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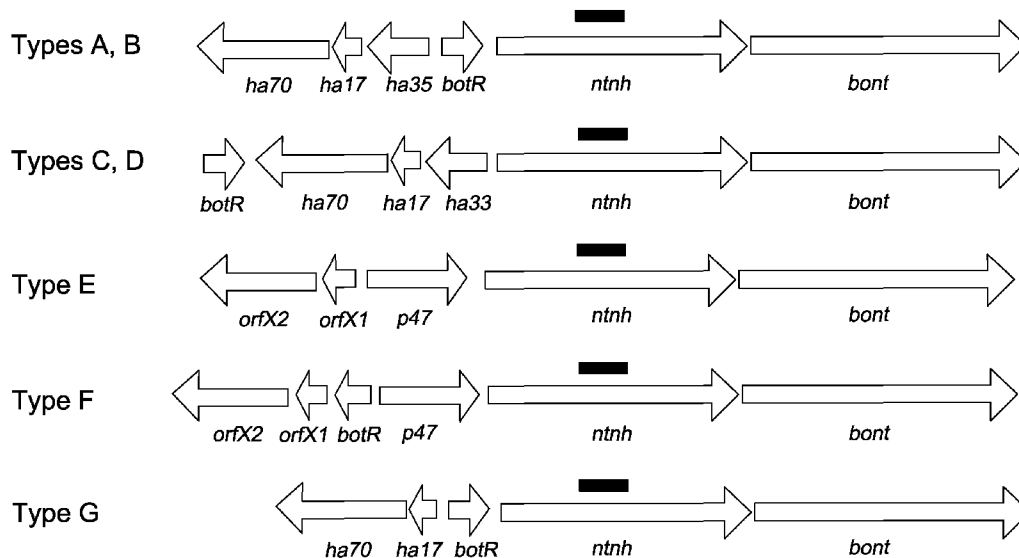
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[Continued on next page]

(54) Title: METHOD FOR RAPID DETECTION OF CLOSTRIDIUM BOTULINUM

FIG. 1



(57) Abstract: The invention provides a rapid sensitive, specific and robust universal assay for detecting all *C. botulinum* toxin types in a sample by (a) PCR amplifying DNA in the sample by use of particular primers, (b) detecting the presence or absence of amplified DNA, and (c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *C. botulinum* in the sample. The invention further provides a method for type-specific analysis of *C. botulinum* toxin gene types A-G in a sample utilizing particular primers and optional probes that are specific to each *C. botulinum* toxin type.

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METHOD FOR RAPID DETECTION OF CLOSTRIDIUM BOTULINUM

SEQUENCE LISTING

[0001] Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 66,258 byte ASCII (text) file named "702402ST25_Sequence.TXT," created January 10, 2008.

BACKGROUND OF THE INVENTION

[0002] *Clostridium botulinum* ("*C. botulinum*") is an anaerobic spore-forming bacterium that produces the seven "types" of botulinum neurotoxin that are distinguishable by the inability of antitoxin against one toxin type to neutralize any of the other six toxin types. The seven types of botulinum toxin have been arbitrarily assigned the letters A-G. *C. botulinum* toxin subtypes exist and are defined both by variations in their neurotoxin gene sequences and by variations in the ability of monoclonal antibody combinations to neutralize individual subtypes (Hill, *J. Bacteriology*, November 17, 2006 (epub); Smith, *Infectious Immunology*, 73(9): 5450-7 (2005); see also *Clostridia: Biotechnology and Medical Applications*. Ch. 7 (pp. 211-250), Bahl H, Durre P eds. Wiley-VCH, New York, 2001). Botulinum toxin subtypes are named by adding an Arabic numeral to the toxin type, e.g., A1, A2, etc., B1, B2, etc.

[0003] *C. botulinum* presents a three-fold danger to public health. First, food contamination with the toxin is a major health hazard that is constantly monitored by the food industry. Second, infant botulism infection, though rare, hospitalizes about 100 infants each year. Third, the use of the toxin as an agent for bioterrorism presents a real danger (see Consensus Statement entitled "Botulinum Toxin as a Biological Weapon: Medical and Public Health Management", Arnon, *J. Amer. Med. Assn.*, 285: 1059-1070 and 285: 2081 (2001)). Although treatment with human Botulism Immune Globulin (BabyBIG®, California Department of Health Services) or EQUINE antitoxins is based on clinical findings, and should be instituted as rapidly as possible, definitive microbiological diagnosis can take up to 4 days. This is because detection of the bacterium and its toxin is often difficult and relies on lengthy culture assays and toxicity

assessment in mice. Moreover, the availability of the mouse bioassay is geographically limited. The availability of a rapid, sensitive, specific and robust diagnostic test would be invaluable for detection of and public health and medical management of foodborne, wound, or bioterrorist botulism outbreaks. Such an array would also allow the limited supply of antitoxin to be directed to affected persons only, thereby allowing exposed but unaffected individuals to be reassured and spared unnecessary exposure to antitoxin.

BRIEF SUMMARY OF THE INVENTION

[0004] The invention provides a method of detecting the presence or absence of an NTN gene in a sample comprising (a) subjecting a sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers, such as primers comprising a sequence having at least 90% homology to SEQ ID NO: 22, and (ii) one or more reverse primers, such as primers comprising a sequence having at least 90% homology to SEQ ID NO: 23, (b) detecting the presence or absence of amplified DNA resulting from step (a), and (c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of the NTN gene in the sample.

[0005] The invention also provides a method of detecting one or more genes of botulism toxin types A-G in a sample utilizing a combination of primers and optional probes specific to each toxin type.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0006] Figure 1 is a schematic diagram of the relative positions of the PCR target sequence with respect to the non-toxin nonhemagglutinin gene of *C. botulinum* toxin types A-G for the universal assay for detecting *C. botulinum*.

[0007] Figure 2 is a schematic diagram of the relative positions of the PCR target sequence with respect to the botulinum neurotoxin region of *C. botulinum* toxin types A-G for the type-specific assay for separately detecting *C. botulinum* types A-G.

DETAILED DESCRIPTION OF THE INVENTION

[0008] The invention provides a method of detecting the presence of *C. botulinum* toxin gene(s) in a sample. The invention is predicated, at least in part, on the discovery of a conserved nucleic acid region among *C. botulinum* toxin types A-G and the construction of suitable pairs of primers (i.e., forward primers and reverse primers) and suitable probes to allow for the detection of *C. botulinum* in general, and/or the specific detection of *C. botulinum* toxin genes type A-G, in the sample through the use of the PCR nucleic acid amplification procedure.

[0009] Thus, the invention provides both a universal assay for detecting all *C. botulinum* toxin types and type-specific assays for detecting any particular toxin type of *C. botulinum* that will also detect any one or more subtypes of the *C. botulinum* toxin gene (such as A1-4, B1-5, E1-4, etc, detectable as types A, B, and E, respectively). The universal assay is a rapid, first-pass assay that determines whether a sample is contaminated with any strain of *C. botulinum*. The universal assay takes advantage of a region of the non-toxin non-hemagglutinin (NTNH) gene that is highly conserved in all *C. botulinum* toxin types and subtypes (see Figure 1), which allows the universal assay to detect all known and as yet unknown *C. botulinum* strains. The toxin type-specific assays take advantage of a botulinum neurotoxin ("BoNT") gene region of *C. botulinum* that differs between each of the seven (A-G) *C. botulinum* toxin types (see Figure 2) by providing an individual assay for each of the toxin types of *C. botulinum* A-G. The type-specific assays can be conducted either independently of, or in conjunction with, the universal assay. Desirably, the universal assay is performed on a sample, and then, if a *C. botulinum* toxin gene is detected in the sample, one or more of the type-specific assays is conducted to determine the particular toxin type(s) of the *C. botulinum* toxin gene in the sample. In addition, the type-specific assays can be conducted individually, or one or more of the type-specific assays can be conducted concurrently. Thus, for example, a sample can be assayed for all seven toxin types of *C. botulinum* toxin gene concurrently.

