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Pentaplexed Quantitative Real-Time PCR Assay for the Simultaneous Detection and Quantification of Botulinum Neurotoxin-Producing Clostridia in Food and Clinical Samples

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Botulinum neurotoxins are produced by the anaerobic bacterium Clostridium botulinum and are divided into seven distinct serotypes (A to G) known to cause botulism in animals and humans. In this study, a multiplexed quantitative real-time PCR assay for the simultaneous detection of the human pathogenic C. botulinum serotypes A, B, E, and F was developed. Based on the TaqMan chemistry, we used five individual primer-probe sets within one PCR, combining both minor groove binder- and locked nucleic acid-containing probes. Each hydrolysis probe was individually labeled with distinguishable fluorochromes, thus enabling discrimination between the serotypes A, B, E, and F. To avoid false-negative results, we designed an internal amplification control, which was simultaneously amplified with the four target genes, thus yielding a pentaplexed PCR approach with 95% detection probabilities between 7 and 287 genome equivalents per PCR. In addition, we developed six individual singleplex real-time PCR assays based on the TaqMan chemistry for the detection of the C. botulinum serotypes A, B, C, D, E, and F. Upon analysis of 42 C. botulinum and 57 non-C. botulinum strains, the singleplex and multiplex PCR assays showed an excellent specificity. Using spiked food samples we were able to detect between 10^3 and 10^5 CFU/ml, respectively. Furthermore, we were able to detect C. botulinum in samples from several cases of botulism in Germany. Overall, the pentaplexed assay showed high sensitivity and specificity and allowed for the simultaneous screening and differentiation of specimens for C. botulinum A, B, E, and F.

Botulinum neurotoxins (BoNTs), the causative agents of botulism, are produced by the anaerobic bacterium *Clostridium botulinum* and are divided into seven serotypes, A to G. While the botulinum neurotoxins BoNT/A, BoNT/B, BoNT/E, and BoNT/F are known to cause botulism in humans, BoNT/C and BoNT/D are frequently associated with botulism in cattle and birds. Despite its toxicity, BoNT/G has not yet been linked to naturally occurring botulism (26).

Botulism is a life-threatening illness caused by food contaminated with BoNT (food-borne botulism), by the uptake and growth of *C. botulinum* in wounds (wound botulism), or by colonization of the intestinal tract (infant botulism) (14). In addition, *C. botulinum* and the botulinum neurotoxins are regarded as potential biological warfare agents (8).

The gold standard for the detection of BoNTs from food or clinical samples is still the mouse lethality assay, which is highly sensitive but rather time-consuming. In addition to various immunological assays for BoNT detection, several conventional and real-time PCR-based assays for the individual detection of *bont* genes have been reported (2, 9–12, 15, 20, 23, 27–30). A major improvement is the simultaneous detection of more than one serotype, which results in a reduction of effort and in the materials used. In recent years, both conventional and real-time PCR-based multiplex assays have been developed for the simultaneous detection of *C. botulinum* serotypes (1, 6, 22, 24). To date,

however, no internally controlled multiplex real-time PCR assay for the simultaneous detection and differentiation of all four serotypes relevant for humans has been reported.

We describe here a highly specific and sensitive multiplex real-time PCR assay based on the 5'-nuclease TaqMan chemistry (17) for the simultaneous detection of the *C. botulinum* types A, B, E, and F, including an internal amplification control (IAC). Furthermore, we developed six different singleplex assays based on the TaqMan chemistry for the detection of *C. botulinum* serotypes A to F. Assays were validated on 42 *C. botulinum* strains, 57 non-*C. botulinum* strains, on spiked food samples, and on real samples from cases of botulism in Germany.

Materials and Methods

Bacterial strains and culture conditions.
The bacterial strains used in the present study are listed in Table 1. Clostridial strains were cultured in reinforced clostridia medium (RCM; Sifin, Berlin, Germany) or in tryptone-peptone-glucose- yeast (TPGY) broth for 3 days in an anaerobic workstation (Don Whitley Scientific, Ltd., West Yorkshire, United Kingdom). The titer of the *C. botulinum* strains 2292 (serotype A), 1029 (serotype B), 1032 (serotype E), and 1033 (serotype F) was determined on blood agar plates. One milliliter of 10-fold dilutions of the cultures was spread on blood agar plates, and colonies were counted after 24 h of incubation, under anaerobic conditions. Bacteria were stored at -20°C in RCM or TPGY broth until use.

PCR primers and probes.
The primers and probes used here are given in Table 2. Primers and probes were based on the published DNA sequences from GenBank database (http://www.ncbi.nih.gov/GenBank/) for the neurotoxin genes *bont/a*, *bont/b*, *bont/c*, *bont/d*, *bont/e*, and *bont/f* (Table 2). All primers and LNA probes were obtained from TIB Molbiol (Berlin, Germany) or Sigma-Aldrich (Munich, Germany); MGB probes were obtained from Applied Biosystems (Foster City, CA).

Standard plasmids and internal amplification control.
As positive controls and for evaluation of the real-time PCR assays, plasmids containing the PCR target regions pBoNT/A, pBoNT/B, pBoNT/C, pBoNT/D, pBoNT/E, and pBoNT/F were constructed. The amplicon for each real-time PCR was amplified by conventional PCR using the same primers as for the TaqMan PCR (Table 2). Amplicons were cloned into pCR2.1-TOPO vectors and used for the transformation of TOP10 cells (both from Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. Serial dilutions of the plasmids were prepared in H_2O (Roth, Karlsruhe, Germany) supplemented with 100 µg of salmon-sperm DNA (AppliChem, Darmstadt, Germany)/ml and stored at -20°C until use.

