



Genetic Characterization of *Clostridium botulinum* Isolated from the First Case of Infant Botulism in Korea

Jun Ho Jeon , Ph.D.^{1*}, Chi-Hwan Choi , Ph.D.^{2*}, Jeong Hyun Kim , M.D.³, Junghee Hyun , M.S.⁴, Eun-Sun Choi , Ph.D.¹, Sang-Yoon Choi , Ph.D.¹, Yong-Woo Shin , Ph.D.¹, Seong Wook Pyo , M.S.¹, Dae-Won Kim , Ph.D.², Byung Hak Kang , Ph.D.¹, Young Joon Park , M.D.⁵, and Gi-eun Rhie , Ph.D.¹

¹Division of High-risk Pathogens, ²Division of Zoonotic and Vector Borne Disease Research, ³Division of Zoonotic and Vector Borne Disease Control, ⁴Division of Emerging Infectious Disease Response, ⁵Director for Epidemiological Investigation Analysis, Korea Disease Control and Prevention Agency, Cheongju, Korea

Botulism is a neuroparalytic disease caused by a neurotoxin produced by *Clostridium botulinum*. This study aimed to genetically characterize *C. botulinum* strain isolated from the first case of infant botulism in Korea reported on June 17, 2019. We isolated *C. botulinum* strain CB-27 from a stool sample of the patient and analyzed the toxin types and toxin gene cluster compositions of the strain using a mouse bioassay, real-time PCR, and genome sequencing. Toxin gene cluster analysis showed that strain CB-27 possesses a *C. botulinum* neurotoxin type A harboring an unexpressed B gene. Although the nucleotide and amino acid sequences of toxin genes as well as the toxin gene cluster arrangements in strain CB-27 were identical to those of the known strain CDC_69094, the total nucleotide sequences of the toxin gene clusters of CB-27 differed from those of CDC_69094 by 0.47%, indicating genetic diversity of toxin gene clusters of CB-27 among other previously reported *C. botulinum* strains. To our knowledge, this is the first description of a *C. botulinum* strain with two separate toxin gene clusters in Korea.

Received: July 14, 2020

Revision received: October 14, 2020

Accepted: March 20, 2021

Corresponding author: Gi-eun Rhie, Ph.D. Division of High-risk Pathogens, Korea Disease Control and Prevention Agency, 187 Osongsaengmyeong 2-ro, Cheongju 28159, Korea
Tel: +82-43-719-8270
Fax: +82-43-719-8309
E-mail: gerhie@korea.kr

*These authors contributed equally to this study.



© Korean Society for Laboratory Medicine

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<https://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Key Words: *Clostridium botulinum*, Toxin type, Toxin gene cluster, Genetic diversity, Neurotoxin

Botulism is a neuroparalytic disease caused by the seven immunologically distinct toxins (A to G) produced by *Clostridium botulinum*, a gram-positive, anaerobic, spore-forming bacterium [1, 2]. Botulinum toxin types A, B, E, and F cause botulism in humans [2]. There are three main forms of botulism: foodborne botulism, infant botulism, and wound botulism [3]. From 2002 to June 16 2019, eight cases of human botulism have been reported in Korea, and all were presumed to be foodborne botulism [4–6]. In the USA, more than 1,500 cases of infant botulism have been confirmed between 1976 and 2007 [7]. By contrast, in Korea, the first documented case of infant botulism

was reported in 2019, after botulism was designated as a notifiable infectious disease in 2002 [8]. In addition, no *C. botulinum* strains with two toxin gene clusters have been reported in Korea. This study aimed to characterize the *C. botulinum* strain CB-27 isolated from a stool sample from the first case of infant botulism in Korea. We found that the strain possessed two different toxin gene clusters, showing genetic diversity compared with other previously reported *C. botulinum* strains.

On June 7, 2019, a 4-month-old Korean baby with suspected infant botulism was admitted to Ajou University Hospital, Suwon, Korea, and on June 13, clinical samples, including serum and

stool samples, were submitted to the Korea Centers for Disease Control and Prevention (KCDC) to identify *C. botulinum* and its toxins [8]. We conducted a mouse bioassay to identify botulinum toxins in the stool and serum samples [9]. Based on neutralization of sample toxicity by monovalent antitoxin A (National Institute for Biological Standards and Control, Potters Bar, UK), only the stool sample was positive for botulinum neurotoxin (boNT) A.