[0010] The invention provides a universal assay for detecting an NTNH gene in a sample. Utilizing PCR amplification, (i) one or more forward primers complementary to the NTNH gene, and (ii) one or more reverse primers complementary to the NTNH gene are used to amplify the gene. The presence or absence of the NTNH gene is subsequently detected, and its presence or absence is correlated with the presence or absence, respectively, of the NTNH gene in the

sample. By complementary it is meant that the primers hybridize with high specificity to a DNA sequence of the NTN gene.

[0011] In a preferred embodiment, the universal assay for the NTN gene is a method of detecting the presence or absence of *C. botulinum* in a sample that comprises (a) subjecting a sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 22 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 23, (b) detecting the presence or absence of amplified DNA resulting from step (a), and (c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of a *C. botulinum* toxin gene in the sample. The presence or absence of amplified DNA is correlated with the presence or absence, respectively, of a *C. botulinum* toxin gene in the sample.

[0012] The invention further provides a type-specific assay for *C. botulinum* toxin genes A-G. In the type-specific assay, one or more (e.g., two, three, four, five, six, seven, eight, nine, ten, or more) forward primers and one or more (e.g., two, three, four, five, six, seven, eight, nine, ten, or more) reverse primers complementary to the BoNT gene of botulism toxin types A-G are used to PCR amplify the BoNT gene in a sample. The presence or absence of the amplified BoNT gene of botulism toxin A-G is then detected, and correlated with the presence or absence, respectively of one or more types of botulism toxin genes A-G in the sample. By complementary it is meant that the primers hybridize with high specificity to a DNA sequence of the genes of botulism toxin type A-G.

[0013] The invention further provides a subtype-specific assay. In the subtype-specific assay, one or more forward primers and one or more reverse primers complementary to the one or more genes of botulism toxin A-G subtypes are used to PCR amplify the gene in a sample. The presence or absence of the amplified gene of botulism toxin A-G subtypes is then detected, and correlated with the presence or absence, respectively of one or more types of botulism toxin genes A-G subtypes in the sample. By complementary it is meant that the primers hybridize with high specificity to a DNA sequence of the genes of botulism toxin type A-G subtypes.

[0014] The type-specific assay for *C. botulinum* toxin gene A is a method of detecting the presence or absence of *C. botulinum* toxin A gene in a sample that comprises (a) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more

forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 1 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 8, (b) detecting the presence or absence of amplified DNA resulting from step (a), and (c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of the *C. botulinum* toxin A gene in the sample. Detecting the presence or absence of amplified DNA resulting from step (a) can be carried out in any suitable manner in the type-specific assay for *C. botulinum* type A. The presence or absence of amplified DNA resulting from step (a) is optionally, but desirably, detected by contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 15. The amplified DNA may also be quantified.

[0015] The type-specific assay for *C. botulinum* B toxin is a method of detecting the presence or absence of *C. botulinum* B toxin gene in a sample that comprises (a) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 2 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 9, (b) detecting the presence or absence of amplified DNA resulting from step (a), and (c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of the *C. botulinum* type B toxin gene in the sample. Detecting the presence or absence of amplified DNA resulting from step (a) can be carried out in any suitable manner in the type-specific assay for *C. botulinum* type B. The presence or absence of amplified DNA resulting from step (a) is optionally, but desirably, detected by contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 16. The amplified DNA may also be quantified.

[0016] The type-specific assay for *C. botulinum* type C toxin is a method of detecting the presence or absence of the *C. botulinum* type C toxin gene in a sample that comprises (a) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 3 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 10, (b) detecting the presence or absence of amplified DNA resulting from step (a), and (c) correlating the presence or absence of amplified DNA with the presence or absence,

respectively, of the *C. botulinum* type C toxin gene in the sample. Detecting the presence or absence of amplified DNA resulting from step (a) can be carried out in any suitable manner in the type-specific assay for *C. botulinum* type C. The presence or absence of amplified DNA resulting from step (a) optionally, but desirably, is detected and quantified by contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 17.

[0017] The type-specific assay for *C. botulinum* type D toxin is a method of detecting the presence or absence of the *C. botulinum* type D toxin gene in a sample that comprises (a) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 4 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 11, (b) detecting the presence or absence of amplified DNA resulting from step (a), and (c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of the *C. botulinum* type D toxin gene in the sample. Detecting the presence or absence of amplified DNA resulting from step (a) can be carried out in any suitable manner in the type-specific assay for *C. botulinum* type D. The presence or absence of amplified DNA resulting from step (a) is optionally, but desirably, detected by contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 18. The amplified DNA may also be quantified.

[0018] The type-specific assay for *C. botulinum* type E is a method of detecting the presence or absence of the *C. botulinum* type E toxin gene in a sample that comprises (a) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 5 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 12, (b) detecting the presence or absence of amplified DNA resulting from step (a), and (c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of the *C. botulinum* type E toxin gene in the sample. Detecting the presence or absence of amplified DNA resulting from step (a) can be carried out in any suitable manner in the type-specific assay for *C. botulinum* type E. The presence or absence of amplified DNA resulting from step (a) is optionally, but desirably, detected by contacting the sample with a probe

comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 19. The amplified DNA may also be quantified.

[0019] The type-specific assay for *C. botulinum* type F is a method of detecting the presence or absence of the *C. botulinum* type F toxin gene in a sample that comprises (a) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 6 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 13, (b) detecting the presence or absence of amplified DNA resulting from step (a), and (c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of the *C. botulinum* type F toxin gene in the sample. Detecting the presence or absence of amplified DNA resulting from step (a) can be carried out in any suitable manner in the type-specific assay for *C. botulinum* type F. The presence or absence of amplified DNA resulting from step (a) is optionally, but desirably, detected by contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 20. The amplified DNA may also be quantified.

[0020] The type-specific assay for *C. botulinum* type G is a method of detecting the presence or absence of the *C. botulinum* type G toxin gene in a sample that comprises (a) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 7 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 14, (b) detecting the presence or absence of amplified DNA resulting from step (a), and (c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of the *C. botulinum* type G toxin gene in the sample. Detecting the presence or absence of amplified DNA resulting from step (a) can be carried out in any suitable manner in the type-specific assay for *C. botulinum* type G. The presence or absence of amplified DNA resulting from step (a) is optionally, but desirably, detected by contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 21. The amplified DNA may also be quantified.

[0021] The universal and type-specific assays rely on PCR (i.e., polymerase chain reaction) technology. PCR amplification is a well known in the art and is described in, for example, U.S.

Patents 4,683,202, 4,683,195, 4,800,159, and 4,965,188, as well as PCR Primer: A Laboratory Manual, Dieffenbach and Dveksler, eds., Cold Spring Harbor Laboratory Press, 1995. As used herein, PCR refers to any suitable PCR technique, including, but not limited to, traditional PCR techniques, quantitative real-time PCR (qPCR) techniques, and improvement to current PCR techniques as may in the future be developed. PCR techniques are used to amplify (e.g., repeatedly copy) a specified sequence of nucleotides of a DNA template (also referred to as a DNA target) in a sample, thereby resulting in an increase in the amount of the specified sequence of nucleotides relative to other nucleotide sequences in the same sample.

