

Prevalent toxin types of *Clostridium botulinum* in South Korean cattle farms

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ABSTRACT

Clostridium botulinum produces neurotoxic substrates that can cause fatal flaccid paralysis called botulism. These neurotoxins are classified into types A–G. Several botulism cases were recorded in 2012–2013 in the Gyeonggi province, South Korea. We assessed the distribution of *C. botulinum* types B, C, and D in several South Korean farms. A total of 184 samples collected in 2012–2013, including feces ($n = 72$), hay and silage ($n = 50$), soil ($n = 26$), water trough ($n = 21$), and stomach contents ($n = 15$), were subjected to multiplex polymerase chain reaction (PCR) to screen for types B, C, and D. Twenty-four samples tested PCR-positive as follows: type B ($n = 11$), type C/D ($n = 4$), and type D ($n = 18$). Eight of the 11 type B samples were detected in hay and silage. Sixteen of the 18 type D samples were detected in fecal and stomach content samples. PCR-positivity was observed in fecal ($n = 9$, 12.5%), hay and silage ($n = 10$, 20.0%), water trough ($n = 2$, 9.5%), and stomach content ($n = 12$, 80.0%) samples. Fourteen (42.4%) *C. botulinum*-positive samples were isolated from the PCR-positive samples (type B [$n = 8$], type C/D [$n = 1$], and type D [$n = 5$]). Our findings demonstrate that *C. botulinum* types B, C/D, and D were prevalent in South Korean cattle farms between 2012 and 2013.

Abbreviations

PCR polymerase chain reaction
TPGY trypticase-peptone-glucose-yeast

1. Introduction

Clostridium botulinum is an obligate anaerobic, spore-forming bacterium that produces the botulinum neurotoxin (BoNT), and is the causative agent of botulism (Huss, 1980). BoNTs can be distinguished based on their antigenic and genetic features into seven types, A to G. Among them, types A, B, C, and D are reported to cause botulism in cattle. Type B botulism in cattle is usually associated with the feeding of poor-quality silage produced at a pH above 4.5 (Whitlock & Williams, 1999). Surveys in the USA and Europe have revealed a high prevalence of *C. botulinum* type B in cattle-related samples, hay and silage (Divers, Bartholomew, Messick, Whitlock & Sweeny, 1986; Gray & Bulgin, 1982; Kelch, Kerr, Pringle, Rohrbach & Whitlock, 2000; Notermans, Dufrenne & Oosterom, 1981; Whitlock & Williams, 1999; Wilson, Boley & Corwin, 1995). A recently performed survey in Sweden showed a high prevalence (62%) of *C. botulinum* type B in fecal samples from animals (Dahlenborg, Borch & Rådström, 2001). Specifically, animal botulism cases are commonly

associated with group III *C. botulinum* strains, which produce type C or D neurotoxin (Smith, 1979). Type C and D botulism in cattle results from the ingestion of feeds containing ensiled bones from dead animals or carcasses contaminated with *C. botulinum* (Neill, McLoughlin & McIlroy, 1989; Smart, Jones, Clegg & McMurtry, 1987). Formidable outbreaks of type D cattle botulism have occurred in beef cattle fed chicken litter in Australia and Israel (Egyed, 1987). However, diverse types of toxin, such as types A, C/D, and D/C, have been reported in related samples during outbreaks in cattle (Frye et al., 2020; Hartley et al., 2009; Otaka et al., 2020; Souillard et al., 2021).

The ingestion of the preformed botulinum toxin, usually types B, C, and D, has been shown to produce disease in most botulism cases in cattle. Several such cases were recorded to have occurred sporadically in the Gyeonggi province, South Korea, in 2012 and 2013. In this study, cattle farms in areas with continuous outbreaks in South Korea were investigated to determine the toxin types prevalent in various epidemiologically related samples.

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2. Materials and methods

2.1. Bacterial strains

Three *C. botulinum* strains, namely S-B-14-12-3 (group I type B), CKIII-U (group III type C), and CVI16878 (group III type D), were used in the inoculation study. The reference strains were cultured in Trypticase-peptone-glucose-yeast extract (TPGY) broth (Lilly, Harmon, Kauter, Solomon & Lynt, 1971) for three days after which the spores were precipitated from the broths and washed using distilled water.

