TABLE 1. *C. botulinum* type B strains and sequences used in this study[a]

Strain identifier	BoNT/B subtype[b]	GenBank accession no.	Origin (country, yr)	Reference or source
Bacterial strain				
111	B2	**AB302854**, AB084152	Infant botulism (Japan, 1995)	15
Osaka05	**Osaka05**	**AB302852**	Infant botulism (Japan, 2005)	In this study
Osaka06	**B2**	**AB302853**	Infant botulism (Japan, 2006)	In this study
89E00061-2	**B1**	**AB302861**	Infant botulism (United States, 1989)	In this study
89E00067-4	**B1**	**AB302862**	Infant botulism (United States, 1989)	In this study
89E00123-1	**B1**	**AB302963**	Infant botulism (United States, 1989)	In this study
90E00001-3	**B1**	**AB302864**	Infant botulism (United States, 1990)	In this study
3129-2-77	**B1**	**AB302865**	Infant botulism (United States, 1990)	In this study
Okra	B1	AB232927	Food borne (unknown)	23
326	**B1**	**AB302858**	Pork meat (Japan)	In this study
407	**B1**	**AB302856**	Pork meat (Japan)	In this study
Ginger	**B2**	**AB302857**	Ginger (Japan)	In this study
7H215S	**B2**	**AB302860**	Honey (Japan)	In this study
9B	**B1**	**AB302859**	Stocked strain (United States)	In this study
67B	**B1**	**AB302855**	Stocked strain (United States)	In this study
GenBank				
CDC1758	B1	EF033127	Unknown	13
Danish	B1	M81186	Unknown	41
Hall6517(B)	B1	EF028399	Unknown	13
CDC1656	B1	EF028396	Unknown	13
Prevot25 NCASE	B2	EF033129	Unknown	13
213B	B2	EF028395	Unknown	13
Smith L-590	B2	EF028398	Unknown	13
Prevot59	B2	EF033128	Unknown	13
CDC1828	B2	EF051571	Unknown	13
CDC6291	B2	EF028401	Unknown	13
Korean soil 1	B2	DQ417353	Korean soil	GenBank
Korean soil 2	B2	DQ417354	Korean soil	GenBank
CDC795	B3	EF028400	Unknown	13
CDC593	Bivalent, A(B)[c]	AF300466	Dog feces (United States, 1976)	19
CDC1436	Bivalent, AB[d]	AF295926	Stool sample (United States, 1977)	19
657Ba	Bivalent, Ba[d]	EF033130	Unknown	13, 36
CDC588	Bivalent, Ab[d]	AF300465	Food borne (United States, 1976)	19
CDC3281	Bivalent, Bf[d]	Y13630	Infant botulism (United States, 1980)	33
ATCC 17844	Nonproteolytic	EF028394	Unknown	13
Eklund17B (B257)	Nonproteolytic	EF051570	Unknown	13
10068	Nonproteolytic	EF028402	Unknown	13
Eklund17B	Nonproteolytic	X71343	Unknown	14

[a] The subtypes and GenBank accession numbers for strains determined in this study are indicated in boldface.
[b] Subtyped by Hill et al. (13); the other strains were subtyped in this study.
[c] BoNT/A producing and unexpressed *boNT/B* gene possessing.
[d] Dual toxin-producing strains; the major toxin type is indicated in uppercase letters and the minor type is indicated in lowercase letters.

comparison of the nucleotide sequences of *boNT/B* and nontoxic component genes, the pulsed-field gel electrophoresis (PFGE) genotypes, the *boNT/B* gene location by PFGE and Southern blot hybridization, and the antigenicity of the new BoNT/B subtype. We developed multiplex PCR assays for the detection and identification of the BoNT/B subtypes.

MATERIALS AND METHODS

Type B infant botulism in Japan. (i) Case 1. The first case of type B infant botulism occurred in a female infant aged 6 months in Ishikawa Prefecture in 1995, and type B strain 111 was isolated from this case (18, 42). Strain 111 was provided by S. Nakamura (Kanazawa University School of Medicine, Ishikawa, Japan).

(ii) Case 2. In Osaka City in October 2005, a previously healthy breast-fed female infant aged 3 months with a 2-day history of fever, pituita, and poor feeding was hospitalized for 45 days and then discharged, and she recovered fully. BoNT/B and type B strain Osaka05 were detected in stool samples on the third hospital day (28).

(iii) Case 3. In Osaka Prefecture in May 2006, a previously healthy mainly breast-fed female infant aged 5 months with a 1-week history of constipation, poor feeding, and weakness was hospitalized for 32 days and then discharged, and she recovered fully. BoNT/B and type B strain Osaka06 were detected in the stool on the fifth hospital day (1). Strain Osaka06 was provided by T. Asao (Osaka Prefectural Institute of Public Health, Osaka, Japan).

C. botulinum was not detected in food, drink, or house dust samples obtained from the patients' homes for cases 2 and 3. In all cases, the patients had no history of honey consumption.

Bacterial strains and DNA extraction. Fifteen proteolytic *C. botulinum* type B strains, including 3 strains associated with infant botulism in Japan, were used in this study (Table 1). The biochemical characteristics, including the proteolytic activities, of all strains were tested by use of an API 20A kit (Biomerieux, Marcy l'Etoile, France). No strains with any unexpressed *boNT* genes were detected by PCR assay (40). Individual strains were cultured in 10 ml cooked meat medium (Difco, Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 0.3% glucose and 0.2% soluble starch (Difco, Becton Dickinson and Co.) under anaerobic conditions at 30°C for 18 h. Bacterial DNA was extracted from 1 ml culture with a DNeasy tissue kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. Finally, DNA was eluted with 100 μl elution buffer and stored at 4°C until use.

Nucleotide sequencing of *boNT/B* and nontoxic component genes. The *boNT/B* gene and nontoxic component genes (*ha70*, *ha17*, *ha33*, *p21*, and *ntnh*) were amplified by using the overlapping primer pairs listed in Table 2, which were designed on the basis of the nucleotide sequences available from GenBank. PCR was performed with a 50-μl reaction mixture containing 10 ng extracted DNA,

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TABLE 2. Primers used for amplification of *boNT/B* and nontoxic component genes

Gene(s) included in amplified fragment	Primer pair sequences (5′–3′)[a]	Location (positions)[a,b]
ha70, *ha17*, and first half of *ha33*	CAAAATATGATTTCCTTGT/AGCAGCATACCAGTTTT	2725–2743/5578–5562
Latter half of *ha33*, *botR*, and first half of *ntnh*	CGCGTAGATTAGTAATTG/AAGTGCATTATTAAATCTATCT	5406–5423/8166–8145
Latter half of *ntnh*	AGGAAATAATGCCATTG/CTTTATAATATCTCCCCGT	8022–8038/10826–10808
boNT/B light chain	TTTATGGGCATTAAAAG/CATCTGAAAAACTATTTTTAT	10671–10687/12116–12096
boNT/B heavy chain	AGAGGTCAGAATAAAGCTA/CAAAATTTAGCTACATCCT	11948–11966/14682–14664

[a] Sequences and positions are for forward primer/reverse primer.
[b] Location of primer sequence of *boNT/B* and nontoxic component genes in the sequence reported under DDBJ accession no. AB232927.

0.5 μM of each primer, 2.5 U LA *Taq* (TaKaRa Shuzo, Kyoto, Japan), 5 μl LA *Taq* buffer, 2.5 mM $MgCl_2$, and 400 μM of each deoxynucleoside triphosphate (dNTP). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 65°C for 3 min and was repeated 25 times. Final extension was carried out at 65°C for 5 min. The amplicons were directly sequenced by primer walking, and the sequence in each direction was confirmed with an ABI Prism BigDye cycle sequencing kit (Applied Biosystems Inc., Foster City, CA).

Nucleotide sequencing of the C-terminal region of BoNT/B. The nucleotide sequences of the C-terminal region (400 bp) of BoNT/B were determined by direct sequencing with the following primers: forward primer 5′-GAAAGTCA AATTCTCAATC-3′ (positions in the coding region, bases 3445 to 3463) and reverse primer 5′-CAAAATTTAGCTACATCCT-3′ (positions in the coding region, bases 3961 to 3943). PCR was performed with a 50-μl reaction mixture containing 1 ng extracted DNA, 0.5 μM of each primer, 2.5 U LA *Taq* (TaKaRa Shuzo), 5 μl LA *Taq* buffer, 2.5 mM $MgCl_2$, and 200 μM of each dNTP. Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 65°C for 2 min and was repeated 25 times. Final extension was carried out at 65°C for 5 min.

Phylogenetic analysis. The nucleotide sequences were aligned by use of the Clustal X program (version 1.83) with the parameters provided in Clustal W, version 1.6. A phylogenetic tree with 1,000 bootstrap replications was constructed by the neighbor-joining method, and genetic distances were calculated by the Kimura two-parameter method (8). The resulting tree was drawn with NJplot software.

PFGE analysis. PFGE plugs were prepared as described by Hielm et al. (12). DNA was left undigested or was digested with 20 U of SmaI (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, United Kingdom) in 200 μl of optimal buffer at 25°C for 18 h. The plugs were electrophoresed in a CHEF-DRIII apparatus (Bio-Rad Laboratories, Hercules, CA) through a 1% pulse-field-certified agarose gel (Bio-Rad Laboratories) in 0.5× Tris-borate-EDTA buffer at 14°C and 6 V/cm. The switching times were ramped from 0.5 to 40 s, and the bands were visualized by ethidium bromide staining. Dendrogram analysis of the band patterns was performed with Fingerprinting II software (Bio-Rad Laboratories). Similarity analysis was performed by using the Dice coefficient, and clustering was examined by the unweighted pair group method with arithmetic averages. Approximate fragment sizes (kbp) were measured by use of a molecular size marker.

Southern blot hybridization. After PFGE, the DNA fragments in the gel were transferred onto Hybond N^+ nylon membranes (GE Healthcare) with a capillary transfer system and 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 18 h. The DNA was fixed to the membrane by UV irradiation. The *boNT/B* and 16S rRNA gene probes were prepared by using the primers reported by Szabo et al. (39) and Marshall et al. (25), and DNA extracted from strain Okra was labeled by use of a PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany). The membranes were incubated at 37°C for 2 h with hybridization solution (DIG-Easy Hyb; Roche Diagnostics), and hybridizations were performed at 42°C for 18 h with fresh DIG-Easy Hyb containing 20 ng/ml of each probe. The membranes were then washed three times for 15 min each time at room temperature with 1× SSC containing 0.1% sodium dodecyl sulfate and twice for 15 min each time with 0.1× SSC containing 0.1% sodium dodecyl sulfate at 60°C for the *boNT/B*-specific probe and at 65°C for the 16S rRNA-specific probe. Hybridization signals were detected with a DIG luminescent detection kit (Roche Diagnostics).

PCR assays for BoNT/B subtyping. PCR was performed with four primer mixtures, as follows: primer B-forward (5′-GATTTTTGGGGAAATCTTT-3′; positions in the coding region, bases 3256 to 3275), primer B1-reverse (5′-CCA ATTACATCCCAATTTTAAA-3′; positions, bases 3840 to 3819), primer B2-reverse (5′-GTATAGTTTTGTAAAATTCATTAGAATCATA-3′; positions, bases 3625 to 3595), and primer Osaka05-reverse (5′-TCTTCCTTTTTCTTAA AATTTTTAAG-3′; positions, bases 3572 to 3547). These primers were designed on the basis of the *boNT* sequences published by GenBank and determined in this study. PCR was performed with a 25-μl reaction mixture containing 0.3 to 1 ng template DNA, 0.25 μM of each primer, 1.25 U Ex *Taq* (TaKaRa Shuzo), 2.5 μl Ex *Taq* buffer (TaKaRa Shuzo), and 200 μM of each dNTP. Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min and was repeated 25 to 35 times. Final extension was carried out at 72°C for 5 min. The amplicons were visualized in 3% Nu-Sieve agarose gels (Camblex Bio Science, Rockland, ME) stained with ethidium bromide.