The plasmid pKoMa2 was used as an IAC for the real-time PCRs. The IAC consists of a chemically synthesized DNA sequence (Table 2) containing the target sequence for the primers KoMa_F and KoMa_R and the probes KoMa_TM or KoMa_TS (Table 2) cloned into the pPCR-Script vector (Stratagene, La Jolla, CA).

Real-time PCR assays.
All real-time PCRs were performed on the LightCycler 480 II system in a total volume of 20 µl using a white LightCycler 480 Multiwell Plate 96 covered with adhesive sealing foil (Roche, Mannheim, Germany). Thermal cycling was done with a two-step PCR protocol: activation of the *Taq* DNA polymerase at 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 60°C for 40 s. The fluorescence data were collected at the end of every 60°C step, and runs were analyzed using LightCycler 480 software (Roche). All real-time PCRs were performed as duplicates. Primer and probes used for singleplex and multiplex PCR are listed in Table 2. (i) The multiplex reaction mix contained 2 µl of template; 10 µl of 2 x ABsolute QPCR mix (ABgene, Epsom, United Kingdom); and optimized concentrations of primer and probes for *C. botulinum* serotypes A, B, E, and F and KoMa2 (Table 2). Also, 75 copies of pKoMa2, as an IAC, were added to a total volume of 20 µl of H_2O. (ii) The singleplex reactions contained 2 µl of template, 10 µl of 2 x ABsolute QPCR mix, optimized

concentrations of primer and probes for *C. botulinum* serotypes A to F (Table 2), and H2O added to a final volume of 20 µl.

Standard curves and PCR performance.
To determine the performance of the different real-time PCR assays, standard curves were obtained by amplification of 10-fold dilutions of the standard plasmids. The obtained threshold cycles (*CT*) were plotted against the logarithm of copy number. Analyses were done using the Prism 5.0 Software (GraphPad, La Jolla, CA). The PCR efficiency (*E*) of the multiplex and singleplex assays was calculated according to the formula $E = 10^{(-1/\text{slope})} - 1$ (21).

Detection limit.
The detection probability for the standard plasmids was determined by multiplex and singleplex PCR, analyzing serial dilutions of the standard plasmids pBoNT/A, pBoNT/B, pBoNT/E, or pBoNT/F. The 95% detection probability was calculated with Prism 5.0, using the cumulative Gaussian distribution model (Table 3).

Preparation of spiked food samples.
Food samples (vacuum-packed, gilled, smoked mackerel; frozen green beans; vacuum-packed, smoked black pudding with meat) used in the present study were purchased from a local retail shop and stored at -20°C until use. Food samples were homogenized in stomacher bags with an equal volume of gelatin-phosphate buffer (0.2% [wt/vol] gelatin in 28mM Na2HPO4 [pH 6.2]) using a BagMixer (Interscience, Saint Nom la Bretèche, France). Each homogenate was spiked with 10^6, 10^5, 10^4, or 10^3 CFU of *C. botulinum* serotype A (strain 2292), *C. botulinum* serotype B (strain 1029), *C. botulinum* serotype E (strain 1032), or *C. botulinum* serotype F (strain 1033)/ml, respectively.

DNA extraction.
Genomic DNA from bacterial culture was purified with the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA extraction from spiked food samples (Table 4) or real botulism specimens (Table 5) was performed with the DNeasy blood and tissue kit using a modified protocol. After centrifugation (10 min, 7,500 x *g*) of 200 µl of the spiked food slurry, the sediment was dissolved in 180 µl of lysis buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 1.2% [vol/vol] Triton X-100, 20 mg of lysozyme/ml) and lysed for 30 min at 37°C. After the addition of 25 µl of proteinase K (600 mAU/ml; Qiagen) and 180 µl of ATL buffer (Qiagen), the lysate was incubated for 30 min at 56°C, followed by 15 min at 95°C. The following steps were performed according to the manufacturer's instructions for the purification of DNA from animal tissue. The purified template DNA was eluted twice with 50 µl of AE buffer (Qiagen).

Investigation of suspected samples.
Suspected samples of three cases of human food-borne botulism (cases 1, 2, and 3), one case of infant botulism (case 4), and two cases of animal botulism, one in cattle (case 5) and one in ducks (case 6), were analyzed by TaqMan PCR (Table 5). The specimens were collected between 2006 and 2009 in Germany. Suspected specimens were transmitted to our laboratory by the relevant German state institutions for food, drug and epizooties, namely, the Landeslabor Brandenburg (case 1), the Landesamt für Verbraucherschutz Sachsen-Anhalt (case 3) or the Institut für Lebensmittel, Arzneimittel, und Tierseuchen in Berlin (cases 2, 5, and 6), respectively. Samples from case 4 originated from the Children's Hospital of the Charité (Berlin, Germany). For enrichment cultures, 5 ml of TPGY broth and/or cooked meat medium (Oxoid, Hampshire, United Kingdom) were inoculated with 500 µl of homogenized samples and incubated anaerobically at 28°C and/or 37°C for 3 to 5 days. After DNA extraction, real-time multiplex and singleplex assays were performed in two independent experiments as described above.

