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RESEARCH ARTICLE

Post-outbreak monitoring of *Clostridium botulinum* types C and D using Polymerase chain reaction in a dairy farm in Kanchanaburi province, Thailand

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Abstract

Objective: This study aimed to investigate the presence of *C. botulinum* on dairy farms with a history of outbreaks to support animal health monitoring and improve preventive strategies.

Materials and Methods: Fecal samples were randomly collected from 400 dairy cattle, including lactating cows, dry cows, pregnant heifers, heifer weaners, and calves, along with 10 feedstuff samples. Approximately 1-2 g of each sample was inoculated into cooked meat medium and incubated anaerobically at 35 ºC for 5 days. DNA was extracted from the enrichment culture and analyzed for *C. botulinum* types C and D using Polymerase chain reaction. Positive PCR products were confirmed by Sanger DNA sequencing.

Results: *C. botulinum* types C and D were detected in fecal samples at a rate of 2.75% (11/400). These were found in the feces of lactating cows (1.80%, 3/167), dry cows (6.98%, 6/86), pregnant heifers (2.00%, 1/50), and calves (2.70%,1/37). No fecal samples from heifer weaners (0/60) and feedstuffs (0/10) were positive.

Conclusion: The persistence of *C. botulinum* one year after an outbreak suggests possible gastrointestinal colonization and environmental contamination. Given the farm's open system and presence of vectors such as flies, birds, rodents, cats, and dogs, biosecurity measures such as disinfection stations, sanitation equipment, and designated footwear at entrances are recommended to reduce pathogen spread.

Keywords: *Clostridium botulinum*, Dairy cattle, Polymerase chain reaction

Introduction

Clostridium botulinum is an anaerobic, gram-positive, rod-shaped, spore-forming bacterium. Clostridial spores are highly resistant to inactivation, and they can survive for many years in soil and aquatic habitats until favorable conditions allow them to germinate, grow, and produce toxin in anaerobic environments (Center for Food Security and Public Health, 2018; Quinn et al., 1994). *C. botulinum* is classified into several toxin types, A through G, based on the production of specific lethal toxins. Types A, B, E, F and G are responsible for classical botulism in humans, while types C and D cause botulism in animals (Carter et al., 1990). Routes of exposure to botulism generally include ingestion of preformed botulinum toxins in food or water, or when clostridial spores germinate in anaerobic tissues within the body and produce toxins as they grow, absorption from colonized wound, inhalation or accidental injection of toxin (Alemu and Ayele, 2018; CFSPH, 2018). In animals, various avian and mammalian species can be affected by botulism such as birds, cattle, horses, mink, sheep and poultry (WOAH, 2022). Clinical signs of botulism in animals are characterized by progressive motor paralysis. The article by CFSPH (2018) describes the clinical signs of botulism in animals as typically presenting with ascending paralysis that affects the hind legs first. Autonomic dysfunction, difficulties in chewing and swallowing, visual disturbances, generalized weakness, and incoordination may also be apparent. Loss of muscle tone around the eyes and mouth can result in an appearance of lethargy or somnolence, characterized by drooping eyelids, pupil dilation, and slow pupillary reflexes. Sensory defects are absent, and death usually results from paralysis of the respiratory muscles.

In animals, cattle are among the most susceptible species to botulinum toxins, as the ingestion of even minute amounts of toxin can result in the onset of clinical disease (Alemu and Ayele, 2018). The occurrence of botulism in cattle has been reported in several parts of the world, including Europe, the United States, the United Kingdom, South America, South Africa, and Asia (Alemu and Ayele, 2018; Park et al., 2022). In Thailand, an outbreak of botulism caused by *C. botulinum* types C and D recently occurred on a dairy cattle farm in Kanchanaburi province in January 2023. The disease had a significant economic impact due to its high fatality rates, reaching up to 79.67% (Narongwanichgarn et al., 2023). Diagnosis of sick animals during the outbreak period was primarily based on history and clinical findings, while in vitro testing using the PCR technique, as described by Heffron and Poxton (2007), was employed to detect toxin genes in bacterial cultures from 38 fecal samples and internal organs of a dairy cattle, including heart, liver, lung, spleen, kidney, small intestine, large intestine, and lymph node. The results identified *C. botulinum* types C and D exclusively in the fecal samples. A year after the outbreak, no recurrence of the disease was observed. However, *C. botulinum* can still be found in feces of clinically asymptomatic animals (Abdel-Moein and Hamza, 2016; Dahlenborg et al., 2003; Notermans et al., 1981; Souillard et al., 2015). The continued detection of the pathogen one year later raises concerns about the effectiveness of the control measures implemented post-outbreak and underscores the need for ongoing monitoring. This study aimed to investigate the current status of *C. botulinum* types C and D on the previously affected farm, one year after the outbreak, to evaluate pathogen persistence and inform future prevention and surveillance strategies. Additionally, the findings provide microbiological evidence that can support a retrospective assessment of the adequacy and long-term impact of the control measures previously implemented.