Sandwich enzyme-linked immunosorbent assay (ELISA) with rH_C. Recombinant BoNT/B H_C (rH_C) from strain Osaka05 (rH_C/Osaka05), rH_C/Okra, and rH_C/111 were expressed as described in our previous report (15), except that the primers used for the amplification of the DNA fragment encoding BoNT/Osaka05 H_C were as follows: forward primer 5′-CACGGATCCAAAAAATAT AATAGCGAAATTTTAAA-3′ and reverse primer 5′-ATTAAGCTTTTATTC AATCCATCCTTCATCTTT-3′. The rH_C that was expressed was purified with nickel-nitrilotriacetic acid agarose (Qiagen) and by CM-Sephadex C-25 (GE Healthcare) chromatography. Polyclonal antibody against each rH_C was prepared with rabbits. The first subcutaneous injection was performed with purified 50 μg rH_C in Freund's complete adjuvant. After 2 weeks, the animals received three booster injections of the same amount of rH_C in Freund's incomplete adjuvant at 2-week intervals. The animals were bled 2 weeks after the last booster injection. The immunoglobulin G fraction was purified by ammonium sulfate precipitation and was subsequently purified on an rH_C-coupled HiTrap *N*-hydroxysuccinimide-activated high-performance column (GE Healthcare), according to the manufacturer's instructions. Biotinylated antibodies were prepared with EZ-Link sulfosuccinimidyl-6-[biotinamido]hexanoate (Pierce, Rockford, IL).

For the sandwich ELISA, 96-well microtiter plates (Iwaki, Japan) were coated with 0.1 ml polyclonal antibody against rH_C (3 μg/ml) at 37°C for 2 h. After the plates were blocked at 4°C for 18 h with 1.0% Blockace blocking reagent (Dainippon Sumitomo Pharma Co., Japan), rH_C was applied at dilutions ranging from 10 ng/ml to 5,000 ng/ml in duplicate and the plates were incubated at 37°C for 1 h. The plates were washed and incubated with 1 μg/ml biotinylated antibody against rH_C at 37°C for 1 h, followed by washing and incubation with 0.2 μg/ml streptavidin-conjugated horseradish peroxidase (Pierce) at 37°C for 1 h. The plates were developed with 2,2-azinobis-(3-ethylbenzthiazoline sulfonic acid) (Roche) at 37°C for 1 h. The color was monitored by measuring the absorbance at 415 nm on a Labsystem Multiscan mass spectrometer (Labsystems, Finland).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this work were submitted to the DDBJ database and may be found under accession numbers AB302852 to AB302865 (Table 1).

RESULTS

Comparison of *boNT/B* and nontoxic component gene sequences among strains Osaka05, Okra (subtype B1), and 111 (subtype B2). The nucleotide sequences of the *boNT/B* gene (3,876 bp) from two isolates associated with infant botulism in Japan (strains Osaka05 and Osaka06) and nontoxic component genes (*ha70*, 1,881 bp; *ha17*, 441 bp; *ha33*, 879 bp; *botR*, 537 bp; *ntnh*, 3,594 bp) from strains Osaka05 and 111 were deter-

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TABLE 3. Identities among strains Osaka05, Okra (subtype B1), and 111 (subtype B2) in the *boNT/B* and nontoxic components[a]

Strains compared	% Nucleotide/% amino acid identity					
	HA70 (1,881/626)[a]	HA17 (441/146)	HA33 (885[879]/294[292][b])	BotR (537/178)	NTNH (3,594/1,197)	BoNT/B (3,876/1,291)
Osaka05 and Okra (B1)	99.6/99.0	99.3/97.9	95.2/90.1	98.5/97.2	97.9/96.7	97.9/96.2
Osaka05 and 111 (B2)	99.1/98.4	98.6/100.0	92.3/83.6	99.4/98.9	99.6/99.3	99.0/98.5
Okra and 111	98.9/98.1	98.0/97.9	93.1/84.9	98.3/96.6	97.8/96.4	97.6/95.7

[a] Data in parentheses are number of nucleotide base pairs/number of amino acid residues.
[b] Data in brackets are for strains Osaka05 and 111.

mined. The *boNT/B* gene nucleotide sequences of strains Osaka05 and Osaka06 shared 98.8% identity. These sequences were further compared with 24 full-length *boNT/B* gene sequences available in the GenBank database (Table 1). The *boNT/B* gene sequence of strain Osaka06 was identical to that of strain CDC6291, which was classified as subtype B2. Strain Osaka05 was shown to possess a unique sequence in comparison with the sequences of the other *boNT/B* genes.

The nucleotide and amino acid identities of *boNT/B* and nontoxic components among strains Osaka05, Okra (subtype B1), and 111 (subtype B2) are summarized in Table 3. At the nucleotide and amino acid levels in *boNT/B*, Osaka05 had identities of 97.9% and 96.2%, respectively, with Okra and 99.0% and 98.5%, respectively, with 111, while Okra and 111 possessed identities of 97.6% and 95.7% at the nucleotide and amino acid levels in *boNT/B*, respectively. The organization of nontoxic component genes (composed of *ha70*, *ha17*, *ha33*, *botR*, and *ntnh*) and the *boNT/B* gene was shown to be common among the three strains. For all nontoxic components except *ha33*, Osaka05 showed 97.9% to 99.6% nucleotide sequence identities and 96.7% to 99.0% amino acid sequence identities with Okra and 98.6% to 99.6% nucleotide sequence identities and 98.4% to 100.0% amino acid sequence identities with 111, while Okra had 97.8% to 98.9% nucleotide sequence identities and 96.4% to 98.1% amino acid sequence identities with 111. For *ha33*, the three strains shared 92.3% to 95.2% nucleotide sequence identities and 83.6% to 90.1% amino acid sequence identities, which were remarkably lower than the levels of identity for the other nontoxic component genes.

The amino acid substitutions in BoNT/B among strains Okra, 111, and Osaka05 involved 61 residues (2 residues in the light chain, which was 441 amino acids [aa], and 59 residues in the heavy chain [which was 850 aa]), and the substitutions spread throughout the domains or the subdomains within the heavy chain were as follows: 22 in H_N (420 aa), 37 in H_C (430 aa), 12 in H_{CN} (167 aa), and 25 in H_{CC} (263 aa). These substitutions were mostly concentrated in the heavy chain, especially in the H_{CC} subdomain. Osaka05 showed identities of 99.5% (light chain), 94.5% (heavy chain), 95.2% (H_N), 92.8% (H_{CN}), and 94.7% (H_{CC}) with Okra at the amino acid level and identities of 100.0% (light chain), 97.6% (heavy chain), 98.6% (H_N), 100.0% (H_{CN}), and 94.3% (H_{CC}) with 111 at the amino acid level. Okra had identities of 99.5% (light chain), 93.6% (heavy chain), 95.5% (H_N), 92.8% (H_{CN}), and 91.3% (H_{CC}) with 111 at the amino acid level (Fig. 1).

Phylogenetic analysis of full-length *boNT/B* gene. The phylogenetic tree was constructed on the basis of alignment of the full length of the nucleotide sequences of the *boNT/B* genes from strains Osaka05 and Osaka06 with 24 *boNT/B* sequences, including the sequences of the five BoNT/B subtypes, published in GenBank (Fig. 2A). Overall, among the 26 strains producing BoNT/B, 25 (the exception was Osaka05) were classified into five clusters, which was the same classification as the BoNT/B sub-

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Light chain (positions 1-441)

Strain/Position	404	441
B1 (Okra)	D	K
B2 (111)	N	R
Osaka05	N	R

H_N domain (positions 442-861)

Strain/Position	476	485	486	487	489	496	530	599	609	614	690	694	737	768	777	829	831	836	842	846	852	854
B1 (Okra)	N	D	F	P	N	D	I	N	N	I	N	S	R	I	G	N	Y	M	I	D	M	N
B2 (111)	D	R	S	S	D	N	—	D	S	—	D	I	K	V	E	D	H	I	M	N	I	—
Osaka05	D	R	S	S	D	N	F	D	—	L	D	R	K	—	E	D	H	I	M	N	I	K

H_{CN} subdomain (positions 862-1028)

Strain/Position	871	884	905	908	922	956	976	990	997	1011	1012	1026
B1 (Okra)	K	K	A	K	V	M	I	N	E	L	N	T
B2 (111)	R	N	T	E	M	I	T	S	D	S	D	I
Osaka05	R	N	T	E	M	I	T	S	D	S	D	I

H_{CC} subdomain (positions 1029-1291)

Strain/Position	1032	1072	1074	1117	1132	1138	1174	1176	1182	1183	1185	1188	1189	1191	1197	1199	1202	1206	1250	1251	1252	1255	1274	1275	1290
B1 (Okra)	R	E	R	P	K	D	L	Q	T	Y	Y	K	E	E	P	S	D	N	F	E	E	D	L	K	T
B2 (111)	G	K	I	S	N	N	S	R	A	—	D	E	—	K	N	Y	N	K	L	K	D	N	P	N	I
Osaka05	G	K	I	—	N	N	—	—	A	L	N	—	K	—	—	—	—	—	—	K	D	Y	P	N	I

FIG. 1. Summary of BoNT/B amino acid substitutions between strains Osaka05, Okra (subtype B1), and 111 (subtype B2) in each domain (light chain and H_N) or subdomain (H_{CN} and H_{CC} in H_C). Hyphens indicate residues identical to those in strain Okra.

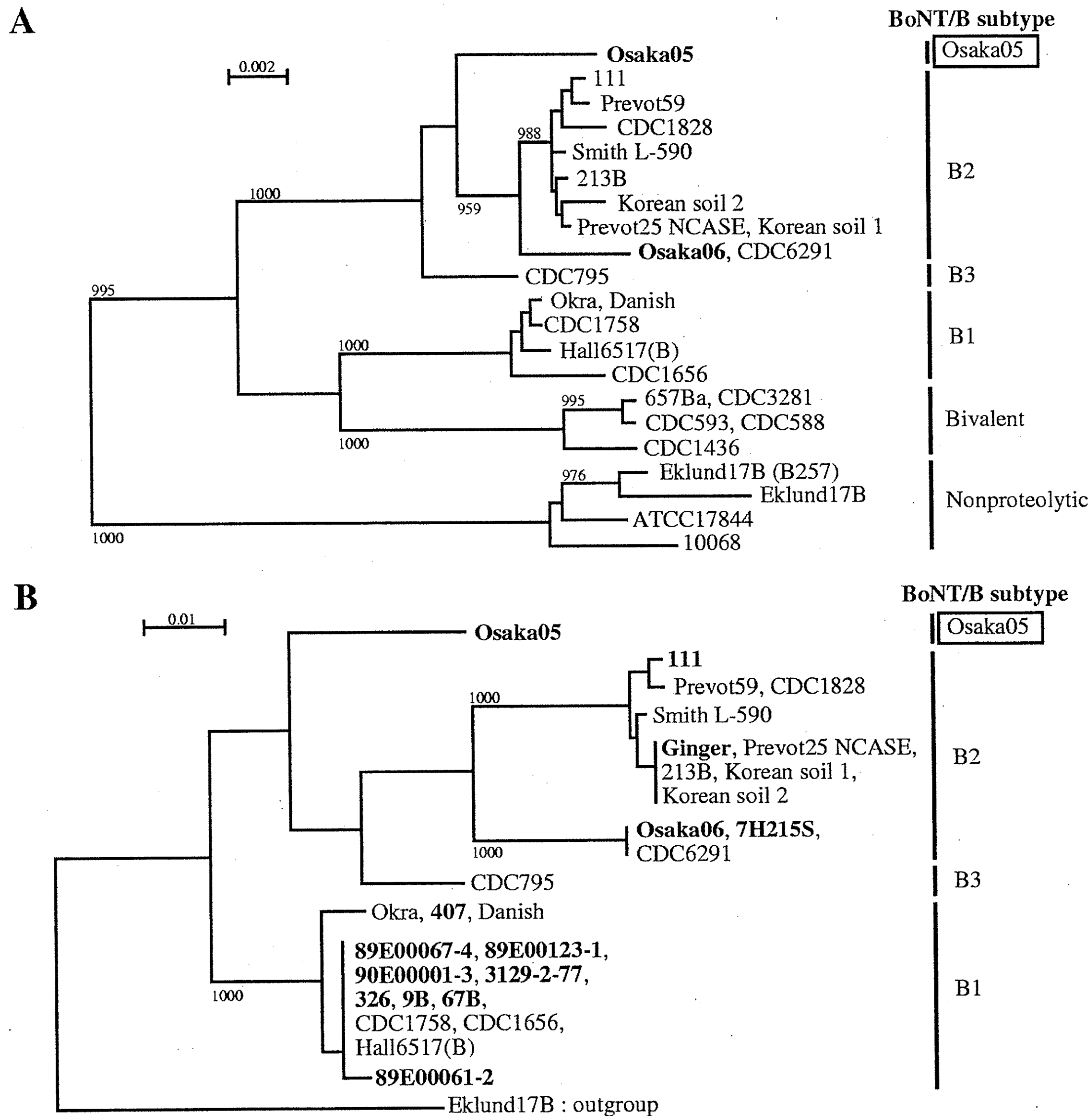

FIG. 2. Phylogenetic trees based on *boNT/B* gene sequences. The full-length *boNT/B* gene nucleotide sequences (3,876 bp) (A) and the nucleotide sequences at the C-terminal region (400 bp) of BoNT/B (B) were constructed by use of the reference sequences listed in Table 1. The sequences determined in this study are indicated in boldface. The outgroup was the neurotoxin sequence of *C. tetani* CN3911 (GenBank accession no. X06214) and was removed from the final figure (A), and nonproteolytic *C. botulinum* Eklund17B type B was the outgroup for the BoNT/B sequences (B). The five BoNT/B subtypes (subtypes B1, B2, B3, bivalent, and nonproteolytic) were described by Hill et al. (13). The BoNT/B subtype newly identified in this study is boxed. Numbers on each branch indicate bootstrap values (>950) for the cluster supported by that branch.

types. Strain Osaka06 was classified into the subtype B2 cluster and showed 99.3% to 100% identity with the subtype B2 strains. The new isolate, strain Osaka05, was different from the strains in the other five clusters and shared 98.8% to 99.1% nucleotide sequence identity with the subtype B2 strains, 98.9% identity with the subtype B3 strains, identities of 97.7% to 98.0% with the subtype B1 strains, identities of 97.4% with the bivalent strains, and identities of 95.7% to 96.2% with the nonproteolytic strains.

