Outbreak Reports

An Outbreak of Foodborne Botulism Caused by *Clostridium botulinum* BoNT/A3 in Pickled Eggs — Weihai City, Shandong Province, China, July 2024

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Summary

What is already known about this topic?

Foodborne botulism is caused by botulinum neurotoxin (BoNT). *Clostridium botulinum* (*C. botulinum*) is a strictly anaerobic, Gram-positive bacterium, which is a key pathogen capable of producing BoNT. BoNTs can be classified into seven serotypes (A to G) based on their antigenic properties. Among these, BoNT/A is one of the most common serotypes.

What is added by this report?

This was a case of foodborne botulism in Weihai caused by homemade pickled eggs contaminated with *C. botulinum*. Five individuals presented with symptoms, while three were asymptomatic. All patients received botulinum antitoxin treatment, and no deaths occurred. During this outbreak, 12 isolates of *C. botulinum* were obtained. The phylogenetic analysis results revealed that all the isolates came from the same origin.

What are the implications for public health practice?

This outbreak indicated that foodborne botulism remains a public health issue in China. We need to strengthen publicity and education efforts to inform people of the potential risk of botulism associated with consuming homemade traditional pickled foods. Heating and boiling homemade foods thoroughly can destroy toxins and prevent foodborne botulism.

On July 15, 2024, two patients suspected of foodborne botulism presented to the Emergency Department of Weihai Municipal Hospital in Shandong Province, China. The patients, who had ingested homemade pickled eggs, presented with symptoms such as foot/wrist drop, difficulty breathing, diplopia, and ptosis. The Huancui District CDC and Weihai CDC immediately initiated epidemiological investigations, *C. botulinum* toxin detection, strain isolation, and molecular identification. Based on the epidemiological investigation, laboratory testing, and toxicological test results, this event was determined to be an outbreak of foodborne botulism caused by consumption of homemade pickled eggs contaminated with *C. botulinum*.

INVESTIGATION AND RESULTS

At 07:01 and 09:39 on July 15, Weihai Municipal Hospital admitted two severely ill patients (Patient A and B) presenting with identical symptoms, including fatigue, foot/wrist drop, nausea, shortness of breath, chest tightness, dyspnea, paralysis, speech difficulties, blurred vision, diplopia, and ptosis (Figure 1). Both patients had consumed homemade pickled eggs. The hospital made a preliminary diagnosis of a foodborne disease outbreak and promptly reported it to the Huancui District CDC. The Huancui District CDC arrived at the hospital at 12:00 to conduct an epidemiological investigation, focusing on the source of the pickled eggs and other individuals who had consumed them. Preliminary judgments indicated a suspected outbreak of foodborne disease caused by botulinum toxin in contaminated pickled eggs. The initial epidemiological investigation report on the incident was submitted to the Huancui District Health Commission and Weihai CDC at 14:30.

From July 16 to 17, the hospital admitted three additional patients (Patients C, D, and E) with the same food exposure history and similar symptoms. The Huancui District CDC investigated the incident. Epidemiological investigation revealed Patient B prepared pickled eggs using home-raised chicken eggs in late June 2024. The eggs, laid by free-range hens in the village, were washed, boiled, cooled, and brined with edible salt at room temperature for approximately two weeks. From July 12 to 14, Patient B gave

