene in the culture of the specimen of the intestine from duck No. 2.

PCR analysis for the determination of A, B, E, and F C. botulinum toxinotypes excluded their role in causing disease.

PCR analysis for the determination of C and D toxinotypes indicated the occurrence of a gene, which determines the production of BoNT/C1 (Fig. 1). Type C strains consist of two distinct subtypes: Cα and Cβ. The subtype Cα produces C1 and lesser amounts of C2 and D toxins, and Cβ produces C2 toxin. Type D produces predominantly type D toxin along with smaller amounts of C1 and C2. The heterogeneity that exists in types C and D has probably arisen from the mutation or recombination of phage genomes that is thought to occur during the cycles of curing and recombination of type C and D strains in the environment. Ability to produce toxins by toxinotypes C and D of C. botulinum is encoded on separate pseudolysogenic bacteriophages (cultures of toxigenic strains can be cured of their prophages and stop producing toxins but can be converted to a toxigenic state by reinfection by phages). The type of the toxin produced is determined by the specific phage with which the bacterium is infected (5, 13).

Computer analysis of the PCR product sequence by using the BLAST algorithm indicated a 98% homology in the sequence of the gene determining the production of BoNT/C1 of C. botulinum C Ekland (http://blast.ncbi.nlm.nih.gov/blast.cgi).

The conducted analyses were specific only for DNA from reference strains of C. botulinum. There were no specific products for DNA from other strains from the genus Clostridium. Specificity of PCR for the determination of C. botulinum toxinotype C was proved by the sequencing analysis of PCR product.

Botulism in birds in Poland is nearly unknown. Lack of information about botulism cases in animals in Poland may be caused by lack of proper laboratory methods for the detection of C. botulinum.
The application of methods for the detection of *C. botulinum*, which are described in Polish Standards (9, 10), and which are used routinely in Poland, does not guarantee a high possibility of the detection of this microorganism. Lack of selective solid media for the isolation of all toxinotypes of *C. botulinum* additionally complicates its isolation and identification.

The application of the described methods of molecular-biology give the possibility of detection of *C. botulinum* at the stage of liquid culture. These methods are not routinely used in laboratory practice in Poland. Using them gives the possibility of limiting difficulties with diagnosis of botulism cases connected with isolation problems from analysed material and approving the affinity of suspected strains to this species.

References