Materials and Methods

Sample Size Determination and Sampling Method

The sample size for estimating a simple proportion (apparent prevalence) was calculated based on a finite population of 1,682 dairy cattle using the n4Studies application (version 1.4.2), developed by Ngamjarus and Chongsuvivatwong (2016). The calculation assumed an expected prevalence of 50%, a 5% margin of error, a 95% confidence level, and a design effect of 1. The minimum required sample size was 313 cattle. However, a total of 400 samples were collected to enhance the precision of

the study and account for potential data loss. A stratified random sampling method was employed to ensure proportional representation of all key production stages and age groups. The dairy cattle population was stratified into five subgroups: lactating cows (n = 701), dry cows (n = 360), pregnant cows (n = 211), heifers (n = 250), and calves (n = 160). The sampling interval was calculated as 1,682/400 = 4.2. Samples were proportionally allocated to each subgroup according to their population sizes, resulting in the collection of 167 samples from lactating cows, 86 from dry cows, 50 from pregnant cows, 60 from heifers, and 37 from calves. Only healthy animals were included in the study. Fecal samples (approximately 150-300 g) were collected directly from the rectum of each animal using sterile gloves. Additionally, 10 feedstuff samples were collected, comprising 1 ground corn, 2 corn husk, 1 corn silage, 1 rice straw, 1 ruzi grass, 1 cassava chips, 1 soybean meal, 1 cassava pulp, 1 brewer's grain. All samples were collected from a dairy farm in Kanchanaburi province, Thailand, in early May 2024.

The study was conducted under the animal use license number U1-01662-2558 and was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Institute of Animal Health (NIAH), Department of Livestock Development (DLD).

Bacterial culture

The bacterial culture method was adopted based on the standards outlined in Bacteriological Analytical Manual (BAM, 2001). The process began with the removal of dissolved oxygen from the Cooked Meat Medium (CMM) (Oxoid, England) by steaming for 10 to 15 minutes, followed by rapid cooling of the medium without agitation before inoculation. Approximately 1 to 2 g of each sample was inoculated into the CMM, and liquid paraffin was added to seal the surface of the medium. The reducing properties of the CMM, combined with the paraffin overlay, prevent oxygen diffusion, thereby creating and maintaining an anaerobic environment suitable for the growth of anaerobic bacteria. The inoculated samples were then incubated at 35 °C for 5 days under these anaerobic conditions.

DNA extraction

DNA extraction was performed using an automated nucleic acid extraction system (IndiMag2, Germany). After 5 days of incubation, 1 ml of each enrichment culture was transferred to a sterile 1.5 ml microcentrifuge tube, centrifuged at 12,000 rpm for 5 minutes, and the supernatant was removed. A total volume of 200 µl of sterile distilled water was added, and the mixture was vortexed thoroughly. The solution was then transferred to well plates, and DNA extraction was conducted following the protocols outlined in the IndiMag Pathogen kit (Indical Bioscience, Germany). The IndiMag Pathogen Kit is known for its high efficiency in extracting DNA from various samples, even those with low DNA concentrations or contaminants. This high-quality DNA extraction method ensures that the extracted DNA is suitable for PCR amplification, thus ruling out the possibility of false-negative results due to insufficient DNA template. To further verify the adequacy of the DNA concentration, we also measured the DNA concentration using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), which confirmed that the extracted DNA was of sufficient quality and concentration for PCR analysis. The successful sequencing results further support the adequacy of the DNA for PCR amplification, confirming that the negative PCR results were not due to low DNA concentration.

