IN-HOUSE VALIDATION OF MULTIPLEX PCR METHOD 
FOR DETECTION OF CLOSTRIDIUM BOTULINUM 
IN FOOD AND FEED

TOMASZ GRENDA, ELŻBIETA KUKIER, ZBIGNIEW SIERADZKI, 
MAGDALEŃA GOLDSZTEJN, AND KRZYSZTOF KWIATEK

Department of Hygiene of Animal Feedingstuffs, 
National Veterinary Research Institute. 24-100 Pulawy, Poland. 
tomasz.grenda@piwet.pulawy.pl

Received: January 3, 2012 Accepted: April 27, 2012

Abstract

The aim of this study was to perform an in-house validation of multiplex PCR method for C. botulinum detection in food and feed samples. The study was carried out on food and feed matrixes artificially contaminated by spores of C. botulinum reference strains. The following characteristic parameters for qualitative detection were estimated: limit of detection expressed as LOD50 according to the Spearman-Kärber formula, specificity, sensitivity, and accuracy according to the PN-EN ISO 16140:2004. The validated method showed high specificity. Specific PCR products were revealed only for DNA obtained from samples contaminated with C. botulinum spores. PCR inhibition was observed, especially during examination of contaminated feed. The calculated LOD50 for feed was nearly 10 times higher than for food. The implemented method enables to obtain test results during 3 d without time-consuming process of isolation and proving the ability of strains to produce botulinum toxins.

Key words: Clostridium botulinum, PCR, in-house validation, food, feed.

The bacteria from the species Clostridium botulinum are spore–forming rods, which have the ability to produce the most potent toxic substances in the environment. They are named botulinum toxins. These toxins are responsible for animal and human botulism, which is caused by the consumption of food or feed contaminated by spores and toxins produced by C. botulinum. In most cases, the methods for detection of this microorganism are based on demonstrating its ability to produce botulinum toxins. (1, 15, 16, 19, 20).

The methods based on PCR techniques enable detection of genes, which determine the production of botulinum toxins. These methods are characterised by high sensitivity and specificity (18 - 20).

Detection of C. botulinum is possible at different stages of culture method or directly from sample without enrichment (in situ). The most PCR protocols are based on detection of bont genes, which determine the production of active form of botulinum toxins (BoNTs), specific for particular toxin types of this pathogen. Some of the known protocols are also based on detection of nth gene, which determines production of non-toxic non-haemagglutinin component in botulinum protoxin. This component is common for all C. botulinum toxin types (19).

Most of PCR protocols are based on detection of single bont genes specific for particular toxin types. The literature is describing also numerous protocols of multiplex PCR (mPCR) methods, which enable the detection of several toxin types in one probe (17, 18).

The aim of this study was to perform an in-house validation of previously improved mPCR for C. botulinum detection in food and feed samples.

Material and Methods

This study was carried out on food (fish salad) and feed (ground grain) matrixes, which were contaminated by spores of C. botulinum reference strains. The following strains were used: C. botulinum NCTC 887 (type A), C. botulinum NCTC 3815 (type B), C. botulinum NCTC 8266 (type E), and C. botulinum NCTC 10281 (type F). The spores of the strains were cropped according to methods described by Fletcher et al. (12).

The 10 g of each contaminated and non-contaminated sample was incubated in 90 ml of TPGY broth during 48 h at 37°C, under anaerobic conditions. Clostridium DNA was extracted from 1 ml of culture using a commercial kit – Genomic Mini AX Bacteria (A&A Biotechnology). The obtained DNA was examined by mPCR method.

For the detection of bont genes of A, B, E, and F toxin types of C. botulinum, the previously improved
mPCR method was used with the set of eight primers, according to the CEN (2). The sequences of primers are shown in the Table 1. These primers allowed detecting the genes, which are responsible for synthesis of active component of BoNT in particular toxin types. The final volume of the PCR mixture was 25 μl and contained: 5 μl of DNA template, 2.5 μl of 10xTaq buffer with KCl (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 (Biometria). Following initial denaturation step at 95°C for 60 s, reaction was subjected to 27 cycles at 95°C for 30 s, 53°C for 30 s, and 72°C for 3 min. Final extension was carried out at 72°C for 3 min.

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

The results of validation were estimated according to the PN – EN – ISO – 16140:2004 (6). The limit of detection and sensitivity values were estimated for three contamination levels (Table 2). At each level, 20 samples of contaminated feed and food matrices were analysed.