2.2. Collection of contaminated field samples

To explore the effect of geographical location on *C. botulinum* distribution, diverse samples were collected from three cattle farms (farms A, B, and C) suspected to have had an outbreak of bovine botulism (Table 1). Farms A (23 samples), B (82 samples), and C (79 samples) were located in the Northern, Western, and Eastern parts of the Gyeonggi province, respectively.

A total of 184 samples including feces ($n = 72$), hay and silage ($n = 50$), soil ($n = 26$), water trough ($n = 21$), and stomach contents ($n = 15$) were collected from cattle farms in the Gyeonggi province, South Korea, in 2012 and 2013. Stomach contents were collected from the carcasses of cattle suspected of botulism (Table 2). Feces ($n = 8$), hay and silage ($n = 15$), soil ($n = 10$), water trough ($n = 8$), and stomach contents ($n = 2$) samples from nine cattle farms without reports of botulism cases in the Gyeonggi, Kangwon, Gyeongbuk, Chungbuk, Jeonbuk, and Jeonnam provinces were used as negative controls (Table 1).

Approximately 10 g of each sample was collected during several outbreaks of botulism in cattle farms, transferred to sterile tubes, and stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

2.3. Investigation of the prevalence of *C. botulinum* in farm samples collected during botulism outbreaks

C. botulinum neurotoxin type was identified via mouse inoculation bioassay (Centers for Disease Control & Prevention, 1998) and polymerase chain reaction (PCR), using the enrichment cultures from hay and silage, soil, water trough, stomach contents, and fecal samples. To confirm the presence of BoNT-producing clostridia, 184 suspected samples were incubated in TPGY broth at $37\text{ }^{\circ}\text{C}$ for three days under anaerobic conditions after heat shock at $70\text{ }^{\circ}\text{C}$ for 15 min. The enrichment cultures were heated at $95\text{ }^{\circ}\text{C}$ for 15 min to break up the cells and release the bacterial DNA and were collected by centrifugation at 4500 rpm for 5 min. Following this, $2\text{ }\mu\text{l}$ of each supernatant was used as a template in the PCR mixture.

Type-specific primer sets for detecting the genes of the B, C, and D toxins were designed based on the toxin gene sequences previously reported (Takeshi et al., 1996), as shown in Table 3. A multiplex PCR was

Table 1
Information on cattle botulism outbreaks in South Korean farms.

Farm	Type of holding	Year of botulism outbreak	Clinical signs of botulism	Region
A	Korean native cattle	2013	Yes	Gyeonggi (Eastern)
B	Dairy cattle	2013	Yes	Gyeonggi (Western)
C	Dairy cattle	2012	Yes	Gyeonggi (Northern)
D ^a	Korean native cattle	2013	No	6 of provinces ^b

^a Cattle botulism have never been occurred at these farms ($n = 8$).

^b Cattle botulism free farms located in six provinces in South Korea, which were Gyeonggi, Kangwon, Chungbuk, Jeonbuk, Jeonnam, and Gyeongbuk.

performed to detect toxin genes; one set of primers was used to detect the type B toxin gene and the other for detecting types C and D toxin genes. The multiplex PCR procedure was carried out as follows: 20 s at $95\text{ }^{\circ}\text{C}$ for denaturation, 30 s at $60\text{ }^{\circ}\text{C}$ for annealing, and 30 s at $72\text{ }^{\circ}\text{C}$ for extension. The cycles were repeated 30 times, followed by a final extension at $72\text{ }^{\circ}\text{C}$ for 7 min. The PCR products were visualized using 1.5% agarose gel electrophoresis, and the gel was visualized using a gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA). The Molecular Analyst Software (Bio-Rad) was used to analyze the gel images (Fig. 1). Positive and negative controls were included in all PCR assays for validation and to rule out sample contamination, respectively.

In case that the strains were identified as C toxin only through mouse inoculation bioassay, the strains were examined with the C/D mosaic toxin PCR (Lindberg, Skarin, Knutsson, Blomqvist & Båverud, 2010) to confirm the result. The forward and reverse primers used were CBOTCF (5'-CACAAGAAGGATTTGGTGCTTTATCA-3') and CBOTCR (5'-CAGACTTAGAAAATCTACCCTCTCTACA-3'). The PCR protocol was as follows: 10 min at $95\text{ }^{\circ}\text{C}$ for denaturation, 45 cycles at $95\text{ }^{\circ}\text{C}$ for 15 s, 1 min at $60\text{ }^{\circ}\text{C}$ for annealing, 30 s at $72\text{ }^{\circ}\text{C}$ for extension, followed by a final extension at $72\text{ }^{\circ}\text{C}$ for 7 min. The PCR products were analyzed as described above.