Polymerase chain reaction

This study employed PCR technique, a methodology previously utilized by Heffron and Poxton (2007), to detect *C. botulinum* by demonstrating botulinal toxin in an enrichment culture. This was accomplished using specific primers for the types C and D toxin genes, as detailed in Table 1.

Table 1. Primer sequences used for the detection of *C. botulinum* types C and D

Toxin gene	Primer	Sequence (5' - 3')	Product size (bp)	Reference
Type C	CI-F	GCGGCACAAGAAGGATTTG	614	Franciosa et al. (1996)
	CII-R	CGCCGTAACCGGAGTATAT		
Type D	ToxD-F	GTGATCCTGTTAATGACAATG	510	Sunagawa et al. (1992)
	ToxD-R	TCCTTGCAATGTAAGGGATGC		

PCR amplification was performed in a 25 µl reaction containing 2.5 µl of 10x PCR buffer (1.5 mM $MgCl_2$, pH 8.7), 0.125 µl of HotStarTaq DNA Polymerase (Qiagen, Germany), 0.5 µl dNTP mix (10 mM of each), 1.0 µl of each primer, 17.375 µl distilled water, and 2.5 µl DNA template. The DNA of *C. botulinum* types C and D, obtained from a previous outbreak was used as the positive control, with distilled water as the negative control. The amplification process was carried out in a thermal cycler under the following conditions: an initial activation step at 95 ºC for 10 minutes, followed by 35 cycles consisting of denaturation at 94 ºC for 1 minute, annealing at 55 ºC for 1 minute, and extension at 72 ºC for 1 minute. A final extension was carried out at 72 ºC for 10 minutes. PCR products were separated by electrophoresis in 1.5% agarose gel at 100 volt 30 minutes and visualized in a UV transilluminator gel documentation (Syngene G: BOX, USA).

DNA extraction and sequencing

Positive PCR products were subjected to nucleotide sequencing at the Bacteriology Laboratory, National Institute of Animal Health (NIAH, Thailand). Genomic DNA was extracted using an agarose gel-based purification method and further purified with the MinElute Gel Extraction Kit (QIAGEN, Germany), following the manufacturer's instructions. The purified DNA was then cleaned using the ZA DNA Sequencing Clean-up Kit (Zymo Research, USA). Both forward and reverse sequencing reactions were performed using the same primers as used in PCR amplification. Sanger sequencing was conducted using a 3500 Genetic Analyzer (Applied Biosystems, Japan)

Genetic sequence data analysis and phylogenetic tree construction

The nucleotide sequences obtained from Sanger sequencing were reviewed and trimmed for quality using Chromas version 2.6.6 (Technelysium Pty Ltd., Australia). Forward and reverse reads were assembled into consensus sequences using BioEdit version 7.2.5. The consensus sequences were exported in FASTA format (.fasta) and subjected to similarity search using BLASTn against the NCBI GenBank database to confirm the identity of *C. botulinum* types C and D. Multiple sequence alignment was performed using the MUSCLE algorithm implemented in MEGA version 12.0. Phylogenetic tree construction was carried out using the maximum likelihood with 1,000 bootstrap replicates to assess the reliability of the tree topology. The phylogenetic tree was visualized and edited in MEGA version 12.0, and evolutionary distances were calculated using the Kimura 2-parameter model.

Results and Discussions

A total of 11 out of 400 fecal samples (2.75%) tested positive for *C. botulinum* types C and D. These samples were collected from dairy cattle that were clinically healthy and showed no signs or symptoms of botulism at the time of sampling. Specifically, the animals included in the study were lactating cows (n = 167), dry cows (n = 86), pregnant heifers (n = 50), heifer weaners (n = 60), and calves (n = 37). All animals were confirmed to be asymptomatic by farm veterinarians prior to sample collection, and all 11 positive cases were identified in animals without any clinical signs from four groups as lactating cows (1.80%, 3/167), dry cows (6.98%, 6/86), pregnant heifers