[0022] PCR amplification of a specified sequence of nucleotides of a DNA template is dependent on the use of appropriate primers. Primers are short, artificial DNA strands, often not more than 50 and usually only 18 to 25 base pairs long that are complementary to the beginning and/or the end of the DNA fragment to be amplified. The primers anneal (or adhere) to the DNA template at the starting and ending points of the sequence of nucleotides to be amplified, where DNA polymerase (e.g., Taq polymerase) binds and begins the synthesis of a new DNA strand complementary to the sequence of nucleotides to be amplified. The choice of the length of the primers and their melting temperature (T_m) depends on a number of considerations. Primers that are too short can anneal at several positions on a long DNA template, which could result in the amplification of sequences of nucleotides not intended to be amplified (i.e., the production of so-called non-specific copies of nucleotide sequences). However, the length of a primer is limited by the maximum temperature that can be applied in order to melt it, inasmuch as the melting temperature of a primer increases with the length of the primer. Melting temperatures that are too high, e.g., above 80° C, can cause problems since DNA polymerase is less active at such temperatures. The optimum length of a primer generally is from 15 to 40 nucleotides with a melting temperature between 55° C and 65° C.

[0023] The forward and reverse primers for use in the universal assay for detecting the presence or absence of *C. botulinum* toxin gene have a least 90% homology or identity to CCHAATATHTGGRTDGCNCC (SEQ ID NO: 22) and RAAAGRAAAYTAGARTCATAWAYTCCHCC (SEQ ID NO: 23), respectively, wherein the symbols representing more than one possible nucleotide are defined in Table 1.

Table 1: Variable Nucleotide Symbols

Symbol	Nucleotides
Y	C or T
R	A or G
S	G or C
M	A or C
W	A or T
H	A or T or C
K	G or T
D	A or T or G
N	A or T or C or G

The location of the forward primer on the NTNH gene is position 108, while the location of the reverse primer on the NTNH gene is position 209. Desirably, the forward and/or reverse primers for use in the universal assay for detecting the presence or absence of the *C. botulinum* toxin gene complex have a least 91% (e.g., at least 92%, at least 93%, or at least 94%) homology or identity to SEQ ID NO: 22 and SEQ ID NO: 23, respectively. Preferably, the forward and/or reverse primers for use in the universal assay for detecting the presence or absence of *C. botulinum* toxin gene complex have a least 95% (e.g., at least 96%, at least 97%, or at least 98%, or at least 99%) homology or identity to SEQ ID NO: 22 and SEQ ID NO: 23, respectively. Most preferably, the forward and/or reverse primers for use in the universal assay for detecting the presence or absence of *C. botulinum* toxin gene have 100% homology or identity to SEQ ID NO: 22 and SEQ ID NO: 23, respectively.

[0024] The forward and reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin A gene have a least 90% homology or identity to TGGTTTTGAGGAGTCACTTGAA (SEQ ID NO: 1) and TCATGTCCCCCAAATGTTCT (SEQ ID NO: 8), respectively. The location of the forward primer on the BoNT gene is position 582, while the location of the reverse primer on the BoNT gene is position 809. Desirably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin A gene have a least 91% (e.g., at least 92%, at least 93%, or at least 94%) homology or identity to SEQ ID NO: 1 and SEQ ID NO: 8, respectively. Preferably,

the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin A gene have a least 95% (e.g., at least 96%, at least 97%, or at least 98%, or at least 99%) homology or identity to SEQ ID NO: 1 and SEQ ID NO: 8, respectively. Most preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin A gene have 100% homology or identity to SEQ ID NO: 1 and SEQ ID NO: 8, respectively.

[0025] The forward and reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin B gene have a least 90% homology or identity to CAAGAAAACAAAGGCGCAAG (SEQ ID NO: 2) and CTGGGATCTTGYCCTCCAAA (SEQ ID NO: 9), respectively, wherein the symbols representing more than one possible nucleotide are defined in Table 1. The location of the forward primer on the BoNT gene is position 619, while the location of the reverse primer on the BoNT gene is position 833. Desirably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin B gene have a least 91% (e.g., at least 92%, at least 93%, or at least 94%) homology or identity to SEQ ID NO: 2 and SEQ ID NO: 9, respectively. Preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin B gene have a least 95% (e.g., at least 96%, at least 97%, or at least 98%, or at least 99%) homology or identity to SEQ ID NO: 2 and SEQ ID NO: 9, respectively. Most preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin B gene have 100% homology or identity to SEQ ID NO: 2 and SEQ ID NO: 9, respectively.

[0026] The forward and reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin C gene have a least 90% homology or identity to CAACTTTAATTATTCAGATCCTGTTGA (SEQ ID NO: 3) and GGCTTGTAACGAGGAGGTT (SEQ ID NO: 10), respectively. The location of the forward primer on the BoNT gene is position 18, while the location of the reverse primer on the BoNT gene is position 199. Desirably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin C gene have a least 91% (e.g., at least 92%, at least 93%, or at least 94%) homology or identity to SEQ ID NO: 3 and SEQ ID NO: 10, respectively. Preferably, the forward and/or reverse primers for use in the type-

specific assay for detecting the presence or absence of the *C. botulinum* toxin C gene have a least 95% (e.g., at least 96%, at least 97%, or at least 98%, or at least 99%) homology or identity to SEQ ID NO: 3 and SEQ ID NO: 10, respectively. Most preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin C gene have 100% homology or identity to SEQ ID NO: 3 and SEQ ID NO: 10, respectively.

[0027] The forward and reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin D gene have a least 90% homology or identity to CCATCATTTGAAGGGTTTGG (SEQ ID NO: 4) and TGGGTCCATCTTGAGARAAA (SEQ ID NO: 11), respectively, wherein the symbols representing more than one possible nucleotide are defined in Table 1. The location of the forward primer on the BoNT gene is position 541, while the location of the reverse primer on the BoNT gene is position 791. Desirably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin D gene have a least 91% (e.g., at least 92%, at least 93%, or at least 94%) homology or identity to SEQ ID NO: 4 and SEQ ID NO: 11, respectively. Preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin D gene have a least 95% (e.g., at least 96%, at least 97%, or at least 98%, or at least 99%) homology or identity to SEQ ID NO: 4 and SEQ ID NO: 11, respectively. Most preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin D gene have 100% homology or identity to SEQ ID NO: 4 and SEQ ID NO: 11, respectively.

[0028] The forward and reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin E gene have a least 90% homology or identity to ATAATGGGAGCAGAGCCTGA (SEQ ID NO: 5) and CCCTTTAGCCCCATATAGTCC (SEQ ID NO: 12), respectively. The location of the forward primer on the BoNT gene is position 448, while the location of the reverse primer on the BoNT gene is position 678. Desirably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin E gene have a least 91% (e.g., at least 92%, at least 93%, or at least 94%) homology or identity to SEQ ID NO: 5 and SEQ ID NO: 12, respectively. Preferably, the forward and/or reverse primers for use in the type-specific assay for

detecting the presence or absence of the *C. botulinum* toxin E gene have a least 95% (e.g., at least 96%, at least 97%, or at least 98%, or at least 99%) homology or identity to SEQ ID NO: 5 and SEQ ID NO: 12, respectively. Most preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin E gene have 100% homology or identity to SEQ ID NO: 5 and SEQ ID NO: 12, respectively.