Phylogenetic analysis of the C-terminal region of BoNT/B. The phylogenetic tree was also constructed on the basis of the alignment of the nucleotide sequences at the C-terminal region (400 bp) of BoNT/B, which partially encoded the H_{CC} subdomain (Fig. 2B). Overall, except for Osaka05, 28 proteolytic type B strains (the 15 bacterial strains and the 13 reference strains; Table 1) were classified into three subtypes (subtypes B1, B2, and B3), which was the same as the classification for the full-length *boNT/B* genes (Fig. 2A). Subtype B1 included five isolates associated with U.S. infant botulism, two isolates from pork meat, two stocked strains, and five reference strains. Subtype B2 included two isolates associated with Japanese infant botulism, isolates from ginger and honey, and eight reference strains. Strains CDC795 (B3 subtype) and Osaka05 were also different from the strains in the other clusters and were not classified in the same cluster.

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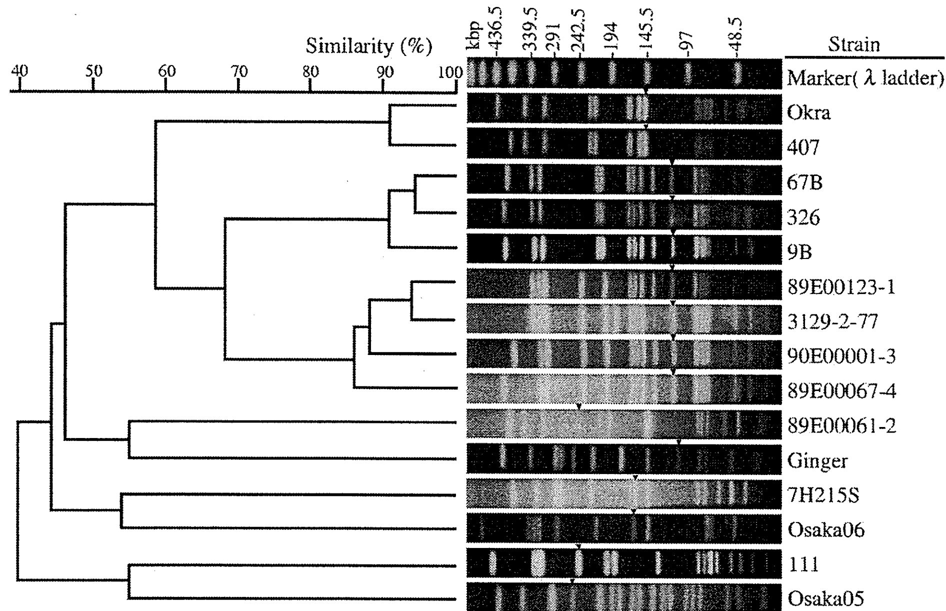

FIG. 3. PFGE genotyping. The dendrogram and PFGE patterns of SmaI-digested DNA from 15 *C. botulinum* type B strains are shown. Arrowheads indicate the DNA fragment containing the *boNT/B* gene detected by Southern blot hybridization.

PFGE and Southern blot hybridization. The PFGE patterns of SmaI-digested DNA from 15 *C. botulinum* type B strains and a dendrogram based on the similarities between normalized PFGE patterns are presented in Fig. 3. All strains were distinguishable, and their similarities ranged from 25.8% to 94.1%. The BoNT/B subtypes were clearly different on PFGE analysis. Strain Osaka05 exhibited a pattern entirely different from the patterns of the other 14 strains, having similarities of 28.6% to 45.7% with subtype B1 strains and similarities of 25.8% to 54.6% with subtype B2 strains. The similarities between subtype B1 and subtype B2 strains ranged from 29.4% to 60.0%. Homologous subtypes of subtype B1 strains showed similarities of 41.2% to 94.1%. Nine of the 10 subtype B1 strains (the exception was strain 89E00061-2) were separated into three clusters (Okra and 407; 326, 9B, and 67B; and 89E00067-4, 89E00123-1, 90E00001-3, and 3129-2-77) with more than 80% similarity. The four subtype B2 strains (strains 111, Osaka06, Ginger, and 7H215S) showed more diversity and had 35.7% to 53.9% similarities.

Fragments containing *boNT/B* genes were detected by Southern blot hybridization and are indicated by arrowheads in the gel in Fig. 3. An approximately 100-kbp fragment was detected in strain Ginger; a 110-kbp fragment was detected in strains 89E0067-4, 89E123-1, 90E00001-3, 3129-2-77, 326, 9B, and 67B; a 150-kbp fragment was detected in strains Okra and 407; a 170-kbp fragment was detected in strain 7H215S; a 175-kbp fragment was detected in strain Osaka06; a 260-kbp fragment was detected in strain 89E00061-2; a 275-kbp fragment was detected in strain 111; and a 280-kbp fragment was detected in strain Osaka05.

DNA fragments smaller than the chromosomal DNA (>970 kbp) were detected from strains 111, Osaka05, 89E00061-2, Okra, and 407 by PFGE of undigested DNA (Fig. 4A). The location of the *boNT/B* gene was confirmed by subsequent Southern blot hybridization with a *boNT/B* probe. The *boNT/B* genes of strains Okra and 407 were on the approximately 150-kbp fragment, the *boNT/B* gene of strain 89E00061-2 was located on the 260-kbp fragment, the *boNT/B* gene of strain 111 was located on the 275-kbp fragment, and the *boNT/B* gene of strain Osaka05 was located on the 280-kbp fragment (Fig. 4B), while the 16S rRNA genes were located on the chromosomal DNA (Fig. 4C). For the remaining 10 strains, the chromosomal DNA fragment was detected only by PFGE of

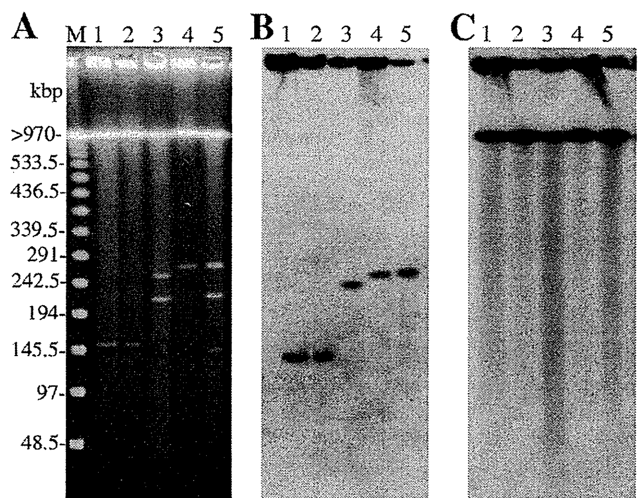

FIG. 4. PFGE of undigested DNA and Southern blot hybridization. The PFGE patterns of undigested DNA (A) and Southern blot hybridization detection of the *boNT/B* genes (B) and the 16S rRNA genes (C) of strains Okra (lane 1), 407 (lane 2), 89E00061-2 (lane 3), 111 (lane 4), and Osaka05 (lane 5) are shown. Lanes M, bacteriophage λ ladder.

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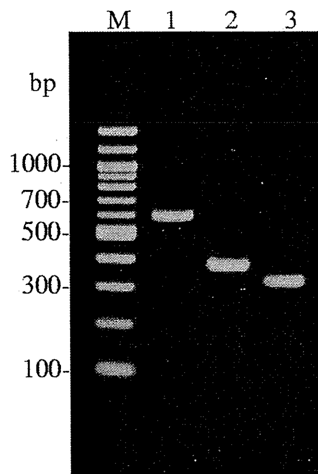

FIG. 5. Multiplex PCR assay for BoNT/B subtyping. The results of the multiplex PCR assay with strains Okra (lane 1), 111 (lane 2), and Osaka05 (lane 3) were visualized. Lanes M, 100-bp ladder.

undigested DNA, and both the *boNT/B* and the 16S rRNA genes were located on the chromosomal DNA.

PCR assays for identification of BoNT/B subtypes. A multiplex PCR assay for the detection of the *boNT/B1*, *boNT/B2*, and *boNT/Osaka05* genes was developed. The results of the PCR assay with strains Okra (subtype B1), 111 (subtype B2), and Osaka05 are shown in Fig. 5.

The *boNT/B1* amplicon (585 bp) from strain Okra was detected in lane 1, the *boNT/B2* amplicon (370 bp) from strain 111 was detected in lane 2, and the *boNT*/Osaka05 amplicon (317 bp) from strain Osaka05 was detected in lane 3. When the other bacterial strains listed in Table 1 were assayed, the *boNT/B1* amplicon was detected in nine strains (strains 89E00061-2, 89E00067-4, 89E00123-1, 90E00001-3, 3129-2-77, 326, 407, 9B, and 67B), and the *boNT/B2* amplicon was detected in three strains (strains Osaka06, Ginger, and 7H215S). The detection limit of the multiplex PCR assay was from 9.5×10 to 2.5×10^2 cells/ml of culture dilution (data not shown). No amplicons were detected from the 11 control strains, as follows: 2 type A strains (strains 62A and Kyoto-F), 1 type C strain (strain CB-19), 1 type D strain (strain 1873), 1 type E strain (strain Iwanai), 1 type F strain (strain Langeland), 1 BoNT/E-producing *C. butylicum* strain (strain 5262), 1 *C. sporogenes* strain (strain ATCC 19404), 1 *C. bifementas* strain (strain ATCC 638), 1 *C. perfringens* strain (strain ATCC 13124), and 1 *C. difficile* strain (strain ATCC 43593) (data not shown).

Comparison of antigenicities of BoNT/B H_C among strains Osaka05, Okra, and 111. In order to characterize BoNT/Osaka05 antigenically, the levels of binding of rH_C/Osaka05, rH_C/Okra, and rH_C/111 to their specific antibodies were measured by a sandwich ELISA (Fig. 6). When the levels of binding of rH_C to the antibody against rH_C/Osaka05 were assayed, rH_C/111 and rH_C/Okra showed low binding affinities, and the binding affinity of rH_C/Okra was lower than that of rH_C/111 (Fig. 6A). rH_C/Osaka05 and rH_C/111 did not react to the antibody against rH_C/Okra (Fig. 6B). rH_C/Osaka05 and rH_C/Okra exhibited a low binding affinity to the antibody against rH_C/111, and the binding affinity of rH_C/Okra was lower than that of rH_C/Osaka05 (Fig. 6C).

DISCUSSION

Two cases of infant botulism occurred in Osaka, Japan, in 2005 and 2006, and type B strains (strains Osaka05 and Osaka06) were successfully isolated from both cases. The full-length *boNT/B* gene sequences of the two isolates were determined and compared with the sequences of the *boNT/B* genes in the GenBank database. Strain Osaka05 possessed a unique *boNT/B* gene. The BoNT subtypes within a serotype were defined as differing by at least 2.6% at the amino acid level (37). Currently, it is usual to determine BoNT subtypes by phylogenetic analysis of full-length *boNT* gene sequences (6, 13). Phylogenetic analysis of the *boNT/B* genes indicated that strain Osaka05 should be classified into a group other than the five BoNT/B subtypes (13), and strain Osaka05 was shown to be a new BoNT/B subtype. On the other hand, the *boNT/B* gene sequence of strain Osaka06 was identical to that of strain CDC6291 and was classified in the B2 subtype (Fig. 2A).