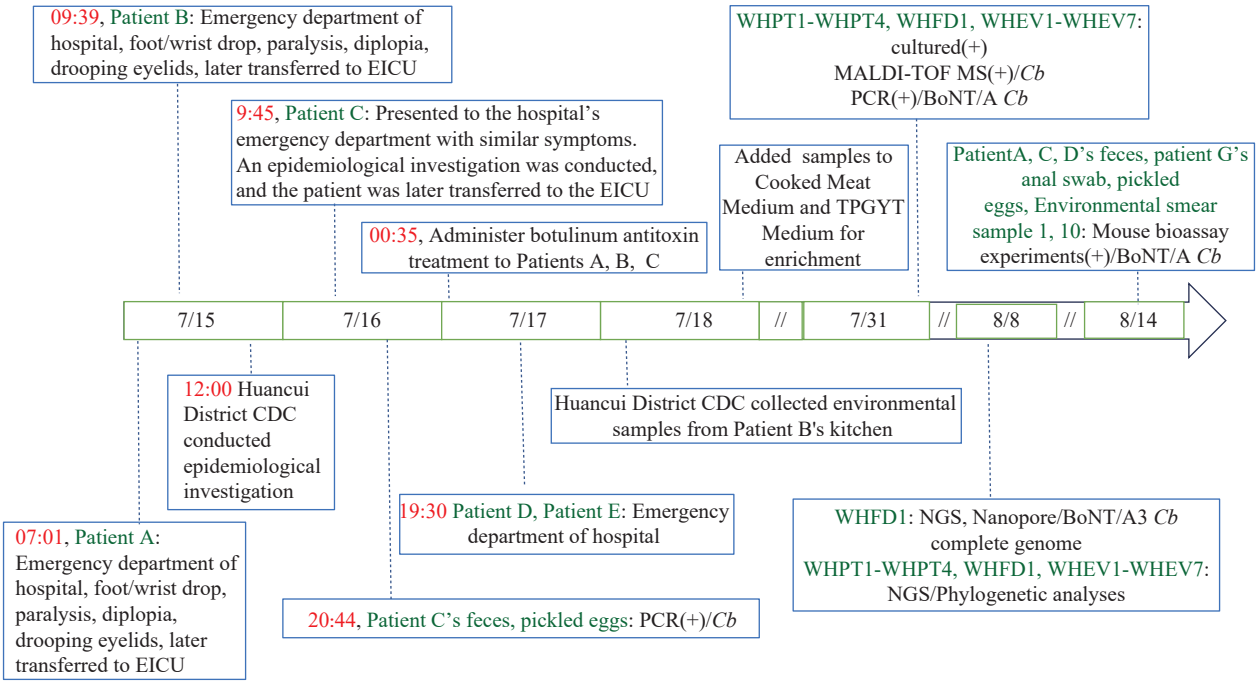

FIGURE 1. Timeline of symptom onset, epidemiological investigation, and pathogen identification in patients.
Abbreviation: EICU=emergency intensive care unit; *Cb=Clostridium botulinum*; MALDI-TOF MS=Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry.

portions of the pickled eggs to her son (Patient D) and daughter-in-law (Patient C), who then shared them with colleagues (Patients A, E, F, G, and H). A total of eight individuals consumed the pickled eggs without heating (Table 1) and subsequently presented with varying degrees of similar symptoms.

On July 16, clinical samples (Patient A's enema solution and Patient C's feces) and pickled eggs collected from the hospital were sent to Weihai CDC for laboratory testing and analysis. Quantitative polymerase chain reaction (qPCR) detection was performed on the clinical samples and pickled eggs using the nucleic acid detection reagent kit for *Clostridium botulinum* (types A, B, E, and F). Patient C's feces and pickled eggs tested positive for *C. botulinum* BoNT/A (Table 2).

At 00:35 on July 17, based on the symptoms and qPCR results, the hospital administered botulinum antitoxin treatment. By July 18, three patients (Patients A, B, and C) were being treated in the Emergency Intensive Care Unit (EICU) of Weihai Municipal Hospital. Two patients (Patients D and E) had mild symptoms; no deaths occurred.

On July 18, to identify and trace the outbreak source, the Huancui District CDC collected 10 environmental samples from Patient B's kitchen and refrigerator. These samples included smears of raw eggs, kitchen utensils used for storing and cleaning pickled eggs, and swabs from the refrigerator and garbage bin (Table 2).

A total of 18 samples, including clinical, pickled egg, and environmental samples collected from July 16 to 18, were processed for enrichment culture and strain isolation according to GB 4789.12-2016 (*1*). Samples were added to Cooked Meat Medium and TPGYT Medium and anaerobically incubated at 36 ℃ and 28 ℃, respectively, for 10 days to enrich for *C. botulinum*. A loopful of enrichment broth that tested positive for the toxin gene by qPCR was streaked onto blood agar and anaerobically incubated at 36 ℃ for 24 hours. Suspected colonies exhibiting flat, smooth, spreading growth with irregular edges were subcultured on blood agar and further identified using qPCR and the Microflex MALDI-TOF MS system (Bruker, Germany).

On July 31, based on qPCR and MALDI-TOF MS results, 12 strains of botulinum bacteria were isolated from the pickled eggs, rectal swabs/feces of patients, and environmental specimens (including smears from plates, sinks, and trash cans). A total of 7 enrichment cultures were selected and sent to the China CDC for mouse bioassay experiments. On August 14, the mouse bioassay showed that all 7 enrichment cultures contained botulinum neurotoxin type A (BoNT/A)

TABLE 1. Basic information of individuals who consumed the pickled eggs, including eating habits and disease conditions — Weihai City, China, July 2024.