Results

Real-time PCR amplification performance.
Starting with singleplex real-time PCR approaches, we developed individual assays for the amplification of *bont/a*, *bont/b*, *bont/c*, *bont/d*, *bont/e*, and *bont/f* using MGB containing oligoprobes (Table 2). Amplification performances of singleplex real-time PCR were determined from the standard curves of the BoNT standard plasmids. As shown in Fig. 1, all real-time PCR assays showed a strong linear correlation ($R2 > 0.99$) between the *CT* value and the template concentration over a range of 5 orders of magnitude.

In order to develop a multiplex assay for the simultaneous amplification of *bont/a*, *bont/b*, *bont/e*, and *bont/f*, together with an IAC, it was necessary, for some of the target genes, to select TaqMan probes different from that of the singleplex assays. It was also necessary to include both MGB oligonucleotides and LNA oligonucleotides in order to increase the annealing temperature and to shorten the probe length (Table 2). The oligoprobes were labeled with different reporter dyes (Cy5, VIC, FAM, TEX, and Cyan500) and were cross validated with the respective forward and reverse primers in a multiplex approach (data not shown). For each *bont* amplicon, the oligoprobes were tested either coupled to an MGB moiety or modified with LNA bases, and additionally with one of the four different colors Cy5, VIC, FAM, or TEX. The optimal MGB-containing or LNA-containing oligoprobes were selected for each *bont* amplicon and finally, the quadruplex assay for *bont/a*, *bont/b*, *bont/e*, and *bont/f* was combined with the IAC assay (i.e., the presence of 75 copies of the synthetic sequence IAC, plasmid pKoMa2). The optimal primer-probe sets for the final pentaplexed approach are given in Table 2. As shown in Fig. 1 for the simultaneous amplification of *bont/a*, *bont/b*, *bont/e*, and *bont/f*, together with IAC, the performances of the multiplex assay and the respective singleplex assays were almost identical. The slopes for both the multiplex and the singleplex assays ranged from -3.889 to -3.243, resulting in PCR efficiencies of 97.9% versus 90.3%, 96.2% versus 92.9%, 103.4% versus 93.8%, and 90.6% versus 88.6%, respectively, for the multiplex versus singleplex assays for *bont/a*, *bont/b*, *bont/e*, and *bont/f*. In addition, the fluorescence spectra of the different reporter dyes used in the multiplex assay were well separated and showed no spectral overlap (data not shown).

Specificity of real-time PCRs.
The specificity of primers and probes was evaluated *in silico* against the published sequences from the GenBank database using the BLAST algorithm. Primer and probe sets were selected to allow amplification of all subtypes of serotypes known thus far: A1, A2, A3, A4, and A5; B1, B2, B3, nonproteolytic B, and bivalent B; E1, E2, E3, E4, E5, and E6; F baratii, F proteolytic, F nonproteolytic, and F bivalent; and C and D, including C/D and D/C mosaic types. To confirm the *in silico* results, we analyzed 42 *C. botulinum* strains and 57 non-*C. botulinum* strains with the singleplex and multiplex real-time PCR assays (Table 1). The multiplex realtime PCR for the simultaneous detection of *bont/a*, *bont/b*, *bont/e*, and *bont/f* showed a high specificity for the predicted target. A total of 4 of the 42 tested *C. botulinum* strains (REB1750, NCTC 2916, NCTC 11199, and NCTC 12265) tested positive for two BoNT genes. As expected, the nontoxic (as determined by mouse assay) *C. botulinum* A strain 2276 and all of the *C. botulinum* C and D strains revealed no PCR signal in the multiplex PCR specific for *bont/a*, *bont/b*, *bont/e*, and *bont/f*.

Similarly, each of the six singleplex real-time PCR was only positive for the predicted target. Strains 2276, 1030, 2141, 2145, and 1739, which had been originally reported to contain the *bont* gene, gave negative results in the six singleplex reactions. The absence of the *bont* genes in these cultures was confirmed by mouse assay. Neither the multiplex PCR nor the singleplex PCR analyses of the non-*C. botulinum* strains was positive for the *bont* genes.

Detection limits of multiplex and singleplex PCR.
To define the sensitivity of the multiplex real-time PCR, we determined the detection probability for different copy numbers between 6 and 800 copies of the standard plasmids in the presence of 75 copies of the IAC pKoMa2. The 95% detection probability per reaction analyzed with the multiplex assay was 32.5 copies for pBoNT/A, 7.4 copies for pBoNT/B, 24.0 copies for pBoNT/E, and 287.2 copies for pBoNT/F (Table 3).

The obtained detection probabilities of 95% for the standard plasmids analyzed with the singleplex PCR were 31.6 copies for pBoNT/A, 7.4 copies for pBoNT/B, 6.4 copies for pBoNT/C, 6.9 copies for pBoNT/D, 16.8 copies for pBoNT/E, and 122.4 copies for pBoNT/F per reaction (Table 3).

Detection of C. botulinum from spiked food samples.
We tested the sensitivity of the multiplex PCR assay directly from food samples without prior enrichment by anaerobic culture. We spiked three different food samples (black pudding, green beans, and smoked mackerel) with 10-fold serial dilutions of *C. botulinum* serotypes A, B, E, and F (10^3 to 10^6 CFU/ml), extracted the DNA, and performed the multiplex PCR assay as described above. The detection limits for *C. botulinum* serotypes A, B, E, and F in smoked mackerel were 10^5, 10^3, 10^4, and 10^4 CFU/ml, respectively (Table 4). These detection limits correspond to 400 bacterial cells per PCR of *C. botulinum* serotype A, 4 cells per PCR for *C. botulinum* serotype B, and 40 cells per PCR for *C. botulinum* serotypes E and F. In green beans and black pudding, we were able to detect as little as 10^4 CFU of *C. botulinum* serotype A/ml, 10^3 CFU of *C. botulinum* serotypes B and E/ml, and 10^4 CFU of *C. botulinum* serotype F/ml (Table 4). This corresponds to detection limits of 40 cells per PCR for *C. botulinum* serotypes A and F, and 4 cells per PCR for *C. botulinum* serotypes B and E. All *C. botulinum*negative samples returned positive signals specific for the IAC pKoMa2. In addition, we were also able to detect *C. botulinum* serotypes A, B, E, and F directly from the spiked green beans and black pudding, without previous DNA extraction (data not shown).