The amino acid substitutions in the BoNT/B subtypes were concentrated in the heavy chain, especially in H_{CC}, the same as in Fig. 1. The sequences in the C-terminal region of BoNT/B, which encoded H_{CC}, were available for BoNT/B subtyping by phylogenetic analysis, as was the sequence of the full-length of *boNT/B* genes (Fig. 2B). We also established a multiplex PCR

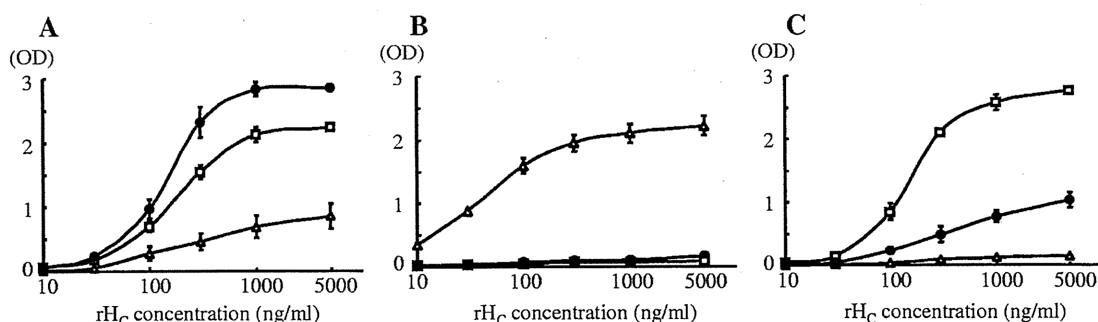

FIG. 6. Sandwich ELISA for determination of the binding affinity of rH_C to specific antibodies. The results are expressed as the means of the optical density (OD) at 415 nm after subtraction of the optical density for the background control in three experiments. Error bars indicate standard errors. Antibodies against rH_C/Osaka05 (A), rH_C/Okra (B), and rH_C/111 (C) were used as capture antibodies; and rH_C/Osaka05 (closed circles), rH_C/Okra (open triangles), and rH_C/111 (open squares) were applied as antigens at various concentrations.

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assay to detect BoNT/B subtypes B1, B2, and Osaka05 (Fig. 5). The PCR results were well correlated with the subtypes identified by phylogenetic analysis of *boNT/B* genes.

Genotyping by PFGE with SmaI-digested DNA revealed genetic diversity among subtypes B1, B2, and Osaka05 (Fig. 3). The diversity within subtype B2 strains was greater than that within B1 strains. The seven isolates from Japan (associated with infant botulism and food samples) were clearly distinct from each other, in contrast to the isolates associated with infant botulism in the United States, which showed high degrees of similarity.

The location of the *boNT* gene and its associated nontoxic component gene cluster varied among serotypes and strains; the *boNT/A*, *boNT/B*, *boNT/E*, and *boNT/F* genes were considered to be located on the chromosome, while the *boNT/C* and *boNT/D* genes were carried on bacteriophages, and the *boNT/G* gene was located on plasmids (4, 30, 41, 43). Recently, sequencing of the complete genome of strain Okra revealed that its *boNT/B* gene was present within the 149-kbp plasmid (36). The *boNT* genes of strain Loch Maree (subtype A3) and strain 657Ba (type B and subtype A4) were also found to be located on the approximately 270-kbp plasmid (25, 36). In this study, we found that the *boNT/B* genes were located on extrachromosomal DNA, assumed to be plasmids, in five strains belonging to distinct BoNT/B subtypes (Fig. 4). The plasmids were approximately 150 kbp, 260 kbp, 275 kbp, and 280 kbp. These findings indicate that the *boNT/B* gene location is not correlated with the BoNT/B subtype. Detailed characterization of the *boNT/B* gene-encoding plasmids is required to understand the mechanisms of *boNT/B* expression and evaluation of the *boNT/B* gene within *C. botulinum*.

The nontoxic component genes encode the proteins that protect the neurotoxin from the acids and proteases in the stomach and assist with transportation of the neurotoxin from the intestine to the bloodstream (30). This study and previous reports (23, 30, 36) indicated that the hemagglutinin genes (*ha70*, *ha33*, and *ha17*), the regulator gene (*botR*), and the nontoxic-nonhemagglutinin gene (*ntnh*) exist upstream of the *boNT/B* gene. The amino acid identities of HA33 were significantly lower than those of the other nontoxic components and BoNT/B (Table 3). It was suggested that HA33 acts as an adhesin, allowing the complex of the neurotoxin and nontoxic components to bind to intestinal epithelial cells and erythrocytes (11, 26); however, the influence of amino acid substitutions in HA33 on symptoms of infant botulism is unknown.

The variation in the BoNT amino acid sequence within serotypes is capable of causing significant differences in the immunological and biological properties of the neurotoxin. We previously indicated immunological differences between BoNT/B1 and BoNT/B2 (15, 22). Briefly, most monoclonal antibodies against the H_C of BoNT/Okra did not react with BoNT/111, while monoclonal antibodies against the light chain and the H_N of BoNT/Okra could react with BoNT/111 by immunoblotting and ELISA. In this study, the antigenicity of BoNT/Osaka05 was suggested to be different from the antigenicities of BoNT/Okra and BoNT/111 by sandwich ELISA with rH_C and their specific antibodies (Fig. 6). Strain Osaka05 was confirmed to be a new BoNT/B subtype by its antigenic specificity, in addition to by subtype classification by phylogenetic analysis of the *boNT/B* gene. The antigenic difference between BoNT/Osaka05 and BoNT/Okra was greater than that between BoNT/Osaka05 and BoNT/111, depending upon the difference in BoNT amino acid similarities. We also previously demonstrated that two different subtypes in BoNT/A (subtypes A1 and A2), which differ by 10% at the amino acid level, had different antigenicities by ELISA with monoclonal antibodies against BoNT/A (21). Similar findings were presented by Smith et al. (37). This information will be important for the development of an immunological BoNT assay, therapy for botulism, and recombinant vaccines for BoNT (34, 35).

In H_{CC}, the level of amino acid replacement between BoNT/Osaka05 and BoNT/Okra was 15 residues, that between BoNT/Osaka05 and BoNT/111 was 14 residues, and that between BoNT/Okra and BoNT/111 was 23 residues. Our previous studies revealed that the toxicity of BoNT/111 was lower than that of BoNT/Okra because of the replacement of 2 residues in H_{CC} which contribute to binding to the receptors (ganglioside GT1b and the synaptotagmin2-GT1b complex) (15, 20, 22). The other 8 residues essential for receptor binding in H_{CC} were also confirmed: 4 residues for binding to ganglioside GT1b and 4 residues for binding to the synaptotagmin2-GT1b complex (20, 32, 38). In BoNT/Osaka05 and BoNT/Okra, those 10 residues were identical. The antigenicity and genetic characteristics of BoNT/Osaka05 were related to those of BoNT/111 rather than to those of BoNT/Okra, but the residues contributing to receptor binding in BoNT/Osaka05 were identical to those in BoNT/Okra. Further investigation into the biological character of BoNT/Osaka05 would give new insight into the receptor binding system of BoNT/B.

In this study, we developed new methods for the subtyping of BoNT/B: phylogenetic analysis of partial *boNT/B* gene sequences and a multiplex PCR assay. The former was also suitable for the identification of a new BoNT/B subtype, and the latter represents the first report of a PCR-based method for the identification of BoNT/B subtypes. The correlation between the BoNT/B subtype and the source of isolation was reported by Hill et al. (13); B1 strains likely originated in the United States and are associated with food-borne disease due to improperly processed vegetables, while B2 strains exist mostly in Europe and are associated with animal cases or meat. The distribution of BoNT/B subtypes in Japan was found to be distinct from the distributions in both the United States and Europe; isolates associated with infant botulism were classified into subtype B2 and the newly identified Osaka05 subtype, and food samples were divided into subtypes B1 and B2. Therefore, further molecular genotyping of type B *C. botulinum* isolates by our BoNT/B subtyping methods will contribute to understanding of the epidemiology of *C. botulinum* and the infectious diseases that it causes.

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REFERENCES

1. **Abe, S., M. Nishino, O. Miyake, N. Hashimoto, T. Nakagawa, E. Satake, T. Asao, Y. Kumada, T. Kawai, and M. Takahashi.** 2006. An infant botulism case in which detected were Clostridium botulinum type B and its toxin, May 2006—Osaka. Infect. Agents Surveill. Rep. **27:**275–276. (In Japanese.)