A small amount of the stool sample was inoculated into cooked-meat medium (Difco, Franklin Lakes, USA) and cultured under anaerobic conditions at 37°C for three days. The culture was mixed 1:1 with 100% ethanol and incubated at 37°C for 1 hour. Serial culture dilutions were plated on egg yolk agar under anaerobic conditions to isolate *C. botulinum*. Five lipase-positive colonies were isolated, and DNA was extracted from each strain using a genomic DNA isolation kit (Intron Biotechnology, Seongnam, Korea). We analyzed botulinum toxin

genes by real-time PCR using previously reported primers [10, 11]. As all strains were positive for both *boNT/A* and *boNT/B* genes, we selected the first isolated strain, named “CB-27,” and cultured it in trypticase peptone glucose yeast extract medium (KisanBio, Seoul, Korea) at 37°C for 24 hours. This study was exempted from the approval by the Institutional Review Board of the KCDC because clinical bacterial strains were used.

The culture filtrate of strain CB-27 was diluted 1:10,000 in

Table 1. Mouse bioassay to identify *C. botulinum* neurotoxin type produced by CB-27 strain

Number of mice alive / Number of mice tested after injection with			
Untreated filtrate	Filtrate plus antitoxin A	Filtrate plus antitoxin B	Filtrate plus antitoxin A/B mixture
0/2	2/2	0/2	2/2