Investigation of suspected samples.
In order to test the multiplex and singleplex real-time PCR assays on real specimens, we analyzed six suspected food, four stool, and several tissue samples from six different cases of botulism in Germany (Table 5). All analyses were performed both on DNA extracted directly from the specimens, without prior anaerobic culture or any other enrichment step, and after enrichment by anaerobic culture. The suspected home-cured ham (case 1), the home-made green bean salad (case 2), and home-marinated herring and one stool sample from case 3 tested positive for BoNT/B, BoNT/A, and BoNT/E, respectively, by both multiplex and singleplex PCRs. The stool sample from a case of infant botulism (case 4) prior to antibiotic treatment returned a positive result for *C. botulinum* serotype A. However, stool specimens taken a week later, after antibiotic treatment, were negative. All tissue specimens from a cow with botulism symptoms (case 5) gave positive results in the singleplex PCR for *C. botulinum* serotype D. Specimens from a symptomless control animal were negative. In gut and liver specimens from three perished ducks taken during an outbreak of avian botulism (case 6) BoNT/C was identified. All samples that were positive for *bont* gave positive results without and after enrichment (Table 5), except for the frozen herring (case 3), which tested positive only after enrichment by anaerobic culture, both with the real-time PCR and in the mouse bioassay. In addition, all of the tested samples demonstrated positive results for the IAC, thus confirming the absence of PCR inhibitors in the extracted DNA. All PCR results were confirmed by mouse bioassays performed at the Robert Koch-Institut, at the Landeslabor Brandenburg (Frankfurt/Oder, Germany) or the Institut für Lebensmittel, Arzneimittel, und Tierseuchen (Berlin, Germany).

Discussion

In the present study we developed a pentaplexed real-time PCR assay for the simultaneous detection and quantification of *bont/a*, *bont/b*, *bont/e*, and *bont/f*, including an internal control IAC in comparison to singleplex real-time PCR assays for all relevant *bont* target genes. We analyzed the assay performance, specificity, and sensitivity and validated the PCR approaches with differently spiked food samples and with specimens from several cases of botulism.

While the detection of single BoNT genes by conventional or real-time PCR methods is well established, a number of PCR assays for the detection of three or more BoNT genes have also been described (11, 13, 15, 23, 27, 28, 29). More recent work has complemented these studies by the description and validation of a conventional multiplex approach for the simultaneous detection of all four human pathogenic *bont* genes (6, 22). Previous approaches have used degenerated primers to detect more than one BoNT gene. Similarly, our pentaplexed PCR assay uses degenerate primers to accommodate sequence variations within the specific binding regions of *bont* genes and therefore is able to detect all human pathogenic serotypes and subtypes described thus far (A1, A2, A3, A4, and A5; B1, B2, B3, nonproteolytic B, and bivalent B; E1, E2, E3, E4, E5, and E6; F baratii, proteolytic F,

nonproteolytic F, and bivalent F) (5, 7, 16). In addition, the pentaplexed PCR approach correctly identifies a novel A subtype identified in our group in the context of a recent food-borne botulism case (strain Chemnitz, Table 1 and also unpublished results).

Generally, major advantages of the real-time TaqMan approach compared to the conventional PCR approach (using gel separation of amplified targets) include the increased assay specificity (by a third gene-specific oligoprobe), reduced assay time (online detection of amplification products, no electrophoresis step), and ease of quantification (using defined concentrations of standard plasmids). From a technical perspective, multiplexing a real-time PCR for more than four target genes is challenging for a number of reasons. (i) To date, only a few instruments are able to detect and differentiate more than four different fluorescent reporter dyes, among them the LightCycler 480 II used in the present study. (ii) An increase in the number of primers and probes per PCR at the same time enhances the problem of nonspecific oligonucleotide interactions. Therefore, shorter oligonucleotides with good hybridiza- tion performance are necessary. For this purpose, alternative probe chemistries have been introduced, such as MGB- and LNA-containing oligoprobes (19). (iii) Some of these improved oligoprobes can only be purchased coupled to a limited number of fluorescent dyes (e.g., MGB-containing probes can be purchased only with up to three different reporter dyes).

To our knowledge, the present study is one of the first to describe a multiplex real-time PCR approach that combines both MGB- and LNA-containing oligoprobes within one reaction. Our study describes only the second reported use of a pentaplexed real-time PCR assay, following a recent description of a pentaplexed assay for the simultaneous quantification of respiratory RNA viruses (25). While setting up our pentaplexed assay, we empirically learned that mixing of MGB- and LNA-containing oligoprobes rendered a superior PCR performance, compared to a multiplex assay with LNA probes only. For each target gene, we carefully selected the optimal probe chemistry (MGB or LNA) and reporter dye (Cy5, VIC, FAM, TEX, and Cyan500) in order to obtain concordant results for the pentaplex approach and the optimal singleplex reaction. In the final pentaplexed assay, both the specificity and the sensitivity were equivalent to the singleplex approach: analysis of 42 *C. botulinum* strains and 57 non-*C. botulinum* strains using the designed multiplex and singleplex real-time PCR assays showed high specificity for their predicted target, with no observed cross-reactivity.