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2. **Arndt, J. W., M. J. Jacobson, E. E. Abola, C. M. Forsyth, W. H. Tepp, J. D. Marks, E. A. Johnson, and R. C. Stevens.** 2006. A structural perspective of the sequence variability within botulinum neurotoxin subtypes A1-A4. J. Mol. Biol. **362:**733–742.
3. **Arnon, S. S., K. Damus, and J. Chin.** 1981. Infant botulism: epidemiology and relation to sudden infant death syndrome. Epidemiol. Rev. **3:**45–66.
4. **Binz, T., H. Kurazono, M. Wille, J. Frevert, K. Wernars, and H. Niemann.** 1990. The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins. J. Biol. Chem. **265:**9153–9158.
5. **Cai, S., B. R. Singh, and S. Sharma.** 2007. Botulism diagnostics: from clinical symptoms to in vitro assays. Crit. Rev. Microbiol. **33:**109–125.
6. **Chen, Y., H. Korkeala, J. Aarnikunnas, and M. Lindstrom.** 2007. Sequencing the botulinum neurotoxin gene and related genes in *Clostridium botulinum* type E strains reveals *orfx3* and a novel type E neurotoxin subtype. J. Bacteriol. **189:**8643–8650.
7. **Cox, N., and R. Hinkle.** 2002. Infant botulism. Am. Fam. Physician **65:**1388–1392.
8. **Efron, B., E. Halloran, and S. Holmes.** 1996. Bootstrap confidence levels for phylogenetic trees. Proc. Natl. Acad. Sci. USA **93:**13429–13434.
9. **Fox, C. K., C. A. Keet, and J. B. Strober.** 2005. Recent advances in infant botulism. Pediatr. Neurol. **32:**149–154.
10. **Franciosa, G., F. Floridi, A. Maugliani, and P. Aureli.** 2004. Differentiation of the gene clusters encoding botulinum neurotoxin type A complexes in *Clostridium botulinum* type A, Ab, and A(B) strains. Appl. Environ. Microbiol. **70:**7192–7199.
11. **Fujinaga, Y., K. Inoue, S. Watarai, Y. Sakaguchi, H. Arimitsu, J. C. Lee, Y. Jin, T. Matsumura, Y. Kabumoto, T. Watanabe, T. Ohyama, A. Nishikawa, and K. Oguma.** 2004. Molecular characterization of binding subcomponents of *Clostridium botulinum* type C progenitor toxin for intestinal epithelial cells and erythrocytes. Microbiology **150:**1529–1538.
12. **Hielm, S., J. Bjorkroth, E. Hyytia, and H. Korkeala.** 1998. Genomic analysis of *Clostridium botulinum* group II by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. **64:**703–708.
13. **Hill, K. K., T. J. Smith, C. H. Helma, L. O. Ticknor, B. T. Foley, R. T. Svensson, J. L. Brown, E. A. Johnson, L. A. Smith, R. T. Okinaka, P. J. Jackson, and J. D. Marks.** 2006. Genetic diversity among botulinum neurotoxin producing clostridial strains. J. Bacteriol. **189:**818–832.
14. **Hutson, R. A., M. D. Collins, A. K. East, and D. E. Thompson.** 1994. Nucleotide sequence of the gene coding for non-proteolytic Clostridium botulinum type B neurotoxin: comparison with other clostridial neurotoxins. Curr. Microbiol. **28:**101–110.
15. **Ihara, H., T. Kohda, F. Morimoto, K. Tsukamoto, T. Karasawa, S. Nakamura, M. Mukamoto, and S. Kozaki.** 2003. Sequence of the gene for *Clostridium botulinum* type B neurotoxin associated with infant botulism, expression of the C-terminal half of heavy chain and its binding activity. Biochim. Biophys. Acta **1625:**19–26.
16. **Infectious Disease Surveillance Center, National Institute of Infectious Diseases.** 2008. Botulism in Japan as of January 2008. Infect. Agents Surveill. Rep. **29:**35–36. (In Japanese.)
17. **Johnson, E. A., and M. Bradshaw.** 2001. *Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective. Toxicon **39:**1703–1722.
18. **Kakinuma, H., H. Maruyama, H. Takahashi, K. Yamakawa, and S. Nakamura.** 1996. The first case of type B infant botulism in Japan. Acta Paediatr. Jpn. **38:**541–543.
19. **Kirma, N., J. L. Ferreira, and B. R. Baumstark.** 2004. Characterization of six type A strains of Clostridium botulinum that contain type B toxin gene sequences. FEMS Microbiol. Lett. **231:**159–164.
20. **Kohda, T., H. Ihara, Y. Seto, H. Tsutsuki, M. Mukamoto, and S. Kozaki.** 2007. Differential contribution of the residues in C-terminal half of the heavy chain of botulinum neurotoxin type B to its binding to the ganglioside GT1b and the synaptotagmin 2/GT1b complex. Microb. Pathog. **42:**72–79.
21. **Kozaki, S., S. Nakaue, and Y. Kamata.** 1995. Immunological characterization of the neurotoxin produced by Clostridium botulinum type A associated with infant botulism in Japan. Microbiol. Immunol. **39:**767–774.
22. **Kozaki, S., Y. Kamata, T. Nishiki, H. Kakinuma, H. Maruyama, H. Takahashi, T. Karasawa, K. Yamakawa, and S. Nakamura.** 1998. Characterization of *Clostridium botulinum* type B neurotoxin associated with infant botulism in Japan. Infect. Immun. **66:**4811–4816.
23. **Lee, J. C., K. Yokota, H. Arimitsu, H. J. Hwang, Y. Sakaguchi, J. Cui, K. Takeshi, T. Watanabe, T. Ohyama, and K. Oguma.** 2005. Production of anti-neurotoxin antibody is enhanced by two subcomponents, HA1 and HA3b, of Clostridium botulinum type B 16S toxin-haemagglutinin. Microbiology **151:**3739–3747.
24. **Lindstrom, M., and H. Korkeala.** 2006. Laboratory diagnostics of botulism. Clin. Microbiol. Rev. **19:**298–314.
25. **Marshall, K. M., M. Bradshaw, S. Pellett, and E. A. Johnson.** 2007. Plasmid encoded neurotoxin genes in *Clostridium botulinum* serotype A subtypes. Biochem. Biophys. Res. Commun. **361:**49–54.
26. **Matsumura, T., Y. Jin, Y. Kabumoto, Y. Takegahara, K. Oguma, W. I. Lencer, and Y. Fujinaga.** 2008. The HA proteins of botulinum toxin disrupt intestinal epithelial intercellular junctions to increase toxin absorption. Cell. Microbiol. **10:**355–364.
27. **Midura, T. F., and S. S. Arnon.** 1976. Infant botulism. Identification of *Clostridium botulinum* and its toxins in faeces. Lancet **ii:**934–936.
28. **Nishida, H., M. Shiota, K. Nakagawa, T. Minamigata, M. Takuwa, T. Morishima, H. Kumakura, T. Yoshioka, A. Uematsu, N. Haneda, D. Hata, K. Umeda, J. Ogasawara, and M. Takahashi.** 2007. An infant botulism case due to Clostridium botulinum type B toxin, October 2005—Osaka City. Infect. Agents Surveill. Rep. **28:**168–169. (In Japanese.)
29. **Noda, H., K. Sugita, A. Koike, T. Nasu, M. Takahashi, T. Shimizu, K. Ooi, and G. Sakaguchi.** 1988. Infant botulism in Asia. Am. J. Dis. Child. **142:**125–126.
30. **Oguma, K., Y. Fujinaga, and K. Inoue.** 1995. Structure and function of *Clostridium botulinum* toxins. Microbiol. Immunol. **39:**161–168.
31. **Pickett, J., B. Berg, E. Chaplin, and M. A. Brunstetter-Shafer.** 1976. Syndrome of botulism in infancy: clinical and electrophysiologic study. N. Engl. J. Med. **295:**770–772.
32. **Rummel, A., S. Mahrhold, H. Bigalke, and T. Binz.** 2004. The HCC-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction. Mol. Microbiol. **51:**631–643.
33. **Santos-Buelga, J. A., M. D. Collins, and A. K. East.** 1998. Characterization of the genes encoding the botulinum neurotoxin complex in a strain of Clostridium botulinum producing type B and F neurotoxins. Curr. Microbiol. **37:**312–318.
34. **Shone, C., J. Ferreira, A. Boyer, N. Cirino, C. Egan, E. Evans, J. Kools, and S. Sharma.** 2006. The 5th International Conference on Basic and Therapeutic Aspects of Botulinum and Tetanus Neurotoxins. Workshop review: assays and detection. Neurotox. Res. **9:**205–216.
35. **Smith, L. A.** 1998. Development of recombinant vaccines for botulinum neurotoxin. Toxicon **36:**1539–1548.
36. **Smith, T. J., K. K. Hill, B. T. Foley, J. C. Detter, A. C. Munk, D. C. Bruce, N. A. Doggett, L. A. Smith, J. D. Marks, G. Xie, and T. S. Brettin.** 2007. Analysis of the neurotoxin complex genes in *Clostridium botulinum* A1—A4 and B1 strains: BoNT/A3, /Ba4 and /B1 clusters are located within plasmids. PLoS One **2:**e1271.
37. **Smith, T. J., J. Lou, I. N. Geren, C. M. Forsyth, R. Tsai, S. L. Laporte, W. H. Tepp, M. Bradshaw, E. A. Johnson, L. A. Smith, and J. D. Marks.** 2005. Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect. Immun. **73:**5450–5457.
38. **Swaminathan, S., and S. Eswaramoorthy.** 2000. Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. Nat. Struct. Biol. **7:**693–699.
39. **Szabo, E. A., J. M. Pemberton, and P. M. Desmarchelier.** 1993. Detection of the genes encoding botulinum neurotoxin types A to E by the polymerase chain reaction. Appl. Environ. Microbiol. **59:**3011–3020.
40. **Takeshi, K., Y. Fujinaga, K. Inoue, H. Nakajima, K. Oguma, T. Ueno, H. Sunagawa, and T. Ohyama.** 1996. Simple method for detection of Clostridium botulinum type A to F neurotoxin genes by polymerase chain reaction. Microbiol. Immunol. **40:**5–11.
41. **Whelan, S. M., M. J. Elmore, N. J. Bodsworth, J. K. Brehm, T. Atkinson, and N. P. Minton.** 1992. Molecular cloning of the *Clostridium botulinum* structural gene encoding the type B neurotoxin and determination of its entire nucleotide sequence. Appl. Environ. Microbiol. **58:**2345–2354.
42. **Yamakawa, K., T. Karasawa, H. Kakinuma, H. Maruyama, H. Takahashi, and S. Nakamura.** 1997. Emergence of *Clostridium botulinum* type B-like nontoxigenic organisms in a patient with type B infant botulism. J. Clin. Microbiol. **35:**2163–2164.
43. **Zhou, Y., H. Sugiyama, H. Nakano, and E. A. Johnson.** 1995. The genes for the *Clostridium botulinum* type G toxin complex are on a plasmid. Infect. Immun. **63:**2087–2091.

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NOTE

A novel multiplex PCR method for *Clostridium botulinum* neurotoxin type A gene cluster typing

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ABSTRACT

A rapid, simple and sensitive multiplex PCR method for *boNT/A* gene cluster typing was developed by combining the results of BoNT/A subtype (*boNT/A1* or */A2*) gene detection with *ha33* and/or *p47* gene detection. Ten isolates associated with infant botulism in Japan were examined and divided into *boNT/A* gene cluster types 2 and 3 by origin (honey feeding or not) and period (1986–1987 or 1999–2007). It is suggested that this multiplex PCR method will be be useful for epidemiological studies of botulism.

Key words *boNT/A* gene cluster typing, infant botulism, multiplex PCR, PFGE.

Clostridium botulinum is an anaerobic spore-forming bacterium producing BoNT, which is the cause of botulism in humans and animals (1) and is divided into seven serotypes (A to G) (2). Some strains harbor two different serotypes of *BoNT* genes in their genome (3). BoNT is encoded by an approximately 3.8 kb gene, which is preceded by several nontoxic component genes (4). BoNT together with the nontoxic component genes are defined as the *boNT* gene cluster (5). Recently, BoNT was subclassified by BoNT gene sequence analysis, BoNT/A being divided into four subtypes (A1, A2, A3 and A4) (3). There are two types of nontoxic components of gene organization (the HA and Orfx clusters), and *C. botulinum* type A strains were classified according to their harboring of these clusters (4–6). The HA cluster consists of *ha17*, *ha33*, *ha70*, *botR* and *ntnh* genes, and the Orfx cluster consists of *orfx3*, *orfx2*, *orfx1*, *botR*, *p47* (unknown function) and *ntnh* genes (6). Franciosa *et al.* have reported that type A strains possess *boNT/A1* and HA cluster genes to *boNT/A* gene cluster type 1; *boNT/A2* and Orfx cluster genes to *boNT/A* gene cluster type 2; and *boNT/A1* with unexpressed or expressed *boNT/B*, HA cluster and Orfx cluster genes to *boNT/A* gene cluster type 3 (5). *BoNT/A* gene cluster typing has recently been applied for molecular characterization of type A strains (5).

In Japan, 24 cases of infant botulism have been reported: 16 of type A, 3 of type B, 1 of type C and 1 of *C. butyricum* producing BoNT/E. The types of toxin in the other three cases were not described (7–9). During 1986 to 1989, nine cases occurred; all were type A and gave a history of feeding with honey before the onset of symptoms. Since 1990, seven cases of type A have occurred, but none had a history of feeding with honey and the origin was not identified in five cases.

In this study, we developed the multiplex PCR method to easily detect *boNT/A* gene cluster types. In order to better understand the background of infant botulism cases in Japan, we then genotyped *C. botulinum* type A isolates by *boNT/A* gene cluster and PFGE types.

Twenty-seven *C. botulinum* type A strains, including 10 isolates associated with infant botulism in Japan, were cultured in 10 ml cooked meat medium (Difco, Becton

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List of Abbreviations: boNT, botulinum neurotoxin; boNT/A, botulinum neurotoxin A; botR, botulinum neurotoxin regulatory; *C. Clostridium*; ha, hemagglutinin; ntnh, nontoxic-nonhemagglutinin; orf, open reading frame; *orfx* unknown function open reading frame gene; PCR-RFLP, PCR restriction fragment length polymorphism; PFGE, pulsed-field gel electrophoresis.

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Table 1. Summary of molecular typing of *C. botulinum* type A strains

Source and strain	Description† (Reference)	*boNT* gene‡	Multiplex PCR *boNT/ A1, A2*	Multiplex PCR *ha33, p47*	*boNT/A* gene cluster type§	PFGE type
Infant botulism in Japan						
Chiba H	Chiba, 1986, honey feeding (7, 8)	A	*A2*	*p47*	2	S1
Kyoto F	Kyoto, 1987, honey feeding (8)	A	*A2*	*p47*	2	S2
KZ1828	Ishikawa, 1987, honey feeding (8)	A	*A2*	*p47*	2	S1
7I03 H	Osaka, 1987, honey feeding (8)	A	*A2*	*p47*	2	S2
7I05 F	Ehime, 1987, honey feeding (8)	A	*A2*	*p47*	2	S3
7I05 H	Ehime, 1987, honey feeding (8)	A	*A2*	*p47*	2	S4
Y8036	Kanagawa, 1987, honey feeding (8)	A	*A2*	*p47*	2	S5
Hiroshima1	Hiroshima, 1999, unidentified (8, 16)	A, B	*A1*	*ha33, p47*	3	S6
Miyagi2006	Miyagi, 2006, well water (9)	A, B	*A1*	*ha33, p47*	3	S6
Iwate2007	Iwate, 2007, unidentified (9)	A, B	*A1*	*ha33, p47*	3	S7
Food-borne botulism in Japan						
Renkon	Kumamoto, 1984, karashi renkon (8)	A, B	*A1*	*ha33, p47*	3	S8
CB111	Tokyo, 1999, unidentified	A	*A1*	*ha33*	1	S9
CB121	Chiba, 1999, vacuum-packed hashed beef (17)	A, B	*A1*	*ha33, p47*	3	S6
Osaka99	Osaka, 1999, unidentified	A, B	*A1*	*ha33, p47*	3	S10
Infant botulism in the USA						
89E00033-1	California, 1989	A	*A1*	*ha33*	1	S11
89E00035-1	California, 1989	A	*A1*	*ha33*	1	S12
89E00064-3	California, 1989	A, B	*A1*	*ha33, p47*	3	S13
89E00086-1	California, 1989	A, B	*A1*	*ha33, p47*	3	S14
83E00080	California, 1990	A	*A1*	*ha33*	1	S15
2137-1-77	California, 1990	A	*A1*	*ha33*	1	S16
Others						
802-1	Germany, 1988, red pepper	A	*A1*	*ha33*	1	S17
804-1H	Brazil, 1988, honey	A	*A2*	*p47*	2	S18
Denken	Stocked strain	A	*A1*	*ha33*	1	S19
97A	Stocked strain	A	*A1*	*ha33*	1	S20
62A	Stocked strain	A	*A1*	*ha33*	1	S21
33A	Stocked strain	A	*A1*	*ha33*	1	S17
36A	Stocked strain	A	*A1*	*ha33*	1	S22

†, Location, year, cause of botulism or origin; ‡ , detected by PCR assay for *boNT/A* to *boNT/G* genes (11); §, determined by multiplex PCR assay.

