Highly sensitive real-time PCR detection of neurotoxin-forming Clostridia in foods

Botulism is a potentially fatal disease that is caused by the botulinum toxin, the most toxic naturally occurring substance [1]. The neurotoxin is formed by various strains of the anaerobic, Gram-positive and spore-forming genus Clostridium (C. botulinum, C. baratii and C. butyricum) [2]. The botulinum toxin is classified in eight serotypes A-H [2][3], of which the serotypes A, B, E and F (BoNT A, B, E, F) are relevant for humans [4].

Food-induced botulism

Under the aspect of food safety two forms of botulism are significant – food-related botulism and infant botulism. Illness transmissible by foods is the classic form of botulism. Causes of poisoning are frequently the consumption of preserves contaminated by the toxin, such as sausage and vegetables preserves, but also canned fish products. In order to avoid botulism poisoning among infants, they should not be fed any honey in the first year of their life as Clostridia can be contained in the product in the form of spores. The spores can reproduce in the infant intestine, which is not yet matured, and form the life-threatening toxins. Alongside honey, contaminated infant formula milk powder is also connected with infant botulism [4][5].

In recent years Clostridia have been an international trade issue. In 2012/2013 major food companies such as Danone and Coca-Cola had to remove products from the market due to suspicion of C. botulinum. Initially it was only reported that "toxic bacteria" had been found in dairy products from New Zealand. Then it became known that the bacterium Clostridium botulinum had been "detected" in three deliveries of a whey protein from the New Zealand dairy Fonterra and the intermediate product had thus become a risk. The whey concentrate is processed for instance in powdered milk (baby food) and sports beverages. Ultimately this was a case of mistaken identification. C. sporogenes was subsequently identified as the contaminant [6][7].

To date the gold standard for detecting contamination and for toxin typification has been a mouse bioassay. In Germany, this represents the reference method in accordance with § 64 of the German Food and Feed Code (LFGB) for public food surveillance authorities. In this method the subject sample is injected into the animal intraperitoneally. In the case of positive samples, the animal initially develops a wasp waste as a typical symptom, before it then dies as a result of respiratory distress or cardiac arrest. The toxin type can be identified through survival of the mice when the corresponding antitoxin is administered.

New quick method to ISO/TS 17919:2012

The example of Fonterra shows impressively that there is a need for quick methods for specific analyses in the food sector. The mouse bioassay is not only questionable on ethical grounds, but also involves a high security outlay, so that only few laboratories can carry out botulinum diagnoses. That is why a new, more specific and faster method for detecting the neurotoxins was developed. Detection is carried out via molecular biological processes using real-time PCR. With the help of specific hydrolysis probes, the four toxins relevant for humans can be
detected. The toxin types are identified via specific detection in the fluorescence channels FAM and HEX and via a subsequent melting curve analysis. Figure 1 shows the amplification and melt curve analyses of BoNT A, B, E and F on the LightCycler® 480 (LC480) with the real-time PCR kit by way of example.

Fig. 1 Detection of the botulinum neurotoxins (BoNT) in the fluorescence channels FAM and HEX with the foodproof® Clostridium botulinum Detection LyoKit.

Top row: Detection of BoNT A, B, E and F using amplification curves in the FAM and HEX channel. Bottom row: Identification of BoNT E (left) and BoNT F (right) using subsequent melting curve analysis.

Purified DNA samples (DNA, approx. 25 genome equivalents of C. botulinum) and infant formula milk powder contaminated with 104 colony-forming units (CFU)/ml were used as food samples (LM) for the detection.
This method is extremely sensitive and the detection limit lies at 25 genome equivalents and also complies with ISO/TS 17919:2012 [8]. By contrast with the mouse bioassay which takes several days, with the real-time PCR method a result is obtained after max 3 hours (excluding enrichment).

The evaluation scheme is summarised in Table 1. In order to rule out false negative results due to PCR inhibition, the kit has an internal amplification control that is detected in the ROX channel. False positive results due to DNA contamination are limited by the presence of the enzyme Uracil-N-glycosylase in the lyophilised ready-to use PCR mixture.

Tab. 1 Summarising evaluation overview of the detection of the toxin genes BoNT A, B, E und F

<table>
<thead>
<tr>
<th>Botulinum-Neurotoxin Type (BoNT)</th>
<th>FAM Channel</th>
<th>HEX Channel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amplification</td>
<td>Melting point</td>
</tr>
<tr>
<td>A</td>
<td>positive</td>
<td>none</td>
</tr>
<tr>
<td>B</td>
<td>negative</td>
<td>none</td>
</tr>
<tr>
<td>E</td>
<td>positive</td>
<td>68°C ± 2°C</td>
</tr>
<tr>
<td>F</td>
<td>negative</td>
<td>none</td>
</tr>
<tr>
<td>Positive control</td>
<td>positive</td>
<td>(64°C ± 2°C)</td>
</tr>
<tr>
<td>Negative control</td>
<td>negative</td>
<td>none</td>
</tr>
</tbody>
</table>

Examination of food samples

A wide range of food samples such as e.g. initial infant formula milk powder, luncheon meat, smoked salmon and canned stews were examined to validate the kit. For this purpose at least 25 g of the sample was weighed out, if necessary minced (in the case of meat or fish products) and added to or (in the case of milk powder) dissolved in the medium suitable for Clostridia (tryptone-peptone-glucose-yeast extract (TPGY) bouillon). The dilution ratio is 1:10, i.e. 25 g sample are added to approx. 225 ml TPGY broth. In order to keep the oxygen input low, strong shaking of the samples or the medium is avoided. Enrichment was carried out anaerobically at 30 °C. The ISO standard recommends initial cultivation for 24 hours, after which the first analysis can be conducted already. In the event of a negative result the sample should be incubated anaerobically for a further 48 hours, and then 1 ml of the suspension be transferred into 9 ml fresh enrichment broth and incubated for a further 18 hours. After this a final PCR analysis is conducted.

After briefly shaking the bottle, 800 µl are withdrawn from the enrichment broth and the DNA extraction is carried out e.g. using foodproof® StarPrep Two Kit (S 400 08). This takes approx. one hour. 25 µl of the extract can be used directly for the PCR analysis.
The actual PCR analysis comprises adding the DNA extract to the ready Lyomix in the PCR strips, brief mixing and a centrifuging step, before it is measured in a suitable real-time PCR Cycler. The kit was validated for the Roche LightCycler® 480, but many other cyclers can be used.

Conclusion

With the foodproof® Clostridium botulinum Detection LyoKit, real-time PCR-based 5’-Nuclease detection of botulinum neurotoxins or their genes is now possible. This is a multiplex assay for detecting all BoNT A, B, E and F serotypes of the bacteria strains Clostridium botulinum, Clostridium baratii and Clostridium butyricum that are relevant for humans.

The foodproof® Clostridium botulinum Detection LyoKit has been validated for a large number of food samples. Alongside infant formula milk powder and preserves, the kit can also be used for detecting toxin-forming Clostridia in honey, various types of sausage products such as salami, luncheon meat, black pudding and bierwurst, and for smoked or cured fish and ready sauces.

References


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