With respect to the analysis of clinical, environmental, or food samples, the use of an internal amplification control is mandatory for any diagnostic PCR method. An internally controlled PCR would demonstrate failure of the assay, e.g., due to the presence of PCR inhibitors in the complex sample material, and therefore avoids false-negative results (18). Consequently, the use of an IAC is included in different international standards, e.g., in ISO 22174, for the analysis of food. Among all PCR assays published on the detection of *C. botulinum*, however, only a limited number include the use of an IAC (1, 3, 6, 24). In our pentaplexed PCR approach, the IAC based on a synthetic sequence was simultaneously amplified with the target sequences *bont/a*, *bont/b*, *bont/e*, and *bont/f*. In order to avoid competition between the *bont*-specific and the IAC-specific amplification, the IAC plasmid pKoMa2 was optimally used at a low concentration (75 copies per reaction). In our description of the multiplex approach, we followed almost all of the recently defined MIQE guidelines for real-time PCR experiments which serve to standardize experimental practice and to promote consistency between laboratories (4).

When we tested the sensitivity of the multiplex PCR assay with *C. botulinum* A, B, E, and F spiked into different food, we found a limit of detection of 10^3 to 10^5 CFU/ml directly from the spiked food samples, without prior enrichment by anaerobic culture. In addition, we successfully applied the multiplex and singleplex real-time PCR assays to specimens from cases of food-borne, infant, and animal botulism (different food, feces, and different organs). Samples that tested positive for *C. botulinum* by mouse bioassay concordantly tested positive by our multiplex and singleplex assays. With one exception, the multiplex and singleplex real-time PCR worked with DNA directly extracted from the suspicious specimens, without prior anaerobic culture, thus yielding results faster than the mouse bioassay. Nevertheless, the independent enrichment step by anaerobic culture is recommended for cases where low concentrations of bacteria are present.

Advancing the existing PCR-based detection of BoNT-producing clostridia, the present study describes a pentaplexed real-time PCR assay for the simultaneous detection of *bont/a*, *bont/b*, *bont/e*, and *bont/f*, including an IAC. Of technical interest is the successful combination of both MGB- and LNAcontaining oligoprobes in the current pentaplex approach. The multiplex assay was found to be as sensitive and specific as the independently developed and validated singleplex real-time PCR assays

for all relevant *bont* genes. Furthermore, the pentaplex assay allows the detection, differentiation, and quantification of all serotypes and subtypes associated with human botulism known thus far, including a novel A subtype identified recently in our group. Most importantly, the assay turned out to be very robust and rapid when analyzing food and clinical specimens from cases of botulism. Therefore, this assay will be of great diagnostic value as a rapid screening tool in surveillance studies or in outbreak situations.

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Tables and Figures

Table 1. Strains tested by singleplex and multiplex real-time PCR

Species	Strain[a]	Source[b]	Other collection name(s)	PCR result (serotype)[c]	
				Singleplex	Multiplex
Clostridium botulinum (serotype A)	1028	Göttingen	NCTC 7272, ATCC 19397, NCTC 4587	A	A
	2267	Göttingen	CECT 551	A	A
	2276*	Göttingen	CCUG 7735	–	–
	2277	Göttingen	CCUG 7968	A	A
	2292	Göttingen	NZRCC 4997	A	A
	NCTC 2012	NCTC	Loch Maree	A	A
	REB 1750	TLLV		A, F	A, F
	NCTC 2916	NCTC	2447	A, B	A, B
	NCTC 3805	NCTC	62	A	A
	NCTC 3806	NCTC	97	A	A
	NCTC 9837	NCTC	Mauritius	A	A
	NCTC 11199	NCTC	F199-200/78	A, B	A, B
	Chemnitz	RKI		A	A
	Friedrichshain	RKI		A	A
Clostridium botulinum (serotype B)	1029	Göttingen	NCTC 7273	B	B
	2278	Göttingen	CCUG 7969	B	B
	2293	Göttingen	75-3074	B	B
	REB 89	TLLV		B	B
	NCTC 12265	NCTC		B, F	B, F
	NCTC 751	NCTC	X	B	B
	NCTC 3807	NCTC	115	B	B
	NCTC 3815	NCTC	6B Nevin, ATCC 443	B	B
	Templin	RKI		B	B
	Waldbrunn	RKI		B	B
	KL34/08	Leipzig		B	B
	KL35/08	Leipzig		B	B
Clostridium botulinum (serotype C)	1030*	Göttingen	NCTC 8264	–	–
	2141*	Göttingen	468C	–	–
	NCTC 8548	NCTC	PR 14	C	–
	2279	Göttingen	CCUG 7970	C	–
	2145*	Göttingen	REB 1455	–	–
Clostridium botulinum (serotype D)	1031	Göttingen	NCTC 8265, D6f	D	–
	1739*	Göttingen	ATCC 9633	–	–
	2142	Göttingen	1873D	D	–
Clostridium botulinum (serotype E)	1032	Göttingen	NCTC 8266, Nanaimo	E	E
	2271	Göttingen	CECT 4611	E	E
	Wittenmoor	RKI		E	E
Clostridium botulinum (serotype F)	REB 1955	TLLV		F	F
	2272	Göttingen	CECT 4612	F	F
	2297	Göttingen	83-4304	F	F
	1033	Göttingen	NCTC 10281, Langeland	F	F
	REB 1072	TLLV		F	F
Bacillus anthracis	UDIII-7 (Jena)	RKI		–	–
Bacillus cereus	DSM 31	RKI	ATCC 14579	–	–
	DSM 4312	RKI	NCTC 11143	–	–
Bacillus thuringiensis	DSM 350	RKI		–	–
Brucella abortus	Biotype 1	RKI		–	–
Brucella melitensis	Biotype 1	RKI		–	–
Brucella ovis	Biotype 1	RKI		–	–
Burkholderia cepacia	ATCC 25416	RKI	NCTC 10743, DSM 7288	–	–
Burkholderia mallei	ATCC 23344	RKI		–	–
Burkholderia pseudomallei	ATCC 23343	RKI		–	–
Burkholderia thailandensis	DSM 13276	RKI	ATCC 700388	–	–
Chromobacterium violaceum	DSM 30191	RKI	ATCC 12472	–	–
Citrobacter youngae	13927/03	RKI		–	–
Clostridium baratii	3027	Göttingen		–	–
Clostridium butyricum	NCTC 3396	Göttingen		–	–
Clostridium chauvoei	1116	Göttingen		–	–