2.4. Isolation of *C. botulinum* strains using immuno-magnetic separation

We isolated *C. botulinum* from the samples in which BoNT was detected. These samples were cultured with 1 g of each sample in TPGY broth containing 0.1% L-cysteine-HCl and 0.14% NaHCO_3 . The isolation of *C. botulinum* type B, C, or D was performed under the same conditions as those mentioned above. Toxic cultures were diluted with phosphate buffered saline (PBS), and $30\text{ }\mu\text{l}$ of 10^{-1} - or 10^{-2} -fold diluted culture was cultured on 5% egg yolk-containing McClung Toabe agar (Becton, Dickson and Company, Sparks, MD, USA).

To improve the isolation, $10\text{ }\mu\text{l}$ of bacterial cells was washed three times with PBS by centrifuging at 5000 rpm for 5 min. After vortex mixing, each supernatant was incubated at room temperature for 1 h with $10\text{ }\mu\text{l}$ of biotin-labeled antibody against *C. botulinum* type B, C, or D spores. Antibody against *C. botulinum* type B, C, or D spores were labeled with biotin with biotinylated protein kit (EZ-Link NHS-Biotin, Thermo Fisher Scientific, Waltham, MA, USA), which were prepared through immunization of each spore to rabbit and purification by Hi-Trap® Protein G HP column (Merck KGaA, Darmstadt, Germany). Then $20\text{ }\mu\text{l}$ of each sample was bound with streptavidin-labeled magnetic beads (Dynabeads T1; Thermo Fisher Scientific, Waltham, MA, USA) in a 1.5-ml microcentrifuge tube.

The tubes were placed in magnetic separator rack (MagnaRack™, Thermo Fisher Scientific, Waltham, MA, USA), and the magnets were placed in position and left for 5 min. The supernatant was removed by aspiration with a Pasteur pipette, the magnetic tube was removed from the rack, the beads were washed by resuspension in $500\text{ }\mu\text{l}$ of PBS containing 0.1% (v/v) Tween-20, and the magnetic tube was replaced for 2 min. This step was repeated with PBS containing 0.1% (v/v) Tween-20 in the same manner. The beads were inoculated onto egg yolk-containing McClung Toabe agar and incubated for three days at $37\text{ }^{\circ}\text{C}$ in anaerobic conditions.

3. Results

3.1. Specificity and sensitivity of the multiplex PCR assay

We designed a set of primers specific to the BoNT genes (Table 3). The specificity of the multiplex PCR assays was evaluated using 14 strains of *C. botulinum*, eight strains of other *Clostridium* spp., and four non-*Clostridium* strains. None of the *C. tetani*, *C. perfringens* and *C. sporogenes*, or other anaerobic bacterial species yielded a PCR product (data not shown). The sensitivity of the PCR assay was tested with genomic DNA from *C. botulinum* type B, C, and D (strains S-B-14-12-3,

Table 2
Summary of the number of detected and isolated types of *C. botulinum* in South Korean farms in 2012–2013.

Region/Breed(Period)	Samples ^a (No.)	Mouse bioassay ^b No. of positive samples	Neutralization ^c			PCR ^c			Isolation
			B	C	D	B	C (C/D)	D	
Northern/Dairy cattle (Sep. 10 – Nov. 23, 2012)	Fecal samples (39)	4	–	–	+	0	0	4	–
	Hay and silage (20)	1	+	–	–	1	0	0	B (1)
	Stomach contents (8)	6	–	–	+	0	0	6	–
	Water trough (8)	1	–	–	+	0	0	1	D (1)
	Soil (4)	ND ^d				0	0	0	–
Eastern or Western/Korean native or dairy cattle (Apr. 12 – Aug. 19, 2013)	Fecal samples (33)	5	+	+	+	1	0 (1)	3	D (3)
	Hay and silage (30)	9	+	+	+	7	0 (1)	1	B (6)
	Stomach contents (7)	6	+	+	+	1	0 (2)	3	C/D (1), D (1)
	Water trough (13)	1	+	–	–	1	0	0	B (1)
	Soil (22)	ND				0	0	0	–
Total (184)	Fecal samples (72)	9	+	+	+	1	0 (1)	7	D (3)
	Hay and silage (50)	10	+	+	+	8	0 (1)	1	B (7)
	Stomach contents (15)	12	+	+	+	1	0 (2)	9	C/D (1), D (1)
	Water trough (21)	2	+	–	+	1	0	1	B (1), D (1)
	Soil (26)	ND				0	0	0	–
Total (184)			+	+	+	11	0 (4)	18	B (16), C/D (2), D (10)