Dickinson, Franklin Lakes, NJ, USA) supplemented with 0.3% glucose and 0.2% soluble starch under anaerobic conditions for 18 hr at 30°C (Table 1). Bacterial DNA was extracted according to our previous report (10). Multiplex PCR assay was performed using two sets of primers: "*boNT/A1, A2*" contained three primers to identify *boNT/A1* or *boNT/A2* genes; and "*ha33, p47*" contained four primers to detect the *ha33* gene, which is specific for the HA cluster, and the *p47* gene, which is specific for the Orfx cluster (Table 2). PCR was performed with

Table 2. Primers for multiplex PCR assays for classification of the *boNT/A* gene cluster type

Primer set	Primer	Sequence (5′-3′)	Product size (bp)	Location on gene (coding region)
boNT/A1, A2	A1-forward	GACTTTACAGGATACTCAGGAAATA	665	2955–2979
	A2-forward	TAGAGATCCACGTAGATACATCAT	440	3180–3203
	A-reverse	TTAGTATTTTTTCTACGCCTGC		3619–3598
ha33, p47	ha33-forward	TGGTAACAATTCATTTATTATTGC	534	303–326
	ha33-reverse	TTAAATACTTGAATAGCAGTTCCGT		836–812
	p47-forward	ACTTATGGTTGGGATATTGTTTA	344	7–29
	p47-reverse	TCATCATTAGACTCAGATCCAA		350–329

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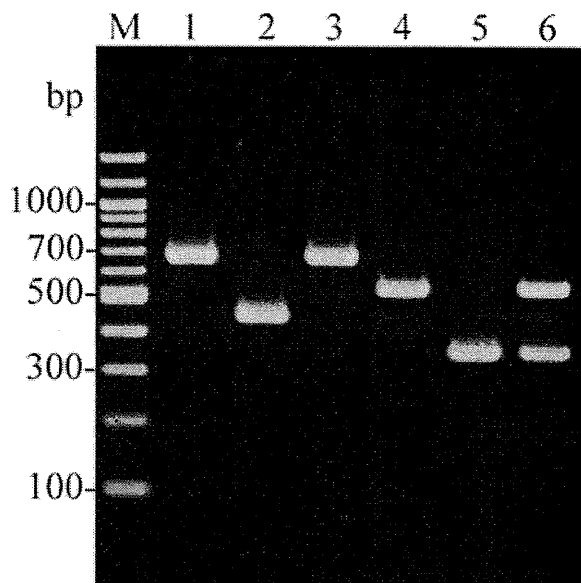

Fig. 1. Multiplex PCR assay for *boNT/A* gene cluster typing. The results of PCR with primer sets "*boNT/A1, A2*" (lanes 1 to 3) and "*ha33, p47*" (lanes 4 to 6) using strain 62A (lanes 1 and 4), Kyoto F (lanes 2 and 5) and Renkon (lane 3 and 6) were visualized on 3% agarose gels stained with ethidium bromide. Lane M: 100-bp ladder.

a 25 μl reaction mixture containing 0.1–1 ng template DNA, 0.25 μM of each primer, 1.25 U Ex *Taq* (TaKaRa Shuzo, Kyoto, Japan), 2.5 μl Ex *Taq* buffer and 200 μM deoxynucleotide-triphosphate. Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and was repeated 30 times. Unexpressed *boNT/B* gene was detected by another PCR assay for *boNT/A* to *boNT/G* genes (11). *Sma* I digested PFGE was carried out as described in our previous report (10). Dendrogram analysis of the band patterns was generated with FPQuest software ver.4.5 (Bio-Rad, Hercules, CA, USA). Another PFGE type was defined where there was more than one fragment difference in the PFGE band pattern.

The results of multiplex PCRs of 27 type A strains are summarized in Table 1 and representative results are depicted in Figure 1. The *boNT/A1* (665 bp) and *ha33* amplicons (534 bp) were detected in 11 strains (CB111, 89E00033-1, 89E00035-1, 83E00080, 2137-1-77, 802-1, Denken, 97A, 62A, 33A and 36A), which were accordingly classified as *boNT/A* gene cluster type 1. The *boNT/A2* (440 bp) and *p47* amplicons (344 bp) were detected in eight strains (Chiba H, Kyoto F, KZ1828, 7I03 H, 7I05 F, 7I05 H, Y 8036 and 804-1H), which were accordingly classified as *boNT/A* gene cluster type 2. The *boNT/A1*, *ha33* and *p47* amplicons were detected in eight strains (Hiroshima1, Miyagi2006, Iwate2007, Renkon, CB121, Osaka99, 89E00064-3 and 89E00086-1). These also harbored the unexpressed *boNT/B* gene (Table 1), and were therefore classified into *boNT/A* gene cluster type 3. No amplicon was detected in the 12 control strains, which were as follows: three type B (Okra, 111 and Osaka05), one type C (CB-19), one type D (1873), one type E (Iwanai), one type F (Langeland), one BoNT/E producing *C. butyricum* (5262), one *C. sporogenes* (ATCC19404), one *C. bifementas* (ATCC638), one *C. perfringens* (ATCC13124) and one *C. difficile* (ATCC43593) by "*boNT/A1, A2*" PCR. The *ha33* amplicon was detected in the three type B strains and the *p47* amplicon in the type F strain by "*ha33, p47*" PCR (data not shown).

The PFGE patterns of *Sma* I digested DNA from 27 type A strains and a dendrogram based on the similarities between normalized PFGE patterns are presented in Figure 2, and PFGE types are listed in Table 1. The 27 strains were divided into 22 PFGE types (S1-S22) and their similarity ranged from 29.6% to 100%. The seven isolates associated with infant botulism in Japan during 1986–1987 were divided into five PFGE types with 78.6–100% similarity, and 81.5% to 89.7% similarity to strain 804-1H, isolated from Brazil honey. The three isolates associated with infant botulism in Japan during 1999–2007 were divided into two PFGE types with 62.1% similarity. Strains Hiroshima1 and Miyagi2006 showed identical PFGE types to strain CB121, which was associated with food-borne botulism in Japan in 1999.

The new multiplex PCR method for *boNT/A* gene cluster typing established in this study is able to classify reference strains (62A and Kyoto-F) into their previously described cluster types (1 and 2, respectively) (5). The detection limit of "*boNT/A1, A2*" PCR was from 5.5 × 10 to 2.8 × 10^2 cells/ml, and of "*ha33, p47*" PCR from 2.1 × 10^2 to 2.1 × 10^3 cells/ml of culture dilutions (data not shown). The PCR-based *boNT/A* gene cluster typing method reported is a combination of BoNT/A subtyping by PCR-RFLP and *ha33* and *p47*gene detection by separate PCR (5); however, our PCR method has the advantages of simplicity, rapidity, specificity and sensitivity, so it would be applicable not only for molecular typing, but also the diagnosis of botulism.

A correlation between *boNT/A* gene cluster types and geographical distribution has been reported (5). Cluster type 1 and 3 strains are predominant in the USA, while cluster type 2 strains are predominant in Europe. Isolates associated with infant botulism in Japan were clearly divided into cluster type 2 and 3 by their time periods, and shown to be related to isolates from honey of South American origin and food-borne botulism, respectively. This is the first report of the genetic relationship between isolates associated with infant botulism and food-borne botulism in Japan. *C. botulinum* type A is rarely found in Japanese

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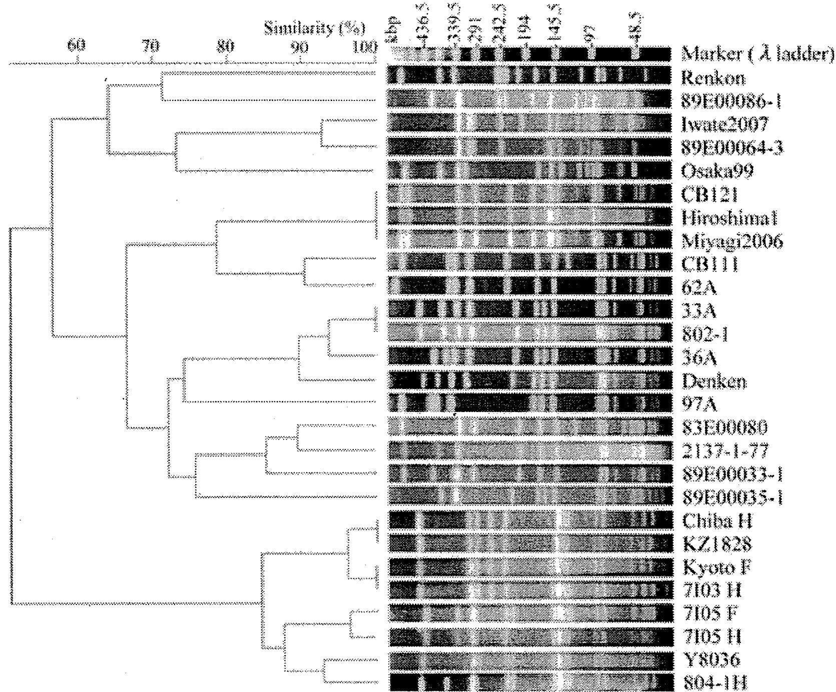

Fig. 2. PFGE genotyping. The dendrogram and PFGE patterns of *Sma* I digested DNA from 27 *C. botulinum* type A strains are shown. Similarity analysis was performed using the Dice coefficient, and clustering was examined by the unweighted pair group method with arithmetic averages.

soil, while type C and E are widely distributed (12). There is a possibility that imported goods are related to botulism cases. In other countries, in addition to honey, powdered infant formula (13), baby food (14) and house dust (15) have been reported as causes of infant botulism. Further risk assessment of several food and environmental samples to prevent infant botulism are warranted.

While *boNT/A* gene cluster typing is less discriminating than PFGE genotyping, it is excellent for genetic comparison among different laboratories or countries. The application of multiplex PCR assays will contribute to understanding the local and geographic epidemiology of *C. botulinum.*

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REFERENCES

1. Cai S., Singh B.R., Sharma S. (2007) Botulism diagnostics: from clinical symptoms to *in vitro* assays. *Crit Rev Microbiol* **33:** 109–25.
2. Lindstrom M., Korkeala H. (2006) Laboratory diagnostics of botulism. *Clin Microbiol Rev* **19:** 298–314.
3. Hill K.K., Smith T.J., Helma C.H., Ticknor L.O., Foley B.T., Svensson R.T., Brown J.L., Johnson E.A., Smith L.A., Okinaka R.T., Jackson P.J., Marks J.D. (2007) Genetic diversity among botulinum neurotoxin producing Clostridial strains. *J Bacteriol* **189:** 818–32.
4. Oguma K., Fujinaga Y., Inoue K. (1995) Structure and function of *Clostridium botulinum* toxins. *Microbiol Immunol* **39:** 161–8.
5. Franciosa G., Floridi F., Maugliani A., Aureli P. (2004) Differentiation of the gene clusters encoding botulinum neurotoxin type A complexes in *Clostridium botulinum* type A, Ab, and A(B) strains. *Appl Environ Microbiol* **70:** 7192–9.
6. Jacobson M.J., Lin G., Raphael B., Andreadis J., Johnson E.A. (2008) Analysis of neurotoxin cluster genes in *Clostridium botulinum* strains producing botulinum neurotoxin serotype A subtypes. *Appl Environ Microbiol* **74:** 2778–86.
7. Noda H., Sugita K., Koike A., Nasu T., Takahashi M., Shimizu T., Ooi K., Sakaguchi G. (1988) Infant botulism in Asia. *Am J Dis Child* **142:** 125–6.
8. Infectious Disease Surveillance Center, National Institute of Infectious Diseases. (2000) Botulism, Japan. *Infectious Agents Surveillance Report (IASR)* **21:** 49–50. (in Japanese).
9. Infectious Disease Surveillance Center, National Institute of Infectious Diseases. (2008) Botulism in Japan as of January 2008. *Infectious Agents Surveillance Report (IASR)* **29:** 35–36. (in Japanese).