Continued on following page

TABLE 1—*Continued*

Species	Strain[a]	Source[b]	Other collection name(s)	PCR result (serotype)[c]	
				Singleplex	Multiplex
Clostridium difficile	FR 460	RKI†		–	–
	NCTC 13287	RKI†	R7404	–	–
	NCTC 13366	RKI†	R20291	–	–
	NCTC 11204	RKI†	N1	–	–
Clostridium perfringens	28	Göttingen	D462	–	–
	1037	Göttingen	NCTC 8732	–	–
	1968	Göttingen	NCTC 3110	–	–
	BT221′7	RKI		–	–
	RKI09057	RKI		–	–
Clostridium septicum	RKI09038	RKI		–	–
	RKI09040	RKI		–	–
	RKI09044	RKI		–	–
Clostridium sordellii	BT221′8	RKI		–	–
Clostridium sporogenes	RKI09041	RKI		–	–
	RKI09043	RKI		–	–
	RKI09045	RKI		–	–
Clostridium tetani	NCTC 3642	Göttingen		–	–
Coxiella burnetii	Nine Mile	RKI		–	–
Enterobacter cloacae	P99	RKI		–	–
Escherichia coli	A002-43 Le10	RKI		–	–
Francisella philomiragia	DSM 7535	RKI	ATCC 25015	–	–
Francisella tularensis subsp. *holarctica*	LVS	RKI		–	–
Francisella tularensis subsp. *tularensis*	ACTC 6223	RKI		–	–
Klebsiella oxytoca	CCUG 15788	RKI		–	–
Klebsiella pneumoniae	ATCC 43816	RKI		–	–
Pseudomonas aeruginosa	ATCC 9027	RKI		–	–
Pseudomonas fluorescens	ATCC 13525	RKI	NCTC 10038, CCEB 546	–	–
Staphylococcus aureus	DSM 20231	RKI	ATCC 12600	–	–
Stenotrophomonas maltophilia	ATCC 13637	RKI	810-2, NCTC 10257	–	–
Yersinia enterocolitica	Ye1	RKI		–	–
Yersinia pestis	EV76	RKI		–	–
Yersinia pseudotuberculosis	Yps1	RKI		–	–

[a] *, nontoxic *C. botulinum* strains according to mouse bioassay.

[b] NCTC, National Collection of Type Culture, London, United Kingdom; Göttingen, Miprolab Mikrobiologische Diagnostik, Göttingen, Germany; TLLV, Thüringer Landesamt für Lebensmittelsicherheit und Veterinärmedizin, Bad Langensalza, Germany; Leipzig, Konsiliarlabor für Anaerobe Bakterien, Leipzig, Germany; RKI, Robert Koch-Institut, Berlin, Germany. †, strains kindly provided by U. Nübel, Robert Koch-Institut, Wernigerode, Germany.

[c] In the multiplex approach, *bont/a*, *bont/b*, *bont/e*, and *bont/f* were simultaneously amplified together with IAC. Isolates producing the toxins C and D were used here as negative controls. In the singleplex approach, the respective individual *bont* genes were tested for comparison. A, B, C, D, E, or F, specific PCR signal for *bont/a*, *bont/b*, *bont/c*, *bont/d*, *bont/e*, or *bont/f*, respectively. –, no PCR signal for *bont/a*, *bont/b*, *bont/c*, *bont/d*, *bont/e*, or *bont/f*.