The specificity of the method was estimated by examination of 20 samples of feed and food matrices, non-contaminated by C. botulinum spores, and by examination of DNA obtained from the other strains of Clostridia: C. chauvoei, C. tetani, C. septicum, C. sporogenes, C. oedematiens, C. sordelli, C. pasteurianum, C. novyi, C. fallax, C. histolyticum (own isolates), and C. perfringens (ATCC 13124).

![Fig 1. Characteristic products of mPCR for the A, B, E and F toxinotypes.](image)

M – molecular mass standard

1 – C. botulinum, toxin type A (101 bp); 2 – Clostridium botulinum, toxin type B (205 bp); 3 – C. botulinum, toxin type E (389 bp); 4 – Clostridium botulinum, toxin type F (543 bp); 5 – C. botulinum A, B, E, F; (C-) – PCR negative control

The accuracy was estimated for samples at the mentioned three levels of contamination (Table 2) and for non-contaminated samples.

The limit of detection, expressed as LOD₅₀, for particular toxin types in feed and food matrices was statistically estimated using Spaerman – Kärber method. LOD₅₀ is the number of microorganisms per gram of sample at which 50% of the tests are positive and 50% are negative. During the examinations, guidelines from AOAC documents (3) and from other publications were used (7, 8, 14). The estimation of LOD₅₀ was conducted using excel calculator of LOD₅₀, available at www.aoc.org/accreditation/DEMO.xls. The obtained results were expressed in the 95% confidential interval.

### Table 1

<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’GGGCCTAGAGGTAGCGTARTG’3</td>
<td>101 bp</td>
<td>11</td>
</tr>
<tr>
<td>A</td>
<td>IA_03_rev</td>
<td>5’TCIIYATTTCCAGAAGCATTTT’3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>CBMLB1</td>
<td>5’CAGGAGAAGTGAGCGAAAA’3</td>
<td>205 bp</td>
<td>17</td>
</tr>
<tr>
<td>B</td>
<td>CBMLB2</td>
<td>5’CTTGGCGCTTTGTTTCTTG’3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CBMLE1</td>
<td>5’CCAAGATTTTCATCCGCCTA’3</td>
<td>389 bp</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CBMLE2</td>
<td>5’GCTATTGATCCAAAACGGTG’3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>CBMLF1</td>
<td>5’CGGCTTCATTAGAAGCGGA’3</td>
<td>543 bp</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>CBMLF2</td>
<td>5’TAACCTCCCCTAGCCCCGTAT’3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R = A, G; Y = C, T
Table 2
Contamination levels

<table>
<thead>
<tr>
<th>Toxin type</th>
<th>Fish salad samples</th>
<th>Ground grain samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Contamination levels Spore (cfu)/g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level I</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Level II</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>Level III</td>
<td>0.003</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Specificity (SP) was estimated as the rate of negative samples, evaluated in the examination as negative (NA) to the total number of negative results (N–):

\[ SP = \frac{NA}{N–} \times 0\%
\]

Sensitivity (SE) was estimated as the rate of positive samples, evaluated in the examination as positive (PA) to the total number of positive results (N+):

\[ SE = \frac{PA}{N+} \times 0\%
\]

Accuracy (AC) was estimated as the rate of sum of positive samples, evaluated in the examination as positive (PA) and negative samples, evaluated in the examination as negative (NA) to the total number of samples (N):

\[ AC = \frac{PA + NA}{N} \times 0\%
\]

For determination of LOD50 the following formulas were used:

- Estimation of LOD50

\[
\mu = \sum_{i}^{k} p_{i} \cdot \log \left( x_{i} + \frac{1}{2} \right)
\]

\[
\log \left( \hat{OD}_{LOD50} \right) = \tilde{t}
\]

\[
LOD_{50} = 0\mu
\]

- Estimation of 95% confidential interval for suspected value of LOD50

\[
\text{var} \tilde{\mu} = \sum_{i}^{k} p_{i} q_{i} / \left( \log(1 + x_{i}) - x_{i} \right) \left( \log(1 + x_{i}) - x_{i} \right)
\]

\[
\hat{\text{var}} \tilde{\mu} = \sqrt{\text{var} \tilde{\mu}}
\]

\[
\ln \left( \hat{OD}_{50} \right) \pm 1.96 \cdot 0.303 (96\%) \ln \left( \hat{OD}_{50} \right) + 0.303 (96\%)
\]