[0029] The forward and reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin F gene have a least 90% homology or identity to GTSAGACAATACCTCAAATATCAAATCG (SEQ ID NO: 6) and CTGGYACTTTTTGTGCATGT (SEQ ID NO: 13), respectively, wherein the symbols representing more than one possible nucleotide are defined in Table 1. The location of the forward primer on the BoNT gene is position 1488, while the location of the reverse primer on the BoNT gene is position 1646. Desirably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin F gene have a least 91% (e.g., at least 92%, at least 93%, or at least 94%) homology or identity to SEQ ID NO: 6 and SEQ ID NO: 13, respectively. Preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin F gene have a least 95% (e.g., at least 96%, at least 97%, or at least 98%, or at least 99%) homology or identity to SEQ ID NO: 6 and SEQ ID NO: 13, respectively. Most preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin F gene have 100% homology or identity to SEQ ID NO: 6 and SEQ ID NO: 13, respectively.

[0030] The forward and reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin G gene have a least 90% homology or identity to ATCCAACCTGGAGCTGAAGA (SEQ ID NO: 7) and GCTGGATCTGCAAATACGC (SEQ ID NO: 14), respectively. The location of the forward primer on the BoNT gene is position 427, while the location of the reverse primer on the BoNT gene is position 674. Desirably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin G gene have a least 91% (e.g., at least 92%, at least 93%, or at least 94%) homology or identity to SEQ ID NO: 7 and SEQ ID NO: 14, respectively. Preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or

absence of the *C. botulinum* toxin G gene have a least 95% (e.g., at least 96%, at least 97%, or at least 98%, or at least 99%) homology or identity to SEQ ID NO: 7 and SEQ ID NO: 14, respectively. Most preferably, the forward and/or reverse primers for use in the type-specific assay for detecting the presence or absence of the *C. botulinum* toxin G gene have 100% homology or identity to SEQ ID NO: 7 and SEQ ID NO: 14, respectively.

[0031] The phrase “at least X% homology,” as used herein, indicates that two nucleic acid sequences share at least X% of the same nucleotides in the same relative positions. Nucleotides (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides) can be added to or removed from the 3' end and/or the 5' end of SEQ ID NOs: 1-14, 22, and 23 to provide suitable primers for the universal and type-specific assays; however, the sensitivity and rapidity of the assays may be adversely affected thereby. Accordingly, in preferred embodiments, the primers utilized in the universal and type-specific assays have the same lengths as SEQ ID NOs: 1-14, 22, and 23 for the particular universal and type-specific assays as described herein.

[0032] Inasmuch as SEQ ID NOs: 6, 9, 11, 22, and 23 contain symbols representing more than one possible nucleotide, as defined in Table 1, each of SEQ ID NOs: 6, 9, 11, 22, and 23 represent multiple similar, but not identical, primers, i.e., they are degenerate primers. The actual nucleotide sequences of the degenerate primers represented by each of SEQ ID NOs: 6, 9, 11, 22, and 23 are set forth in Table 2 as SEQ ID NOs: 24-439. To the extent that the forward and/or reverse primers in the universal and type-specific assays are defined in terms of homology or identity to SEQ ID NOs: 6, 9, 11, 22, and 23, the forward and/or reverse primers in the universal and type-specific assays can be alternatively defined in terms of homology or identity to one or more of the corresponding degenerate SEQ ID NOs as set forth in Table 2.

Table 2: Degenerate Primers

Primer Sequence Containing Variable Nucleotide	Degenerate Nucleotide Sequence	Degenerate SEQ ID NO:
SEQ ID NO: 6	GTCAGACAATACCTCAAATATCAAATCG	24
	GTGAGACAATACCTCAAATATCAAATCG	25
SEQ ID NO: 9	CTGGGATCTTGCCCTCCAAA	26
	CTGGGATCTTGTCCTCCAAA	27
SEQ ID NO: 11	TGGGTCCATCTTGAGAAAAA	28

	TGGGTCCATCTTGAGAGAAA	29
SEQ ID NO: 13	CTGGCACTTTTTGTGCATGT	30
	CTGGTACTTTTTGTGCATGT	31
SEQ ID NO: 22	CCAAATATATGGATAGCACC	32
	CCAAATATATGGATTGCACC	33
	CCAAATATATGGATGGCACC	34
	CCAAATATATGGATAGCCCC	35
	CCAAATATATGGATTGCCCC	36
	CCAAATATATGGATGGCCCC	37
	CCAAATATATGGATAGCGCC	38
	CCAAATATATGGATTGCGCC	39
	CCAAATATATGGATGGCGCC	40
	CCAAATATATGGATAGCTCC	41
	CCAAATATATGGATTGCTCC	42
	CCAAATATATGGATGGCTCC	43
	CCAAATATATGGGTAGCACC	44
	CCAAATATATGGGTTCACC	45
	CCAAATATATGGGTGGCACC	46
	CCAAATATATGGGTAGCCCC	47
	CCAAATATATGGGTTCCCCC	48
	CCAAATATATGGGTGGCCCC	49
	CCAAATATATGGGTAGCGCC	50
	CCAAATATATGGGTTCGCGCC	51
	CCAAATATATGGGTGGCGCC	52
	CCAAATATATGGGTAGCTCC	53
	CCAAATATATGGGTTCCTCC	54
	CCAAATATATGGGTGGCTCC	55
	CCAAATATCTGGATAGCACC	56
	CCAAATATCTGGATTGCACC	57
	CCAAATATCTGGATGGCACC	58
	CCAAATATCTGGATAGCCCC	59
	CCAAATATCTGGATTGCCCC	60
	CCAAATATCTGGATGGCCCC	61
	CCAAATATCTGGATAGCGCC	62

CCAAATATCTGGATTGCGCC	63
CCAAATATCTGGATGGCGCC	64
CCAAATATCTGGATAGCTCC	65
CCAAATATCTGGATTGCTCC	66
CCAAATATCTGGATGGCTCC	67
CCAAATATCTGGGTAGCACC	68
CCAAATATCTGGGTTCACC	69
CCAAATATCTGGGTGGCACC	70
CCAAATATCTGGGTAGCCCC	71
CCAAATATCTGGGTTCCCCC	72
CCAAATATCTGGGTGGCCCC	73
CCAAATATCTGGGTAGCGCC	74
CCAAATATCTGGGTTCGCC	75
CCAAATATCTGGGTGGCGCC	76
CCAAATATCTGGGTAGCTCC	77
CCAAATATCTGGGTTCCTCC	78
CCAAATATCTGGGTGGCTCC	79
CCAAATATTTGGATAGCACC	80
CCAAATATTTGGATTGCACC	81
CCAAATATTTGGATGGCACC	82
CCAAATATTTGGATAGCCCC	83
CCAAATATTTGGATTGCCCC	84
CCAAATATTTGGATGGCCCC	85
CCAAATATTTGGATAGCGCC	86
CCAAATATTTGGATTGCGCC	87
CCAAATATTTGGATGGCGCC	88
CCAAATATTTGGATAGCTCC	89
CCAAATATTTGGATTGCTCC	90
CCAAATATTTGGATGGCTCC	91
CCAAATATTTGGGTAGCACC	92
CCAAATATTTGGGTTCACC	93
CCAAATATTTGGGTGGCACC	94
CCAAATATTTGGGTAGCCCC	95
CCAAATATTTGGGTTCCCCC	96