^a Samples were first incubated for three days at 37 °C, after which 1 ml of each broth was used to extract genomic DNA as described in the materials and methods section.

^b Neutralization test was performed by mixing specific antitoxins of *C. botulinum* with cultures of the isolated strain.

^c Field samples collected from cattle farms were analyzed by *C. botulinum* multiplex PCR, and the C/D or D/C mosaic types were analyzed separately as in Lindberg et al. (2010).

^d ND, not detected

+, neutralized; –, not neutralized.

Table 3
Primers used in the multiplex PCR to detect *C. botulinum* types B, C, and D.

Primer	Nucleotide sequence (5'–3')	Product size (bp) ^a
mBoNTB-F	GGAGCCTCCATTGCGAGAGGT	384
mBoNTB-R	GCTCCACTTCTCTGGATTACTGA	
mBoNTC-F	AATGCGGGTGTTCAGGTGGTT	926
mBoNTC-R	TCGCCTTCTCACTCACTTCTGC	
mBoNTD-F	CAAGTTAAGTAAACCGCCAGACC	603
mBoNTD-R	TCCCTCGCTAACTTGTGGACGA	

^a *C. botulinum* types B, C, and D alone yielded the expected amplification products.

ck II-U, and CVI 16,878) (Fig. 1).

3.2. Detection of genes encoding for type B, C, and D neurotoxins using multiplex PCR on field samples

We examined samples from 15 cattle collected during three different botulism outbreaks in farms A–C (Table 1). Through multiplex PCR assays, we detected a C/D mosaic neurotoxin gene using primers reported previously (Lindberg et al., 2010). The PCR results are summarized in Table 2. In total, 33 samples were PCR-positive for *C. botulinum* type B ($n = 11$), C/D ($n = 4$), and D ($n = 18$). Among the 11 type B positive samples, eight were detected in hay and silage samples. Moreover, five out of the 18 type D positive samples were detected in fecal and stomach content samples. On the other hand, 14 (42.4%) samples yielded positive PCR results for *C. botulinum* and were used for the isolation of *C. botulinum*: type B ($n = 8$), type C/D ($n = 1$), and type D ($n = 5$) (Table 2).

3.3. Prevalence of *C. botulinum* in cattle farm samples

During botulism outbreaks, the prevalence of *C. botulinum* types B, C/D, and D were 33.3% (11/33), 12.1% (4/33), and 54.5% (18/33), respectively (Table 2). Of the 72 fecal samples, nine (8.3%) gave a positive PCR result for the 1-type B, 1-C/D mosaic type, and 7-type D neurotoxin genes. Of the 50 hay and silage samples, 10 (20.0%) gave a positive PCR result for the 8-type B, 1-C/D mosaic type, and 1-type D toxins. Additionally, 15 samples of stomach contents and 21 water trough samples were PCR-positive for the three neurotoxin genes (1-type

B, 2-type C/D, and 9-type D), and two neurotoxin genes (1-type B and 1-type D), respectively. No BoNT types were detected in soil samples (Table 2). Interestingly, none of all samples were positive for the type C neurotoxin gene.

4. Discussion

Bovine botulism can lead to significant economic losses, as mortality rates have been reported to be as high as 35–60% (Abbitt et al., 1984; Galey et al., 2000; Martin, 2003; Kelch et al., 2000; Sharpe et al., 2008). South Korean farms that experienced botulism outbreaks in the past appear to be at a higher risk of further outbreaks following the first one. Therefore, farmers and field veterinarians should remain alert to prevent botulism-associated problems.