\[
\text{Where:}
\]
\[
\hat{\mu} - \text{LOD}_{50} \text{ estimator};
\]
\[
\text{var} \tilde{\mu} - \text{variation of LOD}_{50} \text{ estimator};
\]
\[
k - \text{number of contamination level};
\]
\[
p_{i} - \text{sensitivity for each analysed level (i = 1...k)};
\]
\[
x_{i} - \text{log microorganism number at the given level (x_{i} < x_{k})};
\]
\[
q_{i} = 1 - p_{i};
\]
\[
n_{i} - \text{number of examined samples at given level};
\]
\[
s - \text{standard deviation};
\]
\[
\pm 1.96 - \text{value read out from t - student’s tables for level of relevance } \alpha = 0.05 \text{ and undetermined number of freedom degree at 95\% confidential interval};
\]
\[
2.303 - \text{approximate value of conversion rate of decimal into natural logarithm.}
\]

Results

The examination of food samples contaminated by spores of C. botulinum reference strains showed that the applied method allowed obtaining 100% specificity for each examined toxin type. The sensitivity was different for each level of contamination and toxin type, and ranged from 5% to 100%. The highest value (79%) of accuracy was noted for toxin type E, whilst the lowest (65%) was obtained for toxin type F. The lowest limit of detection was obtained for toxin type A and it was 0.034 (0.021–0.056) spore/g, whilst the highest value of this parameter was observed for toxin type F and it reached 0.102 (0.062–0.168) spore/g. More detailed information about the obtained results for contaminated food matrix samples has been shown in the Table 3.
Table 3
Values of characteristic parameters for detection of *C. botulinum* A, B, E, and F toxin types in fish salad samples

<table>
<thead>
<tr>
<th>Toxin type</th>
<th>Level of contamination (spore/g)</th>
<th>N</th>
<th>N+</th>
<th>N_</th>
<th>PA</th>
<th>NA</th>
<th>SP</th>
<th>SE</th>
<th>AC</th>
<th>LOD&lt;sub&gt;50&lt;/sub&gt; (spore/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>100%</td>
<td>69% (0.021 – 0.056)</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td>0.03</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>65%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.003</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>100%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>0.5</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>100%</td>
<td>70% (0.022 – 0.054)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.005</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>100%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>1</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>100%</td>
<td>79% (0.069 – 0.129)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>100%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>0.7</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>100%</td>
<td>65% (0.062 – 0.168)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.07</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.007</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>100%</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

N - total number of samples; (N+) - total number of positive results; (N_) - total number of negative results; PA – number of positive samples evaluated in the examination as positive; NA – number of negative samples evaluated in the examination as negative; SP – specificity; SE – sensitivity; AC – accuracy; LOD<sub>50</sub> – limit of detection estimated with using Späerman – Kärber method.

Table 4
Values of characteristic parameters for detection of *C. botulinum* A, B, E, and F toxin types in ground grain samples

<table>
<thead>
<tr>
<th>Toxin type</th>
<th>Level of contamination (spore/g)</th>
<th>N</th>
<th>N+</th>
<th>N_</th>
<th>PA</th>
<th>NA</th>
<th>SP</th>
<th>SE</th>
<th>AC</th>
<th>LOD&lt;sub&gt;50&lt;/sub&gt; (spore/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>3</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>100%</td>
<td>77% (0.139 – 0.262)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.03</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>100%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>5</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>100%</td>
<td>65% (0.167 – 0.376)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>16</td>
<td>-</td>
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<td>80%</td>
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<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>20</td>
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<td>3</td>
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<td>20</td>
<td>-</td>
<td>20</td>
<td>100%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>10</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>100%</td>
<td>64% (0.542 – 1.464)</td>
</tr>
<tr>
<td></td>
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<td>1</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>11</td>
<td>-</td>
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<tr>
<td></td>
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<td>0.1</td>
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<td>-</td>
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<td>0%</td>
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<td>-</td>
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<td>-</td>
<td>20</td>
<td>100%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F</td>
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<td>7</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>100%</td>
<td>76% (0.295 – 0.607)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.7</td>
<td>20</td>
<td>20</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<td></td>
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<td>0.07</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>20</td>
<td>100%</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Symbols are explained in the footnote to Table 3.
The analysis of the ground grain samples contaminated by *C. botulinum* spores revealed that specificity for each examined toxin type reached 100%. The sensitivity ranged from 0% to 100% and was different for each toxin type and level of detection. The obtained value of accuracy was the lowest for toxin type B – 64%, and the highest for toxin type A – 77%. The lowest limit of detection was noted for toxin type A and it equaled 0.191 (0.139 – 0.262) spore/g, and the highest value was obtained for toxin type E, which reached 0.891 (0.542 – 1.464) spore/g. The values of LOD<sub>50</sub> obtained for contaminated feed matrices were nearly 10 times higher than for food matrices. More detailed information about the obtained results for contaminated feed matrix samples has been shown in Table 4.