Table 2. Distribution of *Clostridium botulinum* types C and D in the fecal samples of dairy cattle, determined by PCR technique

Source of fecal samples	Total detected/ No. of examined (%)	*C. botulinum type C*	*C. botulinum type D*
		Detected/Total detected (%)	Detected/Total detected (%)
Lactating cows	3/167 (1.80)	2/3 (66.67)	1/3 (33.33)
Dry cows	6/86 (6.98)	4/6 (66.67)	2/6 (33.33)
Pregnant heifers	1/50 (2.00)	1/1 (100.00)	0
Heifer weaners	0/60 (0.00)	0	0
Calves	1/37 (2.70)	0	1 (100.00)
Total	11/400 (2.75)	7/11 (63.64)	4/11 (36.36)

(2.00%, 1/50), and calves (2.70%, 1/37). No positive samples were detected from heifer weaners (0/60) or feedstuffs (0/10). (Table 2)

The results of this study identified *C. botulinum* in fecal samples from healthy dairy cattle that exhibited no clinical signs or symptoms of the disease. This finding is consistent with the study by Notermans et al. (1981), who also detected *C. botulinum* in fecal samples from cows with no clinical symptoms observed on a farm that had previously experienced a botulism outbreak. Similarly, Dahlenborg et al. (2003) reported the presence of *C. botulinum* in fecal samples of cattle collected at Swedish slaughterhouses, suggesting that asymptomatic carriers of the bacterium can be found. Additionally, Souillard et al. (2015) documented the occurrence of *C. botulinum* in healthy cattle following outbreaks of poultry botulism in mixed farms. Moreover, Abdel-Moein and Hamza (2016) also observed the presence of *C. botulinum* in fecal samples from healthy dairy animals. Findings from this study, in conjunction with prior research, suggest that asymptomatic carriage of *C. botulinum* may be more common in cattle than previously recognized. This observation aligns with general evidence indicating that environmental sources and the gastrointestinal tracts of healthy animals frequently harbor *C. botulinum* spores (CFSPH, 2018). Following the January 2023 outbreak reported by Narongwanichgarn et al. (2023), in which clinical signs of botulism were observed in dry cows, heifers, and weaned heifers, while lactating and prepartum cows remained unaffected, several control measures were implemented. These included the disposal of potentially contaminated feed, improvements in feed storage, and cleaning and disinfection of facilities and equipment. Despite these efforts, detection the same toxin types of *C. botulinum* in asymptomatic animals one year later indicates that environmental contamination may have persisted. This suggests that while the interventions may have mitigated clinical disease, they were likely insufficient to eliminate spores already established in the farm environment. As *C. botulinum* spores are known for their environmental resilience, particularly in organic materials such as soil, bedding, and poorly fermented silage, complete eradication remains challenging without long-term monitoring and rigorous biosecurity practices.

In the present study, the higher detection rate in dry cows compared to other groups may be attributed to multiple contributing factors. These include exposure to a broader variety of silages with longer storage durations, which increases the risk of contamination with *C. botulinum* spores from soil, decaying organic matter, or animal remains (Driehuis et al., 2018; Espelund and Klaveness, 2014; Goldsztejn et al., 2020; Relun et al., 2017). Physiological changes during the dry period such as reduced feed intake, heat stress, and alterations in the gut microbiota may also compromise the host's ability to clear ingested spores (Wen et al., 2021; Zhang, 2022). Moreover, the predominance of *C. botulinum* type C over type D in dry cows could reflect