Patients	Sex	Age (years)	Eating time	Amount of eggs consumed	Onset time
Patient A	Female	43	07:00, July 13	1	08:00, July 13
			11:30, July 12	1	10:00, July 13
Patient B	Female	57	17:30, July 12	2	10:00, July 13
Patient C	Female	38	11:30, July 12	1	07:00, July 15
			11:30, July 13	1	
			17:30, July 14	1	
Patient D	Male	39	17:30, July 14	1	13:00, July 16
Patient E	Female	56	11:30, July 13	1/2	20:00, July 17
Asymptomatic patient F	Male	31	11:30, July 13	1	−
Asymptomatic patient G	Female	23	11:30, July 13	1/4	−
Asymptomatic patient H	Female	54	11:30, July 13	1/2	−

Note: "−" means no symptoms have appeared.

TABLE 2. qPCR, enrichment, isolation, MALDI-TOF MS, WGS, and mouse bioassay experiments of samples collected in this study — Weihai City, China, July 2024.

Source	qPCR before enrichment	Enrichment in cooked meat medium and TPGYT medium	qPCR after enrichment	Colony morphology on the blood agar	MALDI-TOF MS	isolated strain	WGS	Mouse bioassay experiments
Patient A (feces)	NEG	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHPT1	BoNT/A3	BoNT/A
Patient C (feces)	BoNT/A	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHPT2	BoNT/A3	BoNT/A
Patient D (feces)	NEG	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHPT3	BoNT/A3	BoNT/A
Patient E (feces)	NEG	Digestion of meat particles	NEG	ND	ND	ND	ND	ND
Asymptomatic Patient F (Anal swab)	NEG	Digestion of meat particles	NEG	ND	ND	ND	ND	ND
Asymptomatic Patient G (Anal swab)	NEG	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHPT4	BoNT/A3	BoNT/A
Asymptomatic Patient H (Anal swab)	NEG	Digestion of meat particles	NEG	ND	ND	ND	ND	ND
pickled eggs	BoNT/A	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHFD1	BoNT/A3	BoNT/A
EV 1 (raw eggs)	ND	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHEV1	BoNT/A3	BoNT/A
EV 2 (Inner pot of rice cooker)	ND	No digestion of meat particles	NEG	ND	ND	ND	ND	ND
EV 3 (refrigerator)	ND	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHEV2	BoNT/A3	ND
EV 4 (Sink)	ND	No digestion of meat particles	NEG	ND	ND	ND	ND	ND
EV 5 (pot)	ND	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHEV3	BoNT/A3	ND
EV6 (kitchen cupboards)	ND	No digestion of meat particles	NEG	ND	ND	ND	ND	ND
EV 7 (Salt jar)	ND	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHEV4	BoNT/A3	ND
EV 8 (casserole)	ND	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHEV5	BoNT/A3	ND
EV 9 (Dining plate)	ND	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHEV6	BoNT/A3	ND
EV 10 (garbage bin)	ND	Digestion of meat particles	BoNT/A	Flat, spreading growth pattern	*C. botulinum*	WHEV7	BoNT/A3	BoNT/A

Abbreviation: qPCR=quantitative polymerasechain reaction; EV=environmental smear sample; ND=not detected; NEG=negative.

(Table 2).

On August 8, we performed sequencing of 12 *C. botulinum* strains isolated in this study. Additionally, 67 complete *C. botulinum* genome sequences were downloaded from the GenBank database. Snippy (v4.6.0, Melbourne, Australia) was used to perform core genome single nucleotide polymorphism (SNP) calling and phylogenetic tree construction on the 12 *C. botulinum* isolates and 67 *C. botulinum* genome sequences. Phylogenetic analysis revealed that the 12 *C. botulinum* strains isolated in this study were located on the same evolutionary branch (Figure 2A), suggesting a common origin.

To investigate the genomic characteristics of *C. botulinum* in this study, we performed whole-genome sequencing (WGS) of the WHFD1 strain isolated from pickled eggs. Sequencing revealed that the WHFD1 genome comprised a circular chromosome and a circular plasmid (Figure 2B), with genome sizes of 3,955,437 bp and 243,977 bp, respectively. The GC contents were 28.19% and 25.48%, respectively. Annotation indicated that these genomes harbored 3,554 and 298 coding DNA sequences (CDS) regions, respectively. Average nucleotide identity (ANI) analysis showed that the chromosomal genome of WHFD1 shared 97.53% identity with the *C. botulinum*