This study shows that outbreaks of botulism in cattle were regularly observed during 2012 and 2013 in South Korean cattle farms, despite the relatively low occurrence of *C. botulinum* (18.0%) in environmental samples.

In many countries, including South Korea, types B, C, or D of *C. botulinum* are responsible for causing botulism in cattle (Lindström, Myllykoski, Sivelä & Korkeala, 2011; Ramírez-Romero et al., 2014). In October 2011, an outbreak of bovine type B botulism affected approximately 150 dairy cattle near the Yeongpyeong River, Gyeonggi-do, South Korea (Byun et al., 2013). In Europe, several cattle botulism cases were recorded between 2003 and 2009 (Payne, Hogg, Otter, Roest & Livesey, 2011).

In this study, we developed a multiplex PCR for the simultaneous detection of *C. botulinum* types B, C, and D in field samples (Table 3). The neurotoxin genes of the 14 *C. botulinum* strains characterized with multiplex PCR assays were either type B, C, or D (Fig. 1c). In addition, we examined 43 samples from nine cattle farms in South Korea without reports of botulism outbreaks, but there were no PCR-positive results. The multiplex PCR was conducted on cattle fecal samples, and the detection limit (3 pg templates per 1 g fecal sample) for *C. botulinum* types B and D was the same as that for the detection of templates in an isolated strain (not from fecal samples), indicating that the detection limit and sensitivity were suitable for the detection of *C. botulinum* types B, C, and D neurotoxin genes in field samples.

C. botulinum types D and B were initially detected in 18 (9.8%) and 11 (6%) samples, respectively, from a total of 184 field samples. From