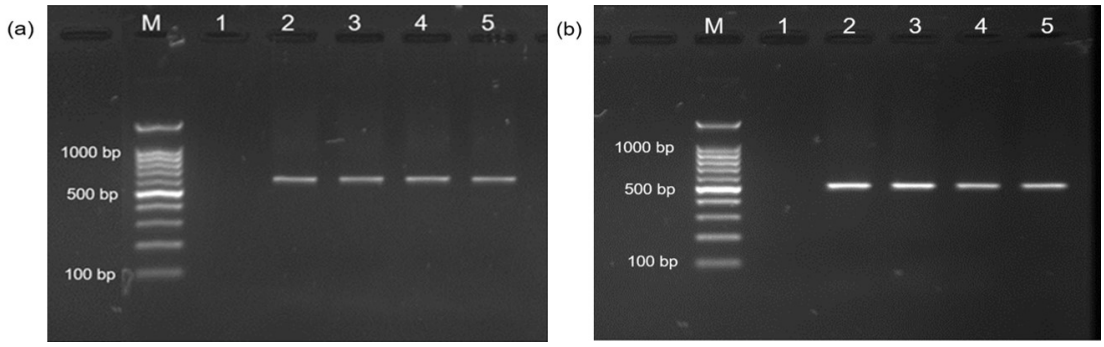


Figure 1. Lane M: DNA ladder; Lane 1: negative control. (a) Lanes 2-5: PCR products for the toxin genes of *C. botulinum* type C showed specific bands at 614 bp. (b) Lanes 2-5: PCR products for the toxin genes of *C. botulinum* type D showed specific bands at 510 bp.

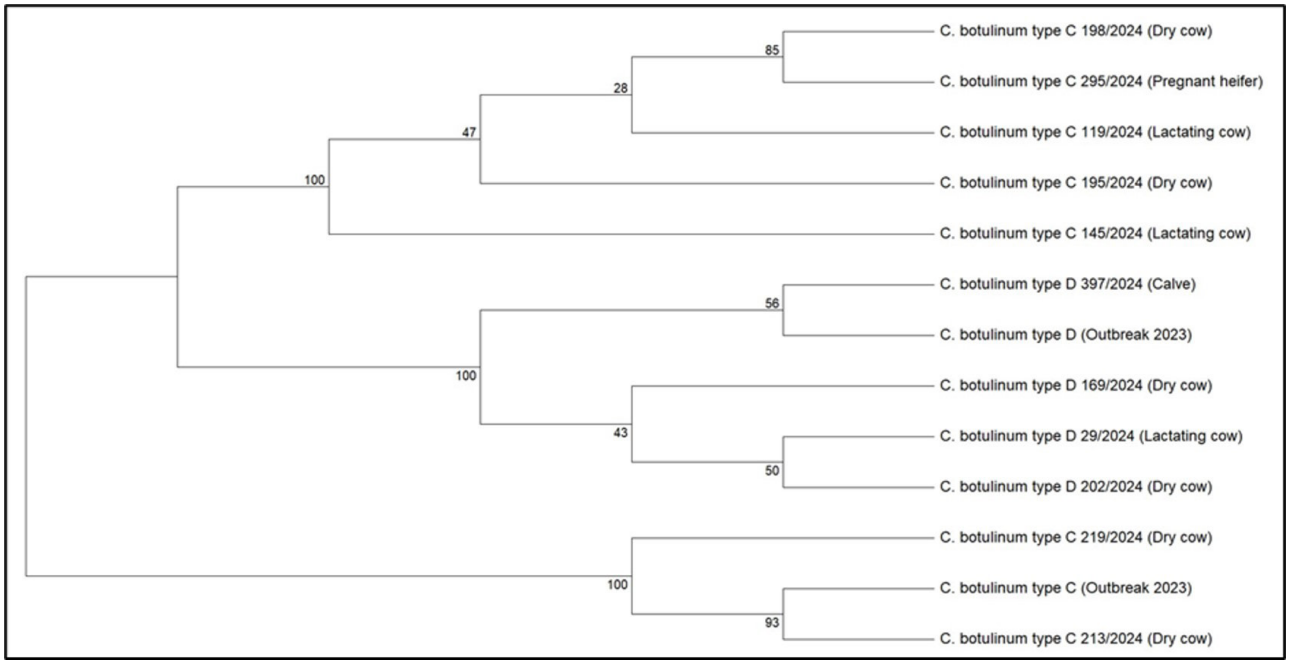


Figure 2. Phylogenetic tree based on nucleotide sequences of *C. botulinum* type C and D isolates from fecal samples of dairy cattle in 2024, compared with outbreak-associated strains from 2023. Bootstrap values (%) are shown at branch nodes. Isolate information includes toxin type, sample ID, year, and animal category. Clustering patterns suggest possible genetic relatedness between outbreak and strains identified in this study.