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10. Umeda K., Seto Y., Kohda T., Mukamoto M., Kozaki S. (2009) Genetic characterization of *Clostridium botulinum* associated with type B infant botulism in Japan. *J Clin Microbiol* **47:** 2720–8.
11. Takeshi K., Fujinaga Y., Inoue K., Nakajima H., Oguma K., Ueno T., Sunagawa H., Ohyama T. (1996) Simple method for detection of *Clostridium botulinum* type A to F neurotoxin genes by polymerase chain reaction. *Microbiol Immunol* **40:** 5–11.
12. Yamakawa K., Kamiya S., Nishida S., Yoshimura K., Yu H., Lu D.Y., Nakamura S. (1988) Distribution or *Clostridium botulinum* in Japan and in Shinkiang district of China. *Microbiol Immunol* **32:** 579–87.
13. Brett M.M., McLauchlin J., Harris A., O'Brien S., Black N., Forsyth R.J., Roberts D., Bolton F.J. (2005) A case of infant botulism with a possible link to infant formula milk powder: evidence for the presence of more than one strain of *Clostridium botulinum* in clinical specimens and food. *J Med Microbiol* **54:** 769–76.
14. Paerregaard A., Angen O., Lisby M., Molbak K., Clausen M.E., Christensen J.J. (2008) Denmark: botulism in an infant or infant botulism? *Euro Surveill* **13:** pii:19072.
15. Nevas M., Lindstrom M., Virtanen A., Hielm S., Kuusi M., Arnon S.S., Vuori E., Korkeala H. (2005) Infant botulism acquired from household dust presenting as sudden infant death syndrome. *J Clin Microbiol* **43:** 511–3.
16. Takeda Y., Toukubo Y., Inoue K., Ogawa H., Okada S., Sato T. (2000) Infant botulism by *Clostridium botulinum* possessing the type A and B botulism neurotoxin genes in Hiroshima prefecture. *Jpn J Food Microbiol* **17:** 143–7. (in Japanese).
17. Kobayashi H., Fujisawa K., Saito Y., Kamijo M., Oshima S., Kubo M., Eto Y., Monma C., Kitamura M. (2003) A botulism case of a 12-year-old girl caused by intestinal colonization of *Clostridium botulinum* type Ab. *Jpn J Infect Dis* **56:** 73–4.

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日集中医誌 2010;17:253～255.

クロストリジウム属菌感染症と抗毒素療法

はじめに

クロストリジウム属菌による代表的な創傷感染症に，*Clostridium tetani*（*C. tetani*）による破傷風がある。破傷風菌（芽胞）は世界中の土壌に存在し，創傷局所に混入した菌が嫌気環境下で発育・増殖する際に産生した毒素により，破傷風を発症する。同様に，*Clostridium perfringens*（*C. perfringens*）をはじめとして，*C. septicum*, *C. oedematiens*, *C. histolyticum* といったガス壊疽の起因菌も広く土壌に存在する。ガス壊疽では，創傷部位に混入したこれらの菌の増殖に伴い，急速，広範囲な筋肉および皮下組織の壊疽を呈する。

上述した毒素産生細菌感染症の治療には，第一次世界大戦以降からウマ抗毒素血清製剤（ウマ免疫グロブリン）が開発され，現在も使用されている[1,2]。破傷風の治療にあっては，破傷風トキソイドの普及により，1970年代からヒト血液由来の免疫グロブリン製剤が開発・市販された。異種タンパクであるウマ抗毒素製剤よりも，治療だけでなく予防的利用が一般化している。

一方，ガス壊疽治療におけるウマ抗毒素製剤は，年間の使用数が限られるため製造所の採算性に乏しく，製造にあたっては馬の管理などの特別な技術や経験が必要であり，国が備蓄供給を管理する国有品となっている。現在は*C. perfringens*, *C. septicum*, *C. oedematiens*の各菌を別々に培養して得た毒素および，精製後にホルマリンで無毒化したトキソイドを用いて高度免疫したウマ血漿を原料として精製した「乾燥ガスえそウマ抗毒素」（乾燥ガスえそ抗毒素）が，（財）化学及血清療法研究所（熊本）で製造されている。本製剤の製造および品質管理方法は，生物学的製剤基準に従って安全性および有効性試験を実施し，さらに国家検定として国による品質確認が行われている[3]。この基準では，各毒素に対する抗毒素価は，1バイアルの製剤中に5,000単位以上を含有することが求められる。添付文書による用法および用量では，添付20 m*l*の溶解液で溶解した後に，「治療にはなるべく早期に10,000～20,000単位（20～40 m*l*）を筋注あるいは静注または生理食塩水で稀釈したものを点滴静注する。また，予防的には5,000～10,000単位（10～20 m*l*）を静脈内あるいは筋肉内に注射する」と示している。

ガス壊疽抗毒素製剤の使用実態調査を，平成14年度の厚生省科学研究費補助金事業の研究班で，過去に本製剤の分与申請を行った医療機関25施設に対し実施した[4]。その結果，わが国のガス壊疽感染の実態は変化しており，糖尿病や慢性肝疾患の増加に伴い，非クロストリジウム属菌によるガス壊疽患者の報告が多くなっていた。そして，このような患者に対しても適応外でガス壊疽ウマ抗毒素製剤が使用されていた。

他方，本報告[5]にもあるように，非外傷性の*C. perfringens*感染症による死亡報告例もあり，感染後の病態に菌が産生した毒素の関与が疑われている。本疾患の治療法も，創傷性ガス壊疽と同様に外科的処置，抗菌薬療法および集中治療が求められている。

医師が*C. perfringens*によるガス壊疽と診断（疑いを含み）し，抗毒素製剤の投与が必要と判断して国家備蓄の抗毒素製剤を請求した場合は，行政側ではガス壊疽の定義に合致すれば申請は受理するのが一般的である。*C. perfringens*による感染が疑われるのであれば，抗毒素製剤投与による早期治療も選択肢の一つであり，抗毒素製剤の申請にあっては，病院検査室などによる当該菌の分離・同定は必須ではない。創傷性ガス壊疽においても抗毒素製剤の有効性の判断は，ガス壊疽の臨床症例が少ないため，各患者の基礎疾患，感染部位および病巣の広がりなどの違いによる比較は困難である。高齢化に伴う非外傷性ガス壊疽に対しては，ウマ抗毒素製剤の迅速な適応による延命効果，治癒機転などの治療効果を見極めることが求められる。国家備蓄品の有効利用による患者救済が，本製剤の目的でもある。

国有ワクチン（抗毒素製剤を含む）の備蓄供給体制

国有ワクチン（抗毒素製剤を含む）の備蓄供給体制とは，国が買い上げることにより，その生産と供給を確保する体制である。患者数が少なくその需要が少ないことから，単独企業では採算がとれず，市場に任せていてはその供給が確保されにくいワクチンおよび抗

Table 1 国有ワクチン・抗毒素製剤の保管連絡先

保管場所	住 所	電話	電話 夜間・休日(緊急時)
(株)ほくやく	〒063-0830 北海道札幌市西区発寒10条3-1-1 札幌西業務センター	011-665-0989	011-665-0989
(株)バイタルネット	〒981-1298 宮城県名取市下余田字鹿島10	022-384-1119	080-3192-5235 080-3146-9870 090-2882-3054
デンカ生研(株)	〒959-1834 新潟県五泉市木越字鏡田1359-1 物流センター	0250-42-0712	0250-43-4111 0250-58-5574 0250-42-6898
(学)北里研究所	〒364-0026 埼玉県北本市荒井6-111	048-593-3939	048-543-7808 048-728-8016
(財)阪大微生物病研究会(大阪)	〒565-0871 大阪府吹田市山田丘3-1	06-6877-4804	06-6850-0715 06-6831-1860
(財)阪大微生物病研究会(観音寺)	〒768-0061 香川県観音寺市八幡町2-9-41	0875-25-4171	0877-21-1257 0875-23-2445 0875-27-7892
武田薬品工業(株)	〒743-8502 山口県光市大字光井字武田4720	0833-71-5511	0833-71-5546
(財)化学及血清療法研究所	〒860-8568 熊本県熊本市大窪1-6-1	096-345-6500	096-344-1211
(株)琉薬	〒901-2686 沖縄県浦添市牧港5-6-5	098-878-3111 内線(1194) 098-878-3314直通	098-878-3358 098-863-3730

厚生労働省連絡先 医薬食品局血液対策課 直通：03-3595-2395 FAX：03-3507-9064，厚生労働省代表番号：03-5253-1111

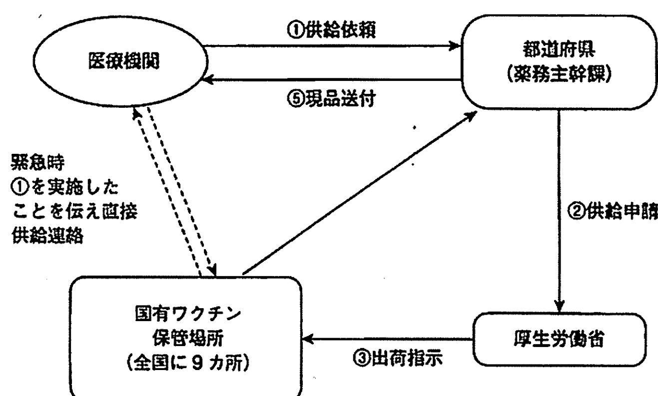

Fig. 1 国有ワクチン・抗毒素製剤の供給体制

毒素製剤が対象となる。それぞれ，コレラワクチン，乾燥組織培養不活化狂犬病ワクチンの2種類，およびガスえそウマ抗毒素，乾燥ジフテリアウマ抗毒素，乾燥ボツリヌスウマ抗毒素(ABEF型およびE型)の4種類の製剤がある。これらは，国が計画的に買い上げて温度管理システムが整っている全国9カ所で保管し，緊急時に速やかに供給されている(Table 1)。なお，過去に患者が発生した都道府県においては，国有ワクチンを国から直接買い上げ，独自に県内に保管し供給体制を構築している場合もある[6),7)]。

標準的な供給方法は，抗毒素製剤を必要とする医師や医療機関が都道府県の薬務所管課に供給申請を行い，薬務主幹課が厚生労働省に供給申請を行う。厚生労働省は，供給要請を受けて，抗毒素製剤の保管先に出荷指示を行い，医療機関に供給が行われる(Fig. 1)。

なお，緊急時にはTable 1に示す保管場所に直接電

話をして，医療機関，必要本数および申請窓口担当者などを連絡することにより，最小限の事務手続きで速やかに適切な対応がとられ，後日の申請処理も可能となっている。

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Antitoxin therapy for Clostridial infection

Key words: ①antitoxin, ②treatment, ③*Clostridium perfringens*

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文　献

1) Robertson M, Felix A. Serological studies in the group of the spore-bearing anaerobes. II. *IN VIVO* experiments with an "O" immune serum to *Vibrion septique* devoid of antitoxin content. Brit J Exp Path 1930;11:14-23.
2) Henderson DW. Studies on *clostridium chauvei*. Brit J Exp Path 1932;13:412-427.
3) (乾燥)ガスえそ抗毒素．細菌製剤協会等監修．生物関連製剤ハンドブック2004. 東京：じほう；2004. p. 24-7.
4) 高橋元秀，杉本　央．わが国におけるガス壊疽抗毒素の使用情況に関するアンケート調査．平成14年度厚生労働科学研究費補助金 安定供給に向けた国内外の抗毒素製剤の品質管理に関する研究 分担研究報告書．2002.
5) 一二三亨，高橋元秀，諸熊一則，他．*Clostridium perfringens*感染患者に対する治療用ウマ抗毒素製剤の存在を知っていますか？ 日集中医誌 2010;17:287-9.
6) 福岡県．国有ワクチンの手引き．国有ワクチンの概要．Available from: http://www.pref.fukuoka.lg.jp/b02/kokuyu.html
7) 北海道保健福祉部．道有医薬品及び国有ワクチン・抗毒素の供給マニュアル(平成20年4月改訂版)．Available from: http://www.pref.hokkaido.lg.jp/NR/rdonlyres/C421EB51-43CC-4D17-8D9A-06B80E5D719D/0/douyuuwakutinn-manyuaru.pdf#search＝'国有ワクチン'

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*Clostridium perfringens*感染患者に対する治療用ウマ抗毒素製剤の存在を知っていますか？