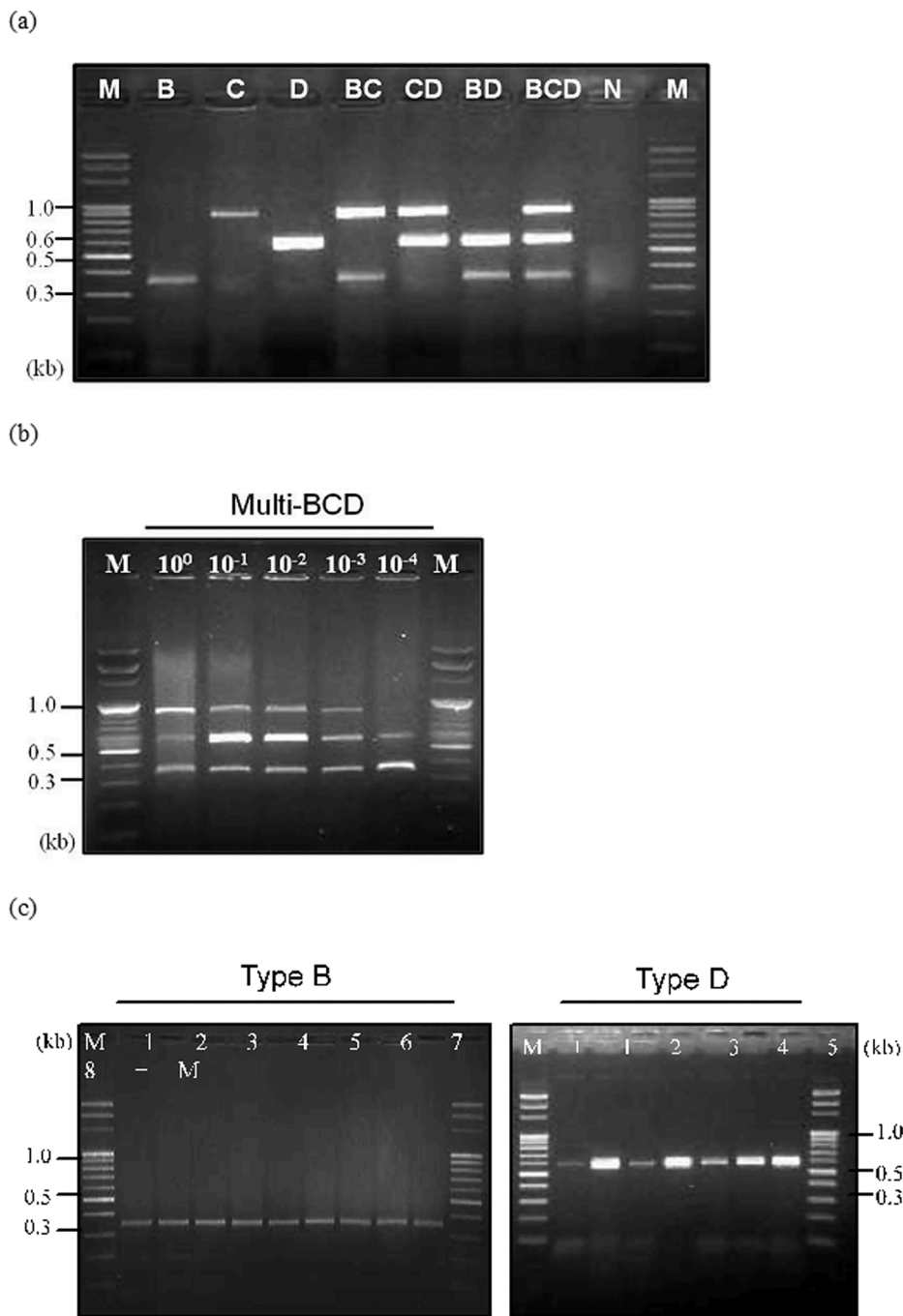


Fig. 1. Multiplex PCR detection of *C. botulinum*. The presence of *C. botulinum* types was assessed for all samples using a multiplex PCR specific for the BoNT B, C, and D genes. DNA extraction was performed by boiling a sample suspension at 95 °C for 20 min. Strains C-B-13-12-3, ck III-U, and CVI16878 were used as references for type B, C, and D, respectively. (a) Lanes: M, 100 bp molecular weight marker; B, *C. botulinum* type B; C, *C. botulinum* type C; D, *C. botulinum* type D; BC, *C. botulinum* types B and C; CD, *C. botulinum* types C and D; BD, *C. botulinum* types B and D; BCD, *C. botulinum* types B, C, and D; and N, negative control. (b) The sensitivity of multiplex PCR from *C. botulinum* types B, C, and D genomic DNA was confirmed via a 10-fold dilution (10^0 – 10^{-4}). (c) Thirteen isolates (Type B 1 to 8: TOM100, 1-23-G, 3-1-G, 4-1-G, 6-1-G, 8-10-G, 8-25-G, and Q47-G; Type D 1 to 5: WT-1, D024-F, Q23-3-F, 8-12-F, and 15-5-S) were subjected to PCR analysis as representative samples of the isolates related to cattle botulism listed in Table 2.

one of these samples in which both types B and D were detected, only type B was isolated. As the *C. botulinum* group III genes are carried by bacteriophages, which can be lost during subculturing and sporulation (Eklund, Poysky, Meyers & Pelroy, 1974; Hunter & Poxton, 2002), an effective isolation method is missing (Le Maréchal et al., 2019).

When we used immuno-magnetic separation to isolate positive samples, type B was isolated in eight of the 11 PCR-positive samples, type C/D was isolated in one out of four, and type D in five out of 11. According to our findings, the isolation rate of *C. botulinum* type B in South Korean cattle is higher than that of type D. The higher isolation rate of *C. botulinum* type B observed in our study is most likely due to inadequate methods for concentration and subsequently growth of *C. botulinum* spores obtained from feed samples. The low isolation rate of type C/D and C was observed because a limited number of samples was obtained during the outbreaks, and a low number and uneven

distribution of *C. botulinum* was observed within the samples. In addition, the type C/D should be further analyzed whether it is novel neurotoxin type in cattle in the future through mouse bioassay and genetic analysis.

In conclusion, botulinum toxin type B was the predominant type in cow feeds, whereas type D was found mainly in cow feces and intestinal contents. Type C/D was also present in cow feeds, feces, and intestinal contents. A total of 14 (42.4%) strains of *C. botulinum* were isolated from PCR-positive samples ($n = 33$). Our findings demonstrate that *C. botulinum* types B and D were highly prevalent in South Korean cattle farms in 2012 and 2013 during botulism outbreaks.

Declaration of Competing Interest

The authors declare that there are no conflict of interests.

Ethical Statement

Postmortem procedures in Korean cattle does not need approval decision due to Korean law and was conducted by qualified veterinarians maintaining animal welfare. Yun Sang Cho

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