The specific mPCR products were only obtained for DNA extracted from samples contaminated by *C. botulinum* spores. There were no positive results for DNA obtained from other strains of *Clostridia*.

## Discussion

The obtained results showed differences in the limits of detection (LOD<sub>50</sub>) of *C. botulinum* spores in the food and feed matrices. The highest obtained values of LOD<sub>50</sub> could be linked with the effect of matrix ingredients, which had inhibitory influence on the growth of *C. botulinum* and mPCR. The relatively high values of LOD<sub>50</sub> reached in the detection of *C. botulinum* toxin type E, could be connected with the influence of culture temperature used in the experiments. Because of the fact that the optimal temperature for growth of *C. botulinum* proteolytic strains is 37°C, this temperature was chosen for incubation. In contrast to the proteolytic strains, the optimal temperatures for growth of toxin type E ranged from 18°C to 25°C. In the most cases, proteolytic strains: A and B are connected with botulism in humans and animals (except C and D toxin types). These strains produce proteases, which release active components of BoNT from the protoxins complexes and do not need action of proteolytic enzymes produced by host organism (animal or human) (15, 16, 19). The problems connected with the inhibitory effect of matrix ingredients during detection *C. botulinum* were also reported by other authors, who described detection of this pathogen after enrichment process of samples. Lindström et al. (17) presented mPCR method for the detection of A, B, E, and F toxin types in food and faeces matrices. The limit of detection for faeces obtained levels from 10<sup>3</sup> to 10<sup>5</sup> spore/g, whilst for fish and meat samples ranged from 10<sup>2</sup> to 10<sup>5</sup> spore/g. Fach et al. (10) reported PCR method for the detection of A, B, E, and F toxin types in food samples with the limit of detection 10<sup>3</sup> cfu/g. Dahlenborg et al. (9) showed difficulties with the detection of *C. botulinum* spores in faecal samples, which was possible only at the higher levels of 10<sup>3</sup> – 10<sup>5</sup> spore/g.

Despite limitation connected with inhibitory influence of matrixes, the described and validated mPCR method improves the detection of *C. botulinum* in food and feed samples. Nowadays, in Poland, there are no data about *C. botulinum* occurrence in the mentioned matrixes. The detection of this pathogen is limited by traditional culture methods, because in the genus *Clostridium* there are strains, which have similar phenotypic properties to *C. botulinum* species, despite the fact that they are unable to produce BoNTs. There are four metabolic groups of *C. botulinum* and other *Clostridia*, which have the same biochemical feathers (16, 17, 20). The toxin genes of isolated strains could be lost during culturing process, which additionally creates difficulties in identification of this pathogen (16, 19). The loss of A, B, E, and F toxin type toxin genes is connected with mutations in bont regions of gene’s cluster, which creates botulinum protoxin. The toxin genes of C and D toxin types are localised on lisogenic bacteriophages. When the bacteriophage is present, the microorganism, with characteristic biochemical properties for the mentioned toxin types, is able to produce BoNTs. There are no selective media for the isolation of this kind of pathogens (19). The presented mPCR enables to detect *C. botulinum* without the isolation process.

The described and validated mPCR method might be used for *C. botulinum* detection in different biological materials and identification of particular toxin types of this anaerobe. Currently, in Poland, there is a lack of proper methods for the detection of this pathogen. The most frequently used method is the ethically controversial and time-consuming mouse bioassay (4, 5). The use of the presently described method allows limiting the time of detection from 10 (mouse bioassay) to 3 d. The described test could be useful for supporting diagnosis of botulism in humans and animals. The use of this method was described in our previous publication dealing with diagnosis of botulism in mallard ducks (13). Nowadays, PCR based methods become standardised. Multiplex PCR for detection of *C. botulinum* has been the subject of CEN/TC 275/WG 6 Technical Committee works in relation to detection of foodborne pathogens in food and feed (2). In spite of the fact that the described method improves laboratory proceedings, it is only a tool for the detection of *C. botulinum*. In order to provide food and feed safety and effective laboratory diagnosis of botulism, development of methods for BoNTs detection is also needed.

## Acknowlegments
This work was financially supported by the Polish Ministry of Science and High Education. Project No N N308 563639 (2010-2012).

## References