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要約：非外傷性の*Clostridium perfringens*（*C. perfringens*）感染症により血管内溶血や代謝性アシドーシスが急速に進行し，短時間の経過で死亡した劇症型の報告例がここ数年散見される。これは，*C. perfringens*のα毒素により血管内溶血が進行し，貧血，腎不全から播種性血管内凝固症候群，多臓器不全へと急激に進行し，死に至ると考えられている。治療法は，必要であれば外科的処置がまず考慮され，それと並行してペニシリン系抗菌薬の大量投与や高圧酸素療法のほか，持続血液濾過透析，エンドトキシン吸着，そして血漿交換などの血液浄化法も，可能性のある治療法として挙げられているが，未だに治療法は確立されていない。*C. perfringens*のα毒素に対する抗毒素製剤の使用は古い歴史をもつ治療法であるが，臨床医には馴染みが薄く，学会発表や論文で治療法として取り上げられていないことが多いため，今回，啓蒙的意義も踏まえて紹介する。

Key words: ①*Clostridium perfringens*, ②antitoxins, ③gas gangrene antitoxin

Ⅰ．はじめに

非外傷性の*Clostridium perfringens*（*C. perfringens*）感染症による死亡報告例が，ここ数年散見される[1),2)]。しかも，そのほとんどが血管内溶血や代謝性アシドーシスが急速に進行し，短時間の経過で死亡した劇症型である。これは，*C. perfringens*のα毒素により血管内溶血が進行し，貧血，腎不全から播種性血管内凝固症候群，多臓器不全へと急激に進行し，いわゆるガス壊疽症状を呈して死に至ると考えられている[3)]。

治療法は，必要であれば外科的処置がまず考慮され，それと並行して早期に集中治療を展開しなければならない。ペニシリン系抗菌薬の大量投与や高圧酸素療法のほか，持続血液濾過透析，エンドトキシン吸着，そして血漿交換などの血液浄化法も，可能性のある治療法として挙げられているが[2),4)]，未だに治療法は確立されていない。

*C. perfringens*のα毒素に対する抗毒素製剤の使用は古い歴史をもつ治療法であるが[5)]，臨床医には馴染みが薄く，学会発表や論文で治療法として取り上げられていないことが多いため，今回，啓蒙的意義も踏まえて紹介する。

> この論文は今号のハイライトで取り上げています。
> 髙橋　元秀．クロストリジウム属菌感染症と抗毒素療法．日集中医誌 2010; 17: 253-255.

Ⅱ．毒素治療の基本 ～抗毒素～

毒素性疾患は，ボツリヌス中毒のようにボツリヌス毒素そのものを含んだ食品を食したり，毒蛇咬傷のように毒牙を通して体内に毒素が注入されたことで，一度に大量の毒素が生体内に入って発病するタイプと，*C. perfringens*感染症などのように病原体が体内に侵入してその部位で菌が増殖し，感染局所で産生された毒素によって初めて発病するタイプの2種類があ

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る[6]。後者の場合は，菌の増殖を抑えない限り引き続いて毒素が産生され，病状が進展しまうことになる。産生された毒素が大きな役割をもつという点で，毒素性疾患は他の感染症とは大きく異なり[6]，我々臨床医は毒素の働きをとにかく止めることに着目しなければならず，できるだけ早期に抗菌薬とともに抗毒素を投与して，毒素を中和しなければならない。

抗毒素とは，細菌の外毒素，植物毒素，動物毒素などの生物から発生した抗原性毒物質に反応して生成される抗体と定義されている[7]。

ここで注意しなければならないのは，抗毒素は毒素を中和して完全に毒作用を抑えることができるが，殺菌作用はなく，菌の増殖を抑える効果はない。また，一度毒素によって傷害された臓器の機能を回復させるものでもない。つまり，*C. perfringens*感染患者に対しては，外科的処置と並行して，できるだけ早期に抗毒素を投与して毒素を中和するとともに，十分な量の抗菌薬を投与し，引き続いて集中治療を行うことが臨床医には要求される。

Ⅲ．*C. perfringens*の産生毒素に対する抗毒素療法

*C. perfringens*に対する抗毒素製剤として現在の日本にあるのは，ガス壊疽患者の緊急治療用として保管されている国家備蓄品（国有品）のみであり，国内市場での流通はない。使用に際しては，厚生労働省の医薬食品局血液対策課または医療機関の所属する都道府県に供給を依頼しなければならない。しかし，国家備蓄品（国有品）の使用に際して制限があるわけでなく，臨床診断に基づいて患者への抗毒素製剤投与が必要であると判断した場合は，通常の保険診療の範囲内で処理される。

国家備蓄品の抗毒素製剤は，1バイアル中に*C. perfringens* Type A, *C. septicum*, *C. oedematiens*の各抗毒素5,000単位を含有した3種混合の凍結乾燥製剤である。免疫用抗原として各毒素をホルマリンで無毒化したトキソイドでウマを免疫し，そのウマから採血・分離した血漿を精製した免疫グロブリン製剤である。

歴史的には，1957年（昭和32年）から日本での製造が開始され，現在は，千葉県血清研究所から継承し，化学及血清療法研究所（化血研）で生産されている。千葉血清研究所では10 ml入りの液状品製剤（有効期間3年）を年間約2,000本製造していたが，現在化血研では，乾燥品製剤（有効期間10年）をほぼ2年間隔で約500本製造している。

用法としては，10,000～20,000単位を数回に分けて静脈内注射するか，生理食塩水などで希釈して点滴静注する[8]。適用は，起因菌が前述した3種の抗毒素の菌と分かっている場合，あるいは起因菌が3種の菌である可能性が高く中毒症状を示している場合である。

2000年から2001年にかけて，実際に抗毒素製剤を患者に投与した医療機関25施設に対して行ったアンケート調査では，13施設より回答があり，抗毒素製剤を実際に投与して「効果あり」と回答した施設が8施設，「効果なし」が2施設，「不明」が3施設であった[9]。しかし，抗毒素製剤を投与した23例中，*Clostridium*属菌は6例に過ぎず，また，血管内溶血や代謝性アシドーシスが急速に進行して死に至る劇症型での抗毒素製剤の使用報告例はなかったことから，その有効性を証明するためにも，今後の症例の蓄積と検討が必要である。

ただし強調するが，抗毒素製剤を投与すれば必ず救命できるという訳ではなく，投与の時期が極めて重要であり，毒素が標的となる組織に結合した後に投与しても効果は期待できない[6]。

また，ウマに免疫して作られるウマ抗毒素製剤は，主な副作用としてアナフィラキシーショックと血清病が挙げられる。アナフィラキシーショックの頻度は1,000人に1人あるいはそれ以下であるが，直ちに治療をしないと死に至る可能性もあるため，投与に際しては酸素，輸液，エピネフリン，気道確保セットを準備しておく必要がある[5]。血清病は，投与数日後に発熱，蕁麻疹様湿疹，顔面浮腫，関節痛，蛋白尿をきたす疾患であるが，多くの場合，予後は良好であり，治療としては抗ヒスタミン薬，ステロイド投与が挙げられる[5]。

これらの問題を解決するため，現在，ウマ抗毒素製剤に代わって，ボツリヌス抗毒素製剤，ジフテリア抗毒素製剤によるヒト型の抗体の製剤化に向けた研究が行われているが[10]，*C. perfringens*に対するヒト型抗毒素製剤に関しては国際的にも行われていない。

したがって我々は，抗毒素製剤の安全性を十分に認識した上で，治療に必要と判断した時には，速やかに投与しなければならない。

Ⅳ．まとめ

①*C. perfringens*感染症に対する治療用抗毒素製剤について情報提供した。しかし，この抗毒素製剤のみで治療できるわけではないことを再度認識しておく必要がある。

②できるだけ早期の抗毒素製剤や抗菌薬の投与に加えて，血液浄化法などを加味した集中治療を展開することが重要である。

③*C. perfringens*抗毒素製剤は国家備蓄品であるが，通常の保険診療の範囲内で処理される。

文献

1) 赤坂威史，橋口浩明，増田和之，他．激しい溶血が特徴的であった劇症型*Clostridium perfringens*感染症の1症例．日集中医誌 2004;11:247-8.
2) 清水敬樹，甲嶋洋平，加藤はる，他．急激な経過をたどり救命し得なかった劇症型*Clostridium perfringens*感染症の1例．ICUとCCU 2005;29:63-70.
3) Ohtani S, Watanabe N, Kawata M, et al. Massive intravascular hemolysis in a patient infected by *Clostridium perfringens*. Acta Med Okayama 2006;60:357-60.
4) Bennet JM, Healey PJM. Spherocytic hemolytic anemia and acute cholecystitis caused by *Clostridium welchii*. N Engl J Med 1963;268:1070-2.
5) 近藤 久．20．抗毒素．国立予防衛生研究所学友会編．日本のワクチン．東京：丸善；1977. p. 279-91.
6) 近藤 久．抗毒素療法のポイント．臨と細菌 1980;7:59-63.
7) 作間 晋．抗毒素の現状．化血研所報黎明 2002;11:19-33.
8) 国立感染症研究所．ガスえそウマ抗毒素（ガスえそ抗毒素）．生物学的製剤基準．2004. p. 23-5.
9) 髙橋元秀，杉本 央．わが国におけるガス壊疽抗毒素の使用情況に関するアンケート調査．平成14年度厚生労働科学研究費補助金 安定供給に向けた国内外の抗毒素製剤の品質管理に関する研究 分担研究報告書．2002.
10) 髙橋元秀．抗毒素製剤の効率的製造方法の開発に関する研究．平成19年度厚生労働科学研究費補助金 総括研究報告書．2007.

Abstract

Do you know the Clostridium perfringens antitoxin?

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There are several reports on non-traumatic *Clostridium perfringens* (*C. perfringens*) infection rapidly progressing to intravascular hemolysis and metabolic acidosis and eventually death, within a few hours after admission. In such cases, *α* toxin of *C. perfringens* is responsible for causing intravascular hemolysis followed by severe anemia, acute renal failure, disseminated intravascular coagulopathy, and multiple organ failure. Surgical debridement is the treatment of choice. High dose of penicillin, hyperbaric oxygen treatment, continuous hemodiafiltration, polymyxin B-immobilized fiber direct hemoperfusion, and plasma exchange are also considered as promising treatment options. However, the optimal therapeutic strategy has not been established thus far. Antitoxin against the *α* toxin of *C. perfringens* has been used as a treatment for *C. perfringens* infection for a long time. However, medical doctors of the current generation are not familiar with this therapeutic option, because it has not been introduced as a treatment of choice in medical papers or congresses. Therefore, in this paper, we introduce the use of antitoxin against the alpha toxin of *C. perfringens* as the optimal therapy.

Key words: ①*Clostridium perfringens*, ②antitoxins, ③gas gangrene antitoxin

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日本産のヘビ類に寄生する吸虫類

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The trematodes parasitic in the snakes of Japan

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最近，シマヘビの口腔内に吸虫の濃厚感染が見られ，調べてみてもこれまでの記録がなく，新たな記録と思われる．一方で，日本におけるヘビに寄生する吸虫類については，影井（1973）によるリストはあるが，すでに古く不完全であるので，そのリストを元に見直しを行った．分類は主に Yamaguti (1971)により，科の分類については，Bray et al. (2008) および Gibson et al.(2002)によった．ウミヘビに感染する吸虫については，一部台湾の記録しかない種があるが，沖縄で見つかる可能性も高く，ここに加えておいた．

1．吸虫のリスト

二生目

Plagiorchiidae Lühe, 1901

Allopharynx japonica Tamura, 1941

宿主：シマヘビ
寄生部位：胆管
分布：日本（山口県）
文献：Tamura, 1941

Encyclometridae Mehra, 1931

Encyclometra japonica Yoshida et Ozaki, 1929

宿主：シマヘビ，ヤマカガシ，ニホンマムシ
寄生部位：腸・胃・食道
分布：日本（本州，四国，九州）
文献：Yoshida and Ozaki, 1929; Yamaguti, 1933; 亀谷他, 1962; 福井, 1963; Kagei and Kifune, 1977; 養命酒中央研究所, 1999; Goldberg et al., 2004
注：*Encyclometra* 属の分類については，研究者により見解が異なり，Yamaguti (1971) は８種を認めているが，Yeh (1958)は本種を含む３種に整理して，本種の分布はアジアからアフリカにまで延びている．Dollfus (1963)はさらに整理を進め，２種だけを認め，本種は *E. colubrimurorum* (Rudolphi, 1819)のシノニムにされた．ここでは Yamaguti (1971)の分類を採用したが，将来変更される可能性がある．

Ochetosomatidae Leão, 1945 (1902)

Ochetosoma sp.

宿主：シマヘビ
寄生部位：口腔・食道・肺