CCAAATATTTGGGTGGCCCC	97
CCAAATATTTGGGTAGCGCC	98
CCAAATATTTGGGTTCGCGC	99
CCAAATATTTGGGTGGCGCC	100
CCAAATATTTGGGTAGCTCC	101
CCAAATATTTGGGTTCGCTCC	102
CCAAATATTTGGGTGGCTCC	103
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CCCAATATATGGATTGCACC	105
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CCCAATATATGGATAGCCCC	107
CCCAATATATGGATTGCCCC	108
CCCAATATATGGATGGCCCC	109
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CCCAATATATGGATTGCGCC	111
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CCCAATATCTGGGTTCACC	141
CCCAATATCTGGGTGGCACC	142
CCCAATATCTGGGTAGCCCC	143
CCCAATATCTGGGTTCCCCC	144
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CCCAATATCTGGGTAGCGCC	146
CCCAATATCTGGGTTCGCC	147
CCCAATATCTGGGTGGCGCC	148
CCCAATATCTGGGTAGCTCC	149
CCCAATATCTGGGTTCCTCC	150
CCCAATATCTGGGTGGCTCC	151
CCCAATATTTGGATAGCACC	152
CCCAATATTTGGATTGCACC	153
CCCAATATTTGGATGGCACC	154
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CCCAATATTTGGATAGCTCC	161
CCCAATATTTGGATTGCTCC	162
CCCAATATTTGGATGGCTCC	163
CCCAATATTTGGGTAGCACC	164

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	CCTAATATTTGGGTTGCTCC	246
	CCTAATATTTGGGTGGCTCC	247
SEQ ID NO: 23	AAAAGAAAAC TAGAATCATAAACTCCACC	248
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	AAAAGAAAAC TAGAATCATAAAATTCTCTCC	253
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GAAAGGAACTAGAATCATATACTCCACC	398
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GAAAGGAAATTAGAGTCATAAATTCTCC	433
GAAAGGAAATTAGAGTCATATACTCCACC	434
GAAAGGAAATTAGAGTCATATACTCCCCC	435
GAAAGGAAATTAGAGTCATATACTCCTCC	436

	GAAAGGAAATTAGAGTCATATATTCCACC	437
	GAAAGGAAATTAGAGTCATATATTCCCCC	438
	GAAAGGAAATTAGAGTCATATATTCCTCC	439

[0033] While a single forward primer and a single reverse primer can be utilized in each of the universal and type-specific assays, more than one forward primer and/or more than one reverse primer can also be utilized in each of the universal and type-specific assays. Multiple forward and/or reverse primers are particularly useful if the same gene from different organisms is to be amplified, as the genes themselves probably are similar but not identical. In a preferred embodiment, multiple degenerate primers set forth in Table 2 are utilized as the forward and/or reverse primers. For example, a mixture of two or more (e.g., three, four, five, six, seven, eight, nine, ten, or more, and preferably all) of the degenerate primers of SEQ ID NOs: 32-247 are used as the forward primers for the universal assay, and a mixture of two or more (e.g., three, four, five, six, seven, eight, nine, ten, or more, and preferably all) of the degenerate primers of SEQ ID NOs: 248-439 are used as the reverse primers for the universal assay. Similarly, a mixture of the two degenerate primers of SEQ ID NOs: 24-25 can be used as the forward primers for the type-specific assay for *C. botulinum* toxin type F, and mixtures of the two degenerate primers of SEQ ID NOs: 26-27, 28-29, and 30-31 can be used as the reverse primers for the type-specific assays for *C. botulinum* toxin types B, D, and F, respectively.

[0034] The invention may be performed using quantitative PCR (qPCR), which allows the user simultaneously to amplify and to quantify a specific part of a given DNA molecule. The inventive method is used to determine whether or not a specific sequence is present in the sample and, if it is present, the number of copies of the sequence that are present in the sample. The procedure follows the general pattern of the polymerase chain reaction, but the DNA is quantified after each round of amplification, which represents the “real-time” aspect of the procedure. Two common methods of quantification are the use of fluorescent dyes that intercalate with double-strand DNA and the use of modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA (e.g., fluorescein, Fam-BHQ1).

[0035] Alternatively, once the DNA is amplified, it may be isolated by any suitable technique, e.g., isolated and visualized using gel electrophoresis. Techniques involving gel electrophoresis are well known in the art. A gel refers to the matrix used to separate the

molecules. In most cases the gel is a cross-linked polymer whose composition and porosity is chosen based on the weight and composition of the target of the analysis. When separating small nucleic acids (DNA, RNA, or oligonucleotides), the gel is usually made with different concentrations of acrylamide and a cross-linker, thereby producing different sized mesh networks of polyacrylamide. When separating larger nucleic acids (greater than a few hundred bases), the preferred matrix is purified agarose.

[0036] “Electrophoresis” refers to the electromotive force (EMF) that is used to push or pull the molecules through the gel matrix. By placing the molecules in wells in the gel and applying an electric current, the molecules will move through the matrix at different rates and towards the anode if negatively charged or towards the cathode if positively charged. After a suitable period of time for the electrophoresis, e.g., when the smallest molecules, which move the fastest, have almost reached the anode or cathode, the molecules in the gel can be stained to make them visible. Suitable staining agents include ethidium bromide, silver, and coomassie blue dye. Other methods also can be used to visualize the separation of the mixture’s components on the gel. If the analyte molecules luminesce under ultraviolet light, a photograph can be taken of the gel under ultraviolet light. If the molecules to be separated contain radioactive atoms, an autoradiogram can be recorded of the gel.

[0037] Desirably, in the inventive method, probes, particularly fluorogenic probes, are utilized in combination with the forward and reverse primers of the *C. botulinum* toxin type gene assays to detect *C. botulinum* toxin genes types A-G. Suitable probes for use in each of the type-specific assays for *C. botulinum* toxin type A-G genes are probes comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 15-21, respectively, as set forth in Table 3.

Table 3: Probes for *C. botulinum* A-G

<i>C. botulinum</i> Subtype	Probe	SEQ ID NO:	Location on BoNT Gene
A	TGCAGGCAAATTTGCTACAGATCCA	15	627
B	CGTGGATATTTTTCAGATCCAGCCTTG	16	652
C	TGAGCCTGAAAAAGCCTTTCGCA	17	93
D	GATTCGTCCACAAGTTAGCGAGGGA	18	744
E	TGCCAAGCAATCACGGTTTTGG	19	515
F	TGCCAAGATATGATTCTAATGGAA	20	1551
G	TGGCCATTCCCAATATCAGAAGG	21	534

Desirably, the probes for use in each of the type-specific assays for *C. botulinum* toxin type A-G genes are probes comprising a nucleic acid sequence having at least 91% (e.g., at least 92%, at least 93%, or at least 94%) homology or identity to SEQ ID NOs: 15-21, respectively.

Preferably, the probes for use in each of the type-specific assays for *C. botulinum* toxin type A-G genes are probes comprising a nucleic acid sequence having at least 95% (e.g., at least 96%, at least 97%, or at least 98%, or at least 99%) homology or identity to SEQ ID NOs: 15-21, respectively. Most preferably, the probes for use in each of the type-specific assays for *C. botulinum* toxin type A-G genes are probes comprising a nucleic acid sequence having 100% homology or identity to SEQ ID NOs: 15-21, respectively.