Table 2. Primers and probes for singleplex and multiplex real-time PCR assays

Target[a]	Primer or probe	Sequence (5′→3′)[b]	Position[c]	Application[d]	Concn (nM)[e]
bont/a	BoNT_A_F	ATTAGAGGTTATATGTATCTTAAAGGGC	79–106 (S)	M, S	300
	BoNT_A_R	CTAACAATATTATCTTYATTTCCAGAAGC	215–187 (A)	M, S	300
	BoNT_A_T_{MGB}	FAM-TTTTATAATAAATTTTGTCCCC-MGB-NFQ	180–159 (A)	S	150
	BoNT_A_T_{LNA}	Cy5-TACGCT**ACCTCTA**-BBQ	120–108 (A)	M	150
bont/b	BoNT_B_F	TTGCATCAAGGGAAGGCT	569–586 (S)	M, S	150
	BoNT_B_R	ATCCACGTCTATTAAATATACTTGCG	685–660 (A)	M, S	150
	BoNT_B_$T1_{MGB}$	FAM-CCAGAATATGTAAGCGTATT-MGB-NFQ	616–635 (S)	S	100
	BoNT_B_$T2_{MGB}$	VIC-CCAGAATATGTAAGCGTATT-MGB-NFQ	616–635 (S)	M	100
bont/c	BoNT_C_F	AGAGAAAACATTATAGAYCCAGAAACTT	629–656 (S)	S	250
	BoNT_C_R	GACTTAGAAAATCTACCCTCTCCTACAT	795–768 (A)	S	250
	BoNT_C_T_{MGB}	FAM-CACCAAATCCTTCTTGT-MGB-NFQ	707–691 (A)	S	150
bont/d	BoNT_D_F	GGGTAATACCAGAAAGATTTTCATC	177–201 (S)	S	150
	BoNT_D_R	AGGATCATAATAACTTTGATACTTTGAAGT	268–239 (A)	S	150
	BoNT_D_T_{MGB}	FAM-TCCAAGTTTAAGTAAACC-MGB-NFQ	211–228 (S)	S	150
bont/e	BoNT_E_F1	TGAAAATAATGTCAATCTCACCTCTTCA	217–244 (S)	M, S	250
	BoNT_E_R1	AAATAATGCTGCTTGCACAGGTT	340–318 (A)	M, S	250
	BoNT_E_T_{MGB}	FAM-CAGCATTATTAGAACAAC-MGB-NFQ	252–269 (S)	M, S	75
bont/f	BoNT_F_F1	CCGGMTTCATTARAGAACGGAAG	375–397 (S)	M, S	250
	BoNT_F_R1	TGATATTTCTTSTAACAAAACTTYCCCTG	527–499 (A)	M, S	250
	BoNT_F_T_{MGB}	FAM-CTTATTATGATCCTAATTATTTAAC-MGB-NFQ	403–427 (S)	S	100
	BoNT_F_T_{LNA}	TEX-CTTAT**TA**T**GA**TCC**TA**ATTATT**TTA**AC-BHQ2	403–427 (S)	M	100
IAC	KoMa_F	GGTGATGCCGCATTATTACTAGG	198–220 (S)	M	150
	KoMa_R	GGTATTAGCAGTCGCAGGCTT	336–316 (A)	M	150
	KoMa_TS	Cy5-TTCTTGCTTGAGGATCTGTCGTGGATCG-BBQ	224–251 (S)	S	150
	KoMa_TM	Cyan500-TTCTTGCTTGAGGATCTGTCGTGGATCG-BHQ1	224–251 (S)	M	150

[a] GenBank accession numbers: *bont/a*, AY327854; *bont/b*, AB084152; *bont/c*, D49440; *bont/d*, X54254; *bont/e*, AB040128; *bont/f*, X81714.

[b] BBQ, BlackBerry Quencher; BHQ, Black Hole Quencher; FAM, 6'-carboxyfluorescein; MGB, minor groove binder; NFQ, nonfluorescent quencher; TEX, Texas Red; boldfaced nucleotides denote locked nucleic acid (LNA) bases. Nucleotide acronyms: M, A/C; R, A/G; S, G/C; Y, C/T. The IAC synthetic sequence (5'→3') was as follows: CGGCTCTAGCGCTGGTGGAGGTTAGAGTTCTCTGACATACGTGCTTCTGAACGGTAGGGAGTTG ACGGACTGAGGGTAGGAGTGCTTAGCGTAGGAGTATTAGGTGGCGTGCTGTGGTGGTCGCGTG TGGGTTTAAGGAGTAAGTGCATTGAGGATTCAGGTGACACAGTAACGTGCAGTTGACTGGTGATG CCGCATTATTACTAGGCGATTCTTGCTTGAGGATCTGTCGTGGATCGGGGAGCGCAAACCTTACA TGATATGTCTAAAATAGCTTTATGCCCTGCGATCGACCATATTTAAAAGCCTGCGACTGCTAATAC CTATAGACTGAGGAGGGATTGAGAGAGCGAAAAATAGAGCAGACTGTATGATTACTATCGCGTGC CATCTCTAACTTTGCATAAGCGTCGTATTATTGGCAGCTACGAGTATCACGATTAGTCCGAACTAG TGGC (the underlined nucleotides denote the priming sites for the primers KoMa_F and KoMa_R and the probes KoMa_TM or KoMa_TS).

[c] S, sense orientation; A, antisense orientation.

[d] M, multiplex real-time PCR; S, singleplex real-time PCR as indicated in Materials and Methods. In independent experiments, individual singleplex real-time PCRs gave identical results in the presence or absence of 75 copies of the synthetic gene IAC and the probe KoMa_TM, where KoMa_TS was used as depicted with Cy5 as a reporter dye and BBQ as a quencher.

[e] Primer and probe concentrations were optimized for singleplex and multiplex real-time PCR assays.