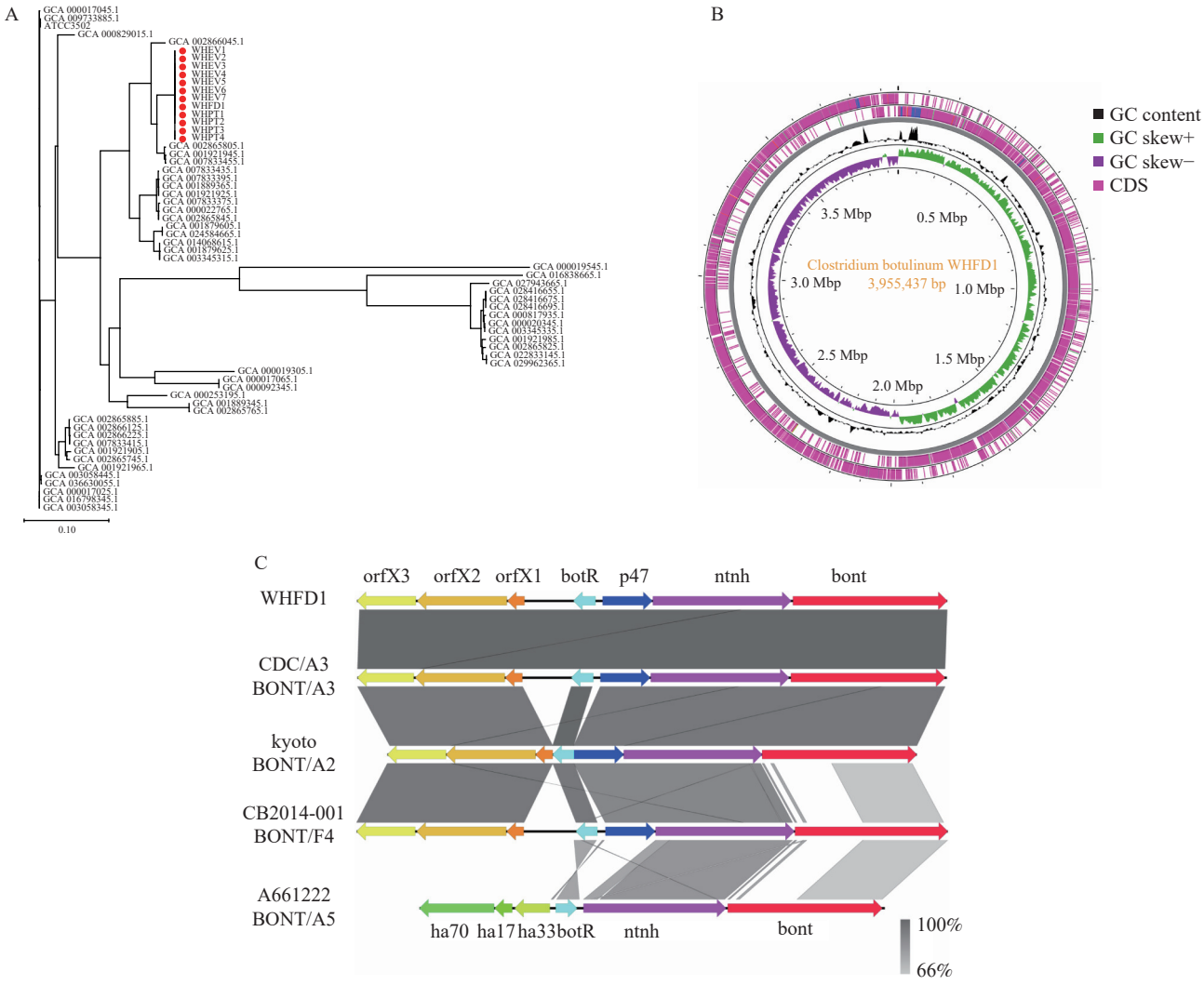

FIGURE 2. The genomic characteristics of *C. botulinum* strains in this study. (A) Phylogenetic tree of *C. botulinum* strains based on coreSNP analysis; (B) Circular map of the WHFD1 isolate strain genome; (C) Comparison of the *bont* gene cluster arrangements in the WHFD1 genome and four *C. botulinum* genomes downloaded from GenBank.
Note: The label names marked with red dots in panel A are the sequences of this study; These circles in panel B represent (from inside to outside): 1) GC skew, 2) GC content, 3) and 4) coding sequences (CDS); the different colors in panel C represent different genes in the bont gene clusters. The gradient of gray color represents the different BLAST identity values.

reference sequence ATCC3502, confirming the identification of WHFD1 as *C. botulinum*. The *bont* gene in the WHFD1 genome was located on the plasmid, with no *bont* gene detected on the chromosome. BLAST comparison showed that the *bont* gene had the highest homology with the *bont/A3* subtype.