[0038] The combination of specific forward and reverse primers in combination with a specific probe for each toxin type gene of *C. botulinum* provides a high degree of selectivity and sensitivity to the inventive method. In this regard, the inventive method is capable of detecting *C. botulinum* in minute quantities in a sample. Concentrations of a *C. botulinum* toxin gene as low as 10 copies, or less than 10 bacteria in a sample undergoing the PCR, may be readily detected, thereby providing a highly sensitive and accurate assay for detecting contamination of a sample with *C. botulinum*. Further, the inventive method provides a vast improvement over current methods of detecting the *C. botulinum* toxin in a sample, which can take days in order to complete. In particular, the inventive method provides a method of detecting *C. botulinum* toxin gene in a sample within a matter of hours, e.g., typically in less than 5 hours and preferably in

less than 3 hours. This shortened time frame for determining whether or not a sample contains a *C. botulinum* toxin gene provides a significant advantage to a physician treating a patient suspected of having a *C. botulinum* infection or an inspector seeking to determine whether or not food has been contaminated (e.g., through a bioterrorist act). The inventive method allows for a definitive diagnosis/determination to be made rapidly and, therefore, allows for proper treatment or prophylactic action to commence more expeditiously.

[0039] The sample to be analyzed in accordance with the inventive method can be any suitable sample. Suitable samples that can be analyzed according to the inventive method include, but are not limited to, bodily fluid (e.g., blood, urine, etc.), serum, emesis, stool, and a wound from a subject. While the subject can be any animal, particularly a mammal, the subject typically will be a human (who can be of any age). Suitable samples also include food products and environmental samples suspected of natural or intentional (e.g., bioterrorism) contamination. Food products include any solid or fluid suitable for ingestion by an animal, especially a mammal such as a human. Food products, therefore, include all foods, beverages, medicaments, additives, etc. In particular, food products include canned goods, fruits, vegetables, meats, and beverages (e.g., water, juices, sodas, beer, wine, etc.).

[0040] Thus, the invention provides a method of diagnosing a subject, especially a human, comprising determining the presence or absence of a *C. botulinum* toxin gene in a sample from the subject by using the universal and/or type-specific assays as described herein, and correlating the presence or absence of the *C. botulinum* toxin gene in the sample with the diagnosis of the human as being or not being infected with the *C. botulinum*. If a determination is made that the subject is infected with *C. botulinum*, then appropriate treatment of the subject can be commenced, e.g., a suitable pharmaceutical agent or composition can be administered to the subject to effect treatment of the subject. Typically, an antitoxin, such as having Botulism Immune Globulin (e.g., BabyBIG®) or equine botulinum antitoxin, is administered to a subject, along with adjunctive therapy as necessary (e.g., a respirator), to manage the symptoms of illness caused by *C. botulinum* and its neurotoxin.

[0041] The invention further provides primers, probes, and kits containing one or more of the same for the detection of *C. botulinum* toxin gene and toxin types and subtypes thereof.

[0042] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

[0043] This example demonstrates the sensitivity and specificity of the inventive method, particularly the universal assay, for detecting the *C. botulinum* toxin gene(s).

[0044] DNA samples from numerous *botulinum* and non-*botulinum* clostridial species (provided by T. Smith, USAMRIID) were obtained and subjected to the universal assay in accordance with the invention to detect the presence of the *C. botulinum* toxin gene. The various *botulinum* and non-*botulinum* clostridial species that served as sources of the DNA samples are set forth in Table 3.

[0045] Each of the DNA samples was subjected to PCR nucleic acid amplification procedures using a mixture of the degenerate primers corresponding to SEQ ID NOs: 22 and 23. The PCR conditions were as follows: 95° C for 5 min, then 35 cycles of 95° C for 15 sec and 57° C for 1 min. The PCR reaction mixture contained 1x PCR buffer, 3.5 µM MgCl₂, 200 nM dNTPs, 1 µM forward primer, 1 µM reverse primer, 0.25 U Platinum Taq polymerase (Invitrogen, Carlsbad, CA). 5 µL of each DNA sample was used in each 25 µL PCR reaction mixture. The PCR products were run on a 2.5% agarose gel and visualized for 101 bp bands by UV illumination. The existence of a 101 bp band evidenced the existence of amplified NTNH in the PCR reaction mixture and, therefore, the existence of the NTNH gene in the DNA sample and the presence of *C. botulinum* toxin gene.

[0046] The results of this testing are set forth in Table 3, where the result is indicated as “+” for the DNA samples evidencing the presence of the *C. botulinum* toxin gene and “–” for the DNA samples evidencing the absence of the *C. botulinum* toxin gene.

Table 3: DNA Sample Test Results for *C. botulinum*

Clostridial Strain	Result
A1-Hall	+
A2 Kyoto-F	+

Clostridial Strain	Result
perfringens A ATCC 12915	–
perfringens A ATCC 12917	–

A3 Loch Maree	+
A1 1757	+
A1 1744	+
A2b 1436	+
B1 1656	+
B2 1826	+
B1 1758	+
Ba4 657	+
B1 okra	+
Bf An 436	+
B2 213B	+
npB Eklund 17B	+
Ba4 Strain 657	+
C Stockholm	+
C/D 6813	+
D ATCC 11873	+
D 1875	+
D/C VPI 5995	+
E1 Beluga	+
E3 Alaska E43	+
E Butyr BL5262	+
protF Langeland	+
npF Eklund 202F	+
Bf AN436	+
G 1354	+

perfringens A ATCC 12918	—
perfringens A ATCC 12919	—
perfringens A ATCC 12920	—
perfringens D ATCC 3629	—
perfringens D ATCC 3630	—
perfringens D ATCC 3631	—
perfringens E ATCC 27324	—
absonum ATCC 27555	—
baratii ATCC 27638	—
histolyticum ATCC 19401	—
novyi A ATCC 2706	—
novyi B ATCC 19402	—
septicum ATCC 12464	—
sordelli ATCC 9714	—
sporogenes ATCC 19404	—
sporogenes ATCC 3854	—
tetani ATCC 10799	—
tetani ATCC 19406	—
haemolyticum ATCC 9650	—
subterminale ATCC 25774	—
C. spp hastiforme ATCC 25772	—
bifermentans ATCC 638	—
C. spp ATCC 17861	—
ramosum ATCC 25582	—
tertium ATCC 14573	—
perfringens A ATCC 3624	—
perfringens B ATCC 3626	—
perfringens A ATCC 13124	—

[0047] As shown in Table 3, positive results were obtained for all *botulinum* species but not for any of the non-*botulinum* species. Accordingly, the NTNH DNA PCR assay of the invention is specific and sensitive to *C. botulinum* and provides a universal *C. botulinum* detection assay.

EXAMPLE 2

[0048] This example demonstrates the sensitivity and specificity of the inventive method, particularly the type-specific assays, for detecting *C. botulinum* toxin A-G.

[0049] DNA samples from numerous *C. botulinum* species (provided by T. Smith, USAMRIID) were obtained and subjected to the seven toxin type-specific assays in accordance with the invention to identify the presence or absence of *C. botulinum* toxin genes A-G. The various *C. botulinum* species that served as sources of the DNA samples are set forth in Table 4.