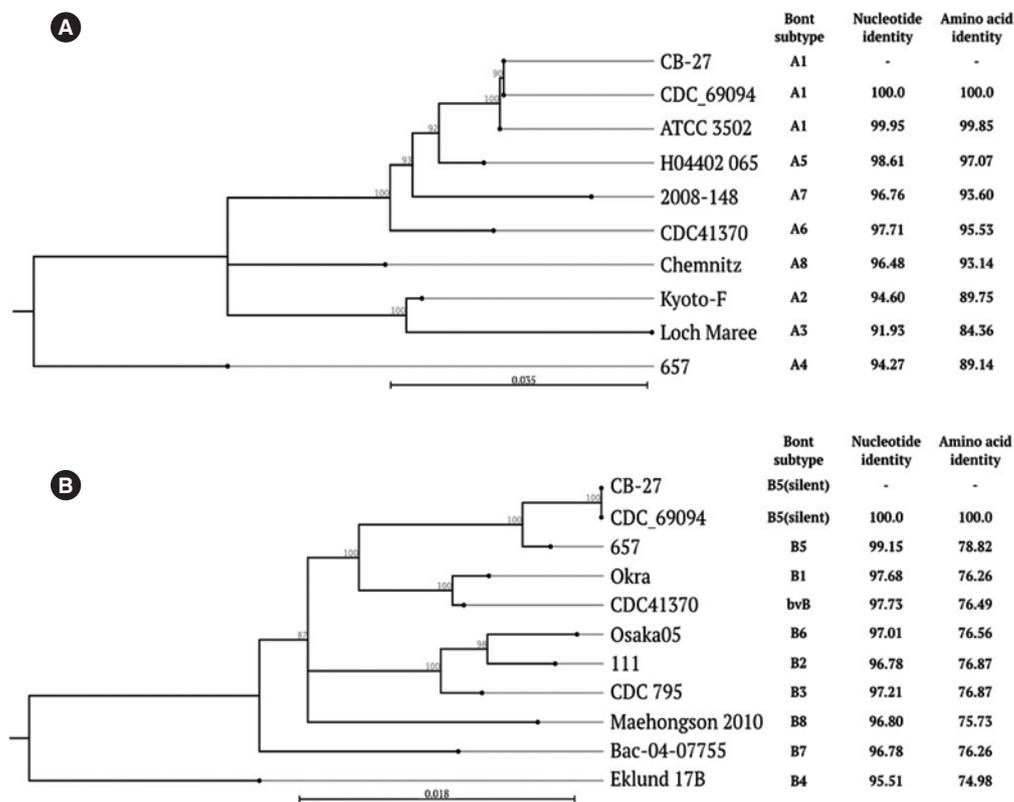


Fig. 1. Comparison of botulinum neurotoxin (*boNT*)/A and *boNT*/B nucleotide sequences. (A) The *boNT*/A sequences of strain CB-27 were compared with those of previously reported strains. (B) The *boNT*/B sequences of strain CB-27 were compared with those of previously reported strains. Nucleotide and amino acid identities of toxin genes between each strain and CB-27 are indicated on the right. The numbers on the tree indicate bootstrap values for branch points; only values >70 are shown. GenBank accession numbers are as follows: CDC_69094 (CP013246), ATCC 3,502 (AM412317), H04402 065 (EU679004), 2,008-148 (JQ954969), CDC41370 (FJ981696, FJ981697), Chemnitz (KM233166), Kyoto-F (X73423), Loch Maree (CP000963), 657 (CP001081), Okra (CP000940), Osaka05 (AB302852), 111 (AB084152), CDC 795 (EF028400), Maehongson 2010 (JQ964806), Bac-04-07755 (JQ354985), Eklund 17B (EF051570), and CB-27(MT199282).

phosphate-buffered saline containing 0.2% gelatin and then analyzed using a mouse bioassay. The mice were injected with 0.05 international units of botulinum antitoxins A and B (National Institute for Biological Standards and Control, Potters Bar, UK). Botulinum antitoxin A and a mixture of antitoxins A and B showed neutralizing ability against the CB-27 culture filtrate (Table 1) in accordance with the mouse bioassay results obtained using stool samples.

To confirm the discrepancy between the mouse bioassay and real-time PCR analysis results, genomic DNA was extracted from the CB-27 strain and subjected to whole genome sequencing using a PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA; <https://www.pacb.com>) and an Ion S5 (Thermo Fisher Scientific, Waltham, MA USA; <https://www.thermofisher.com>) sequencer. The toxin gene cluster sequences of CB-27 were assembled *de novo* using the Hierarchical Genome Assembly Process version 3 (Pacific Biosciences). High-quality Ion S5 reads were used to correct potential sequencing errors in the PacBio long reads in Proovread version 2.14 (<https://github.com/BiolInf-Wuerzburg/proovread>) [12]. The cluster sequence of CB-27 (67,538 bp) has been deposited at GenBank under accession number MT199282. Genes were annotated using rapid prokaryotic genome annotation (Prokka version 1.14.5, <https://github.com/tseemann/prokka>) [13]. Gene annotation

showed that the cluster sequence of CB-27 harbored a *boNT/A* gene and a silent *boNT/B* gene (*boNT/A(B)*).

To phylogenetically characterize the toxin gene clusters of CB-27, we downloaded sequence data of 240 strains representing different toxin types and subtypes from Pathosystems Resource Integration Center (PATRIC) (<https://www.patricbrc.org>) [14]. All-against-all pairwise sequence comparisons were conducted using Basic Local Alignment Search Tool (<https://blast.ncbi.nlm.nih.gov/>) to determine the closest relatives of CB-27. Multiple sequence alignments were conducted using MAFFT version 7.453 (<https://mafft.cbrc.jp/alignment/software/>), with a maximum of 1,000 iterations. We constructed maximum-likelihood phylogenetic trees using RAxML-NG version 0.9.0 (<https://github.com/amkozlov/raxml-ng>) with TVM+F+G4, the best nucleotide substitution model recommended by ModelFinder [15, 16]. The *boNT/A* nucleotide sequence of CB-27 was identical or highly similar to the sequences of previously reported subtype A1 strains, including CDC_69094 (100%) and ATCC 3502 (99.95%) (Fig. 1A). The *boNT/B* sequence of CB-27 showed 100% identity with that of CDC_69094 (Fig. 1B), which contains a nucleotide substitution that can lead to premature termination of *boNT/B* at amino acid position 128 [17].