Table 3. Detection probability for singleplex and multiplex realtime PCR

C. botulinum serotype	No. of copies/assay[a]	Detection probability (%)[b]	
		Singleplex PCR	Multiplex PCR[c]
A	100	4/4 (100.0)	4/4 (100.0)
	50	6/6 (100.0)	5/6 (83.3)
	25	9/10 (90.0)	8/10 (80.0)
	12	3/12 (25.0)	3/12 (25.0)
	6	5/14 (35.7)	2/14 (14.3)
B	100	4/4 (100.0)	4/4 (100.0)
	50	6/6 (100.0)	6/6 (100.0)
	25	10/10 (100.0)	10/10 (100.0)
	12	12/12 (100.0)	12/12 (100.0)
	6	9/14 (64.3)	9/14 (64.3)
C	100	4/4 (100.0)	
	50	6/6 (100.0)	
	25	10/10 (100.0)	
	12	12/12 (100.0)	
	6	13/14 (92.9)	
D	100	4/4 (100.0)	
	50	6/6 (100.0)	
	25	10/10 (100.0)	
	12	11/12 (91.7)	
	6	12/14 (85.7)	
E	100	4/4 (100.0)	4/4 (100.0)
	50	6/6 (100.0)	6/6 (100.0)
	25	10/10 (100.0)	9/10 (90.0)
	12	12/12 (100.0)	7/12 (58.3)
	6	10/14 (83.0)	8/14 (57.1)
F	800	4/4 (100.0)	4/4 (100.0)
	400	6/6 (100.0)	6/6 (100.0)
	200	10/10 (100.0)	7/10 (70.0)
	100	10/12 (83.3)	7/12 (58.3)
	50	5/14 (35.7)	5/14 (35.7)

[a] Tests were performed using the corresponding standard plasmids pBoNT/A, pBoNT/B, pBoNT/C, pBoNT/D, pBoNT/E, or pBoNT/F, respectively.
[b] That is, the number of positive results/number of replicates tested.
[c] Multiplex PCR in the presence of 75 copies of IAC.

Table 4. Detection of *C. botulinum* serotypes A, B, E, and F spiked into food samples

C. botulinum serotype[a]	CFU of spiked *C. botulinum* cells/ml of sample	Multiplex real-time PCR results for three different spiked food samples[b]		
		Smoked mackerel	Green beans	Black pudding
A	1.0×10^6	A	A	A
	1.0×10^5	A	A	A
	1.0×10^4	–	A	A
	1.0×10^3	–	–	–
B	1.0×10^6	B	B	B
	1.0×10^5	B	B	B
	1.0×10^4	B	B	B
	1.0×10^3	B	B	B
E	1.0×10^6	E	E	E
	1.0×10^5	E	E	E
	1.0×10^4	E	E	E
	1.0×10^3	–	E	E
F	1.0×10^6	F	F	F
	1.0×10^5	F	F	F
	1.0×10^4	F	F	F
	1.0×10^3	–	–	–

[a] The strains used were strain 2292 (*C. botulinum* serotype A), strain 1029 (*C. botulinum* serotype B), strain 1032 (*C. botulinum* serotype E), and strain 1033 (*C. botulinum* serotype F).
[b] A, B, E, or F, PCR signal for *bont/a*, *bont/b*, *bont/e*, or *bont/f*, respectively; –, no PCR signal for *bont/a*, *bont/b*, *bont/e*, or *bont/f*. The samples were analyzed in two independent PCR experiments each, yielding the same results.

Table 5. Detection of *C. botulinum* in clinical, food, and tissue samples

Case	Sample	Multiplex/singleplex real-time PCR result[a]		Source (mo/yr)[b]
		Without enrichment	After enrichment	
1	Home-cured ham	B	B	Food-borne botulism, 11/2006
2	Green been salad	A	A	Food-borne botulism, 11/2007
	Black pudding	–	–	
	Liver sausage	–	–	
3	Marinated herring	E	E	Food-borne botulism, 8/2009
	Frozen herring	–	E	
	Stool (patient 1)	E	E	
	Stool (patient 2)	–	–	
4	Stool	A	A	Infant botulism, 4/2009
	Stool (after antibiotic treatment)	–	–	
5	Gut, liver, kidney (animal 1)	D	D	Botulism in cattle, 9/2008
	Gut, liver, kidney (control animal)	–	–	
6	Gut, liver (duck)	C	C	Avian botulism, 10/2008

[a] Both multiplex and singleplex real-time PCR assays were performed on the indicated specimens. "Without enrichment" refers to PCR analysis of DNA directly extracted from the specimen; "after enrichment" refers to PCR analysis of DNA extracted from specimens after 3 to 5 days of anaerobic culture. A, B, D, or E indicates a positive PCR signal for *bont/a*, *bont/b*, *bont/d*, or *bont/e*, respectively; –, no PCR signal for *bont/a*, *bont/b*, *bont/c, bont/d, bont/e*, or *bont/f*. The samples were analyzed in two independent PCR experiments each, yielding the same results. The results were confirmed by mouse bioassay performed at the Robert Koch-Institut (Berlin, Germany; cases 3 and 4), Landeslabor Brandenburg (Frankfurt/Oder, Germany; case 1), or Institut für Lebensmittel, Arzneimittel und Tierseuchen (Berlin, Germany; cases 2, 5, and 6).

[b] Dates indicate the time points at which the corresponding cases were reported to the authorities.

Figure 1. Multiplex and singleplex real-time PCR standard curves. Logarithms of the copy numbers are plotted against their corresponding threshold cycles (*CT*). Multiplex PCR standard curves as measured in duplicates are shown as closed squares (■), and singleplex PCR standard curves are shown as open squares (□). The amplifications of pBoNT/A (A), pBoNT/B (B), pBoNT/C (C), pBoNT/D (D), pBoNT/E (E), and pBoNT/F (F) by singleplex and/or multiplex PCRs are shown. *R*2, regression correlation coefficient.