[0050] Each of the DNA samples was subjected to seven individual PCR nucleic acid amplification procedures using the degenerate type-specific primer/probe sets corresponding to SEQ ID NOs: 1, 8 and 15 (for *C. botulinum* toxin A), SEQ ID NOs: 2, 9 and 16 (for *C. botulinum* toxin B), SEQ ID NOs: 3, 10 and 17 (for *C. botulinum* toxin C), SEQ ID NOs: 4, 11 and 18 (for *C. botulinum* toxin D), SEQ ID NOs: 5, 12 and 19 (for *C. botulinum* toxin E), SEQ ID NOs: 6, 13 and 20 (for *C. botulinum* toxin F), and SEQ ID NOs: 7, 14 and 21 (for *C. botulinum* toxin G). The PCR conditions were as follows: 95° C for 15 sec and 60° C for 1 min. The PCR reaction mixture contained 1x PCR buffer, 3.5 μ M MgCl₂, 200 nM dNTPs, 500 nM forward primer, 500 nM reverse primer, 200 nM Fam-BHQ1 probe, 3 nM BD636 reference dye, and 0.25 U Platinum Taq polymerase (Invitrogen, Carlsbad, CA). 5 μ L (0.25 ng/ μ L) of each DNA sample was used in each 25 μ L PCR reaction mixture. PCR products were visualized on a 1.5% agarose gel.

[0051] To generate plasmid standards, the PCR products corresponding to the DNA samples used to generate the template standards were gel purified and ligated into pGEM T-easy vectors (Promega Corp., Madison, WI). Ligations were transformed into DH5 α *E. coli* bacteria using α -complementation to determine positive colonies. Plasmid DNA was purified and sequenced for determination of the correct type insert sequence. The DNA was quantified by UV spectrophotometry, and serial dilutions were made from 10⁶ to 10² target molecules/5 μ L, thereby allowing for quantification of the *C. botulinum* toxin genes A-G.

[0052] 5' nuclease technology was used for quantitative PCR probe hydrolysis. For absolute quantification, template standards for each of the *C. botulinum* types, i.e., for each of the *C. botulinum* toxin genes A-G, were run alongside the DNA samples for each of the seven PCR procedures.

[0053] The results of this testing are set forth in Table 4, where the result is indicated as “+” for the DNA samples evidencing the presence of the indicated *C. botulinum* toxin gene type (A-G) and “-” for the DNA samples evidencing the absence of the indicated *C. botulinum* toxin gene type (A-G).

Table 4: DNA Sample Test Results for *C. botulinum* toxin A-G

DNA Sample	<i>C. botulinum</i> Type						
	A	B	C	D	E	F	G
A1 Hall	+	-	-	-	-	-	-
A2 Kyoto	+	-	-	-	-	-	-
A3 Loch Maree	+	-	-	-	-	-	-
B1 Okra	-	+	-	-	-	-	-
B2 213B	-	+	-	-	-	-	-
npB Eklund 17B	-	+	-	-	-	-	-
Ba4 657	+	+	-	-	-	-	-
C Stockholm	-	-	+	-	-	-	-
C/D 6813	-	-	+	-	-	-	-
D ATCC 11873	-	-	-	+	-	-	-
D/C VPI 5995	-	-	-	+	-	-	-
E1 Beluga	-	-	-	-	+	-	-
E3 Alaska	-	-	-	-	+	-	-
E Butyr BL5262	-	-	-	-	+	-	-
ProtF Langeland	-	-	-	-	-	+	-
npF Eklund 202F	-	-	-	-	-	+	-
Bf AN436	-	+	-	-	-	+	-
G 1354	-	-	-	-	-	-	+
A1 1757	+	-	-	-	-	-	-
A1 1744	+	-	-	-	-	-	-
A2b 1436	+	-	-	-	-	-	-
B1 1656	-	+	-	-	-	-	-
B2 1826	-	+	-	-	-	-	-

B1 1758	-	+	-	-	-	-	-
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[0054] As shown in Table 4, positive results were obtained only when the type-specific primer/probe set had its correct template to amplify. Strains known to produce or contain the genes for two toxin types were successfully confirmed as such by the assay.

[0055] As confirmation that the inventive assay provides for the sensitive detection and identification of *C. botulinum* toxin types A-G, purified DNA from *C. botulinum* cultures was diluted taking into account genomic size and concentration of the DNA sequence. 5 log dilutions representing 10^5 genomic copies to 1 genome copy of BoNT were made. Each dilution series was run with six replicates to determine reproducibility. Plasmid standards were run along with each dilution series to determine the exact copy number in each reaction.

[0056] As shown in Table 5, BoNTs were consistently detected as sensitive as 10 gene copies per reaction. The results represent the percentage of the six replicates that contained accurate copy numbers in each reaction. Therefore, the detection level of the inventive assay was reliable down to 10 copies of neurotoxin gene.

Table 5: Sensitivity of the Inventive Assay

Toxin Type	Genomic Copies					
	1×10^5	1×10^4	1×10^3	1×10^2	10	1
A	100%	100%	100%	100%	100%	50%
B	100%	100%	100%	100%	100%	33%
C	100%	100%	100%	100%	100%	0%
D	100%	100%	100%	100%	100%	0%
E	100%	100%	100%	100%	100%	33%
F	100%	100%	100%	100%	100%	17%
G	100%	100%	100%	100%	100%	50%

[0057] These results demonstrate that the inventive method provides for the proper and specific detection and identification of *C. botulinum* toxin gene types A-G and also for the

amount of *C. botulinum* bacterial DNA present in the sample to be quantified accurately and sensitively.

[0058] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0059] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0060] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIM(S):

1. A method of detecting the presence or absence of *C. botulinum* in a sample comprising:
 - (a) subjecting a sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 22 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 23,
 - (b) detecting the presence or absence of amplified DNA resulting from step (a).
2. The method of claim 1, wherein the method further comprises correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *C. botulinum* in the sample.
3. The method of claim 1 or 2, which method further comprises:
 - (d) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 1 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 8,
 - (e) detecting the presence or absence of amplified DNA resulting from step (d), and
 - (f) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *C. botulinum* toxin A gene in the sample.
4. The method of claim 3, wherein step (e) comprises contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 15.
5. The method of claim 1 or 2, which method further comprises:
 - (d) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to

SEQ ID NO: 2 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 9,

(e) detecting the presence or absence of amplified DNA resulting from step (d), and

(f) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *C. botulinum* toxin B gene in the sample.

6. The method of claim 5, wherein step (e) comprises contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 16.

7. The method of claim 1 or 2, which method further comprises:

(d) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 3 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 10,

(e) detecting the presence or absence of amplified DNA resulting from step (d), and

(f) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *C. botulinum* toxin C gene in the sample.

8. The method of claim 7, wherein step (e) comprises contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 17.

9. The method of claim 1 or 2, which method further comprises:

(d) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 4 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 11,

(e) detecting the presence or absence of amplified DNA resulting from step (d), and

(f) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *C. botulinum* toxin D gene in the sample.

10. The method of claim 9, wherein step (e) comprises contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 1811.

The method of claim 1 or 2, which method further comprises:

(d) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 5 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 12,

(e) detecting the presence or absence of amplified DNA resulting from step (d), and

(f) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *C. botulinum* toxin E gene in the sample.

12. The method of claim 11, wherein step (e) comprises contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 19.

13. The method of claim 1 or 2, which method further comprises:

(d) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 6 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 13,

(e) detecting the presence or absence of amplified DNA resulting from step (d), and

(f) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *C. botulinum* toxin F gene in the sample.