We examined genomic arrangements of the toxin gene clusters of CB-27 using the multiple genome alignment tool MAUVE

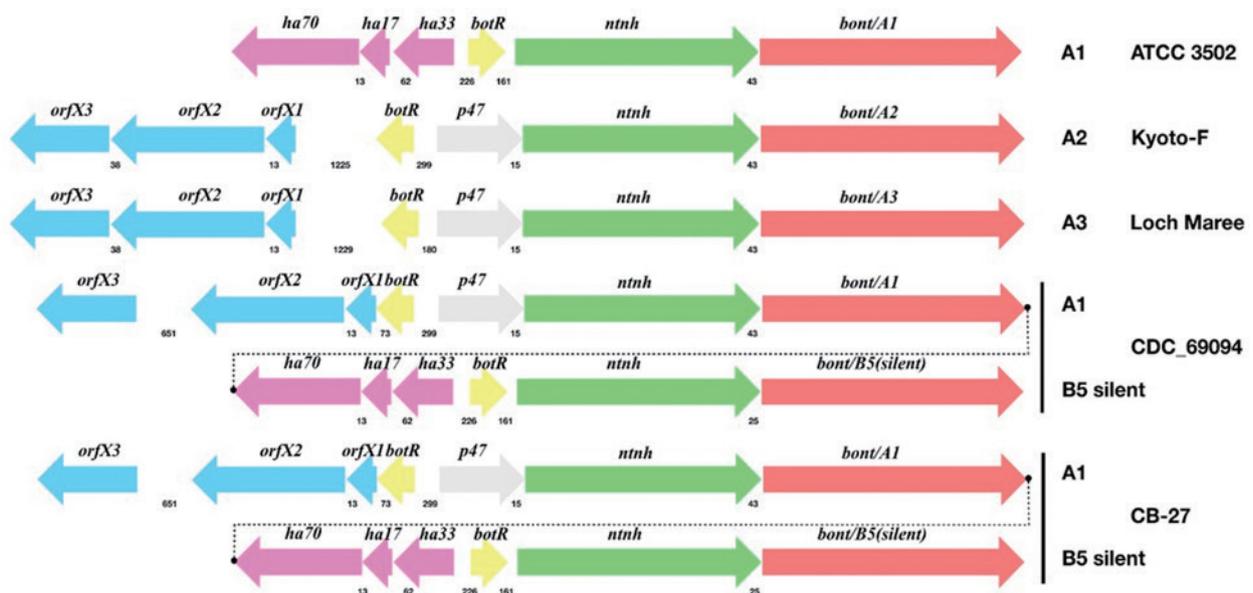


Fig. 2. The toxin gene cluster structures of CB-27 and other representative strains (subtypes A1, A2, A3, and A(B)). Gene locations and directions are indicated by arrows, with gene names presented above the arrows. The numbers between genes below the arrows indicate intergenic spacing. GenBank accession numbers are as follows: ATCC 3502 (AM412317), Kyoto-F (CP001581), Loch Maree (CP000963), CDC_69094 (CP013246), and CB-27(MT199282).

version 2.4.0 (<http://darlinglab.org/mauve/mauve.html>) [18]. Fig. 2 shows a schematic diagram of the toxin A and B gene cluster structures of CB-27 and other representative strains (subtypes A1, A2, A3, and A(B)). The toxin gene cluster structures of CB-27 were identical to those of CDC_69094. Both CB-27 and CDC_69094 possess not only *ha70*, *ha17*, *ha33*, *botR*, *ntnh*, and *boNT/A1* in the HA gene cluster but also *orfX3*, *orfX2*, *orfX1*, *botR*, *p47*, *ntnh*, and *boNT/B5* (silent) in the OrfX gene cluster. However, the toxin gene cluster of CB-27 showed 99.53% nucleotide sequence identity with that of CDC_69094, showing genetic diversity between the toxin gene clusters.

In conclusion, we characterized the toxin gene clusters of *C. botulinum* strain CB-27 isolated from the first case of infant botulism in Korea in 2019. The CB-27 genome possesses *boNT/A1* and unexpressed *boNT/B5*. Although the nucleotide sequences of the toxin gene cluster of CB-27 showed a 0.47% difference from that of CDC_69094, the sequence of the complete genome of CB-27 strain may show a larger difference. Further analysis using whole genome sequencing of CB-27 will be needed to confirm this notion.

AUTHOR CONTRIBUTIONS

Conceptualization: Rhie G. Data curation: Jeon JH, Choi CH, Kim JH, Hyun J, Choi ES, Choi SY, Shin YW, Pyo SW, Kim DW, Kang BH, Park YJ. Writing-review and editing: Rhie G, Jeon JH. Final approval of the manuscript: all authors.

CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

RESEARCH FUNDING

This work was supported by the KDCA (4840-302-210-13).

ORCID

Jun Ho Jeon <https://orcid.org/0000-0003-2612-1081>
Chi-Hwan Choi <https://orcid.org/0000-0002-8560-5230>
Jeong Hyun Kim <https://orcid.org/0000-0003-3918-2676>
Junghee Hyun <https://orcid.org/0000-0002-9499-4143>
Eun-Sun Choi <https://orcid.org/0000-0002-5432-1059>
Sang-Yoon Choi <https://orcid.org/0000-0001-5108-4345>
Yong-Woo Shin <https://orcid.org/0000-0002-0239-7502>
Seong Wook Pyo <https://orcid.org/0000-0001-8775-3571>

Dae-Won Kim <https://orcid.org/0000-0003-0917-1511>
Byung Hak Kang <https://orcid.org/0000-0003-1952-0965>
Young Joon Park <https://orcid.org/0000-0001-9697-4173>
Gi-eun Rhie <https://orcid.org/0000-0002-4212-5501>

REFERENCES

1. Arnon SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, et al. Botulinum toxin as a biological weapon: medical and public health management. *JAMA* 2001;285:1059-70.
2. Hill KK, Smith TJ, Helma CH, Ticknor LO, Foley BT, Svensson RT, et al. Genetic diversity among botulinum neurotoxin-producing clostridial strains. *J Bacteriol* 2007;189:818-32.
3. Sharma SK, Ferreira JL, Eblen BS, Whiting RC. Detection of type A, B, E, and F *Clostridium botulinum* neurotoxins in foods by using an amplified enzyme-linked immunosorbent assay with digoxigenin-labeled antibodies. *Appl Environ Microbiol* 2006;72:1231-8.
4. Chung GT, Kang DH, Yoo CK, Choi JH, Seong WK. The first outbreak of botulism in Korea. *Korean J Clin Microbiol* 2003;6:160-3.
5. Yi HA, Lim JG, Lee JB, Her JH, Kim HA, Shin YE, et al. A familial outbreak of food-borne botulism. *J Korean Neurol Assoc* 2004;22:670-2.
6. Infectious Disease Portal. <http://www.kdca.go.kr/npt/biz/npp/ist/bass/bassDissStatsMain.do> (Updated on Mar 11, 2021).
7. Brook I. Infant botulism. *J Perinatol* 2007;27:175-80.
8. Jang HG, Jang J, Jung HJ, Jung DE. The first reported case of infant botulism in Korea: treatable infantile neuromuscular disease. *J Korean Med Sci* 2020;35:e93.
9. Thirunavukkarasu N, Johnson E, Pillai S, Hodge D, Stanker L, Wentz T, et al. Botulinum neurotoxin detection methods for public health response and surveillance. *Front Bioeng Biotechnol* 2018;6:80.
10. Hill BJ, Skerry JC, Smith TJ, Arnon SS, Douek DC. Universal and specific quantitative detection of botulinum neurotoxin genes. *BMC Microbiol* 2010;10:267.
11. Fach P, Micheau P, Mazuet C, Perelle S, Popoff M. Development of real-time PCR tests for detecting botulinum neurotoxins A, B, E, F producing *Clostridium botulinum*, *Clostridium baratii* and *Clostridium butyricum*. *J Appl Microbiol* 2009;107:465-73.
12. Hackl T, Hedrich R, Schultz J, Förster F. proovread: large-scale high-accuracy PacBio correction through iterative short read consensus. *Bioinformatics* 2014;30:3004-11.
13. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068-9.
14. Wattam AR, Davis JJ, Assaf R, Boisvert S, Brettin T, Bun C, et al. Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center. *Nucleic Acids Res* 2017;45:D535-42.
15. Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics* 2019;35:4453-5.
16. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermini LS. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods* 2017;14:587-9.
17. Lúquez C, Raphael BH, Maslanka SE. Neurotoxin gene clusters in *Clostridium botulinum* type Ab strains. *Appl Environ Microbiol* 2009;75:6094-101.
18. Darling AC, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res* 2004;14:1394-403.