a combination of biological and ecological factors, including differences in environmental distribution, spore germination, toxin production, and host absorption dynamics. While both toxin types are pathogenic in cattle, type D is more frequently associated with outbreaks linked to poultry litter and decomposing carcasses. Type C, on the other hand, may exhibit greater environmental stability and enhanced adaptability within the ruminant gastrointestinal tract (Meurens et al., 2023; Relun et al., 2017). Therefore, the predominance of type C in this study may be attributable to local environmental conditions as well as host pathogen interactions specific to dry cow physiology and management practices. These findings highlight the complex ecology of *C. botulinum* on dairy farms and emphasize the importance of long-term environmental management and targeted surveillance, particularly in susceptible groups

such as dry cows. As noted by WOAH (2022), spores commonly reside in soil and water and may be ingested or acquired through environmental contact, contributing to a self-perpetuating contamination cycle as animals shed spores back into the environment. Effective long-term prevention, therefore, requires not only reactive responses during outbreaks but also proactive strategies aimed at reducing environmental spore loads and disrupting this contamination cycle.

In addition, Meurens et al. (2023) emphasize that the epidemiological cycle of *C. botulinum* on cattle farms significantly contributes to contamination leading to botulism outbreaks, with several pathways involved, including commercial feed, drinking water, litter, silage, forage, and pasture. *C. botulinum* spores can also contaminate litter by absorbing manure from asymptomatic poultry, feces or bodily fluids from cattle, including feces from commensal animals (Meurens et al., 2023; Souillard et al., 2021). However, in our study, the specific source of the pathogen in the fecal samples collected from the rectum of dairy cattle could not be definitively identified, as feedstuff samples showed no evidence of contamination. Nevertheless, we are reasonably certain that it is the same strain responsible for the illness and mortality in dairy cattle during the botulism outbreak in January 2023, as confirmed by DNA sequencing results from positive samples. Notably, the study identified a positive fecal sample from a calf raised on pasture, which is of particular concern. This raises the possibility that feces containing the pathogen could be excreted, potentially leading to widespread environmental contamination and posing a risk of reintroducing the pathogen into the cattle population.

Our study demonstrated that even after one year, the pathogenic organism responsible for the disease could still be detected within the farm. This indicates that *C. botulinum* may persist in the farm environment or in asymptomatic carrier animals. The relatively low detection rate (only 11 out of 400 animals) could be attributed to several possible factors. For instance, intermittent shedding of *C. botulinum* spores by asymptomatic carriers might result in false negative outcomes when fecal samples are collected from a single point in time (Rasetti-Escargueil et al., 2019; Souillard et al., 2021). Additionally, biosecurity and hygiene measures possibly implemented following the 2023 outbreak may have reduced environmental contamination and limited the risk of re-exposure, including the year-long gap between the outbreak and the sampling period also may have allowed natural clearance of spores in gastrointestinal tract of animals.

Conclusion

This study confirmed the persistence of *C. botulinum* types C and D in the feces of clinically healthy dairy cattle one year after a previous outbreak, suggesting possible gastrointestinal colonization and intermittent fecal shedding. These findings highlight the ongoing risk of environmental contamination and the potential for disease recurrence. To mitigate this risk, several measures should be taken. Regular environmental monitoring through systematic sampling of feed, water, soil, and bedding is essential for early detection of contamination. Strengthening biosecurity practices is also necessary and should include restricted movement between animal housing areas, installation of disinfection stations, use of dedicated footwear and clothing, and strict adherence to sanitation protocols. Vector control should be prioritized by implementing pest management strategies to reduce populations of rodents, birds, flies, and other potential carriers of *C. botulinum* spores. Farmer education plays a critical role in prevention and should be supported through ongoing training focused on early recognition of clinical signs, proper carcass disposal, effective disinfection, and emergency response procedures. Additionally, fecal screening of asymptomatic animals should be integrated into routine herd health programs to monitor potential silent carriers. An integrated approach that combines health surveillance, environmental monitoring, enhanced biosecurity, vector control, and farmer education is essential to reducing the risk of future botulism outbreaks in dairy farms.

Conflicts of Interest

The authors declare no conflicts of interest.

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