14. The method of claim 5, wherein step (e) comprises contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 20.15.

The method of claim 1 or 2, which method further comprises:

(d) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers comprising a sequence having at least 90% homology to SEQ ID NO: 7 and (ii) one or more reverse primers comprising a sequence having at least 90% homology to SEQ ID NO: 14,

(e) detecting the presence or absence of amplified DNA resulting from step (d), and

(f) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of *C. botulinum* toxin G gene in the sample.

16. The method of claim 5, wherein step (e) comprises contacting the sample with a probe comprising a nucleic acid sequence having at least 90% homology to SEQ ID NO: 21.

17. A method of detecting the presence or absence of one or more types of *C. botulinum* toxin gene in a sample comprising performing each of the methods of claims 3, 5, 7, 9, 11, 13, and 15 on the sample.

18. A method of detecting the presence or absence of one or more types of *C. botulinum* toxin gene in a sample comprising performing each of the methods of claims 4, 6, 8, 10, 12, 14, and 16 on the sample.

19. The method of any of claims 1-18, wherein the sample is from a mammal.

20. The method of claim 19, wherein the sample is from a human.

21. The method of claim 18 or 20, wherein the sample is selected from the group consisting of serum, emesis, wound, and stool samples.

22. The method of any of claims 1-18, wherein the sample is a food product.

23. The method of claim 22, wherein the sample is a food product suitable for ingestion by a mammal.
24. The method of claim 23, wherein the mammal is a human.
25. The method of claim 24, wherein the food product is selected from the group consisting of canned goods, fruits, vegetables, meats, beverages, and combinations thereof.
26. A method of diagnosing a human comprising determining the presence or absence of a *C. botulinum* toxin gene in a sample from the human according to any of claims 1-18, and correlating the presence or absence of *C. botulinum* toxin gene in the sample with the diagnosis of the human as being or not being infected or intoxicated with a *C. botulinum*.
27. The method of claim 26, which method further comprises administering a *C. botulinum* antitoxin to the human.
28. The method of claim 27, wherein the antitoxin is botulism immune globulin, trivalent botulinum antitoxin, or heptavalent botulinum antitoxin.
29. An isolated polynucleic acid having at least 90% homology to a sequence selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 23.
30. An isolated polynucleic acid having at least 90% homology to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7.
31. An isolated polynucleic acid having at least 90% homology to a sequence selected from the group consisting of SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID NO: 14.
32. An isolated polynucleic acid having at least 90% homology to a sequence selected from the group consisting of SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, and SEQ ID NO: 21.

33. A kit comprising polynucleic acids of claim 29 for use as at least one forward primer and at least one reverse primer for *C. botulinum* toxin gene(s) amplification.

34. A kit comprising at least one polynucleic acid of claim 30 and at least one polynucleic acid of claim 31 for use as at least one forward primer and at least one reverse primer.

35. The kit of claim 34, wherein the kit further comprises at least one polynucleic acid of claim 32 for use as a probe.

36. A method of detecting an NTN gene in a sample comprising

(a) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers complementary to the NTN gene and (ii) one or more reverse primers complementary to the NTN gene,

(b) detecting the presence or absence of amplified DNA resulting from step (a), and

(c) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of the NTN gene in the sample.

37. The method of claim 36, which method further comprises:

(d) subjecting the sample to a PCR nucleic acid amplification procedure involving the use of (i) one or more forward primers complementary to one or more botulism toxin gene A-G and (ii) one or more reverse primers complementary to one or more botulism toxin genes type A-G,

(e) detecting the presence or absence of amplified DNA resulting from step (d), and

(f) correlating the presence or absence of amplified DNA with the presence or absence, respectively, of one or more of the botulism toxin genes A-G.

FIG. 1

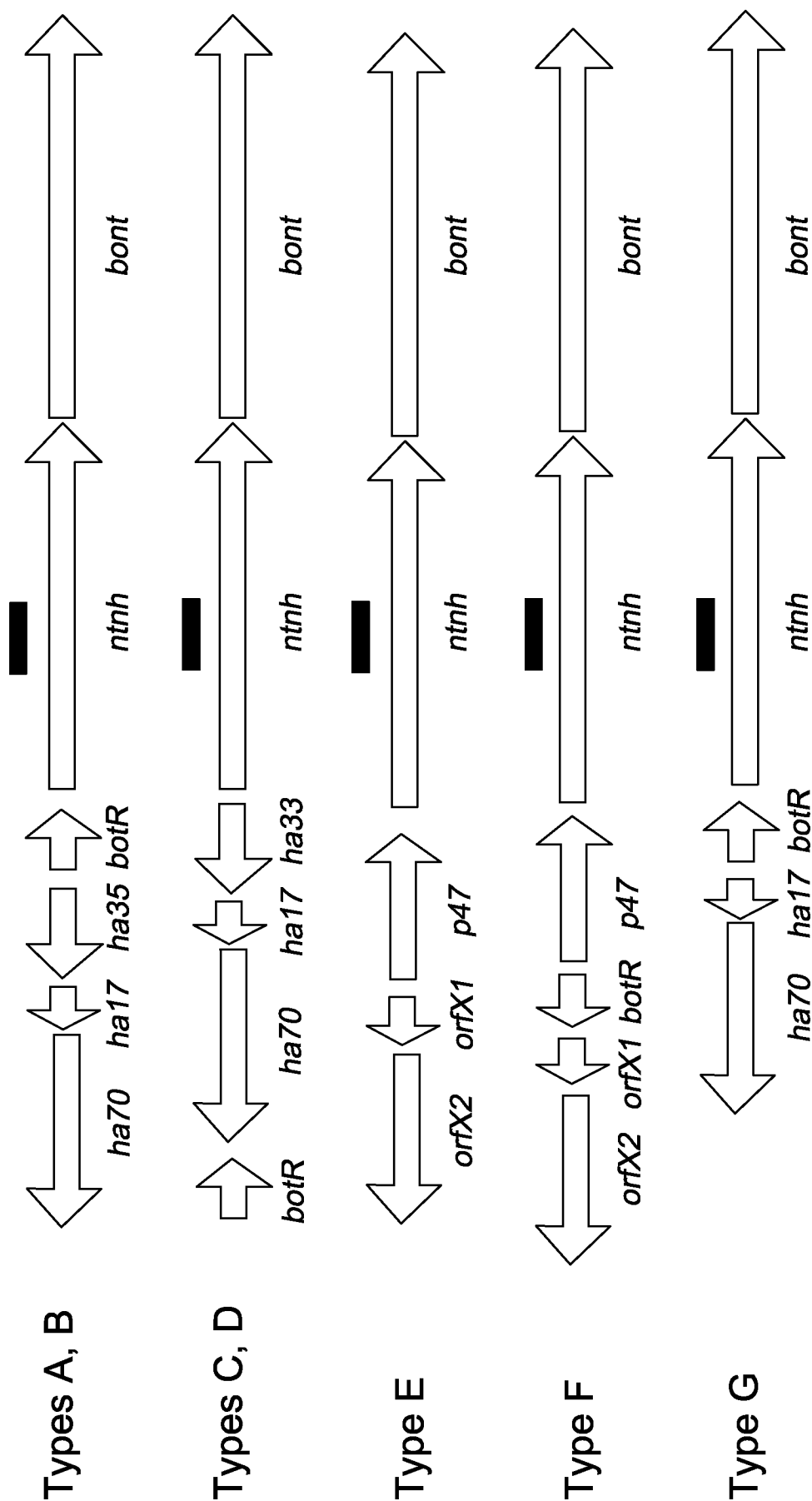


FIG. 2