Furthermore, we analyzed the *bont* gene cluster encoding botulinum neurotoxin in the WHFD1 isolate. We extracted the *bont* gene cluster from the WHFD1 genome and downloaded four distinct *bont* gene clusters (A3, A2, F4, and A5) from the GenBank database. Using Easyfig (version 2.2.5, Brisbane, Australia) software (*2*), a collinearity analysis was performed, revealing that the *bont* gene clusters of the WHFD1 isolate shared the same arrangements and locations as BoNT/A3 CDC/A3 (Figure 2C). They both belong to the typical orfX+ gene cluster, which consists of the *bont* gene and five accessory genes. Although BoNT/A2 Kyoto and BoNT/F4 CB-2014001 also belong to the orfX gene cluster, their *bont* gene sizes and arrangements differ partially from that of the WHFD1 strain. The collinearity analysis confirmed that the WHFD1 strain belonged to the BoNT/A3 subtype, consistent with the BLAST result.

In this incident, five cases presented with similar symptoms, including blurred vision and ptosis as the primary symptoms, accompanied by difficulties swallowing, speaking, and breathing. These findings were consistent with the characteristics of botulinum toxin poisoning. After treatment with botulinum antitoxin, significant improvements were observed.

The qPCR assays conducted on fecal samples from the patient and pickled eggs for BoNT/A *C. botulinum* both yielded positive results. Twelve strains of *C. botulinum* were isolated from the pickled eggs, rectal swabs and feces of patients, and environmental specimens. Phylogenetic analysis confirmed that they originated from the same source. Whole-genome sequencing (WGS) revealed that all strains belonged to the BoNT/A3 subtype.

Based on a comprehensive analysis of the clinical symptoms, epidemiological investigation results, and laboratory test findings, we concluded that this outbreak was a case of foodborne botulism caused by homemade pickled eggs contaminated with *C. botulinum* producing BoNT/A3.

PUBLIC HEALTH RESPONSE

On July 19, the Weihai CDC published "Summer Alarm Bell: Foodborne Disease Crisis Caused by Botulinum Toxin" online, which provided a detailed introduction to botulinum toxin, poisoning symptoms, high-risk food sources, and preventive measures. The publication recommended avoiding homemade fermented and pickled foods.

Additionally, we suggest that medical institutions maintain a reserve supply of botulinum antitoxin for emergency use.

DISCUSSION

C. botulinum is a strictly anaerobic, Gram-positive bacterium whose spores are ubiquitous in soil and livestock feces. When foods such as fruits, vegetables, meat, and grains are contaminated by *C. botulinum* spores, the bacteria can proliferate rapidly and produce toxins under anaerobic conditions. In 1897, van Ermengem first isolated a strain of *C. botulinum* from salted ham associated with a foodborne botulism outbreak in Belgium (*3*). In China, Wu et al. first confirmed the presence of botulism in Xinjiang in 1958 (*4*). As of 2020, a total of 22 PLADs in China have reported cases of foodborne botulism (*5*). Most foodborne botulism cases occurred in northwestern China, including Xinjiang and Qinghai. The most common contaminated sources of botulism were homemade pickled foods, such as stinky tofu and dried beef (*5*).

In this study, the contaminated food source was pickled eggs. Epidemiological investigations revealed that the eggs originated from free-range hens within the village. Their surfaces may have been contaminated by *C. botulinum* from soil or livestock feces. Inadequate cleaning before pickling and the anaerobic conditions produced during the pickling process likely led to botulinum toxin production. Botulinum toxin is heat-labile and can be completely destroyed by heating at 80 ℃ for 30 minutes or 100 ℃ for 10 to 20 minutes. In this case, individuals consumed the pickled eggs without heating. We speculate that this was the primary cause of this foodborne botulism outbreak. We recommend thoroughly heating pickled foods before consumption to eliminate botulinum toxin and avoid similar incidents.

We used WGS to perform source tracing analysis, strain typing, and virulence gene subtyping. Core SNP analysis indicated that the 12 *C. botulinum* isolates originated from the same source. This finding was consistent with epidemiological investigation results and provided evidence for source tracing analysis. *C.*

botulinum can be classified into seven serotypes (A to G) based on antigenic properties. Through WGS and BLAST comparison, all isolated strains were confirmed as BoNT/A3. The BoNT/A3 subtype is frequently found in the Southern Hemisphere, with outbreaks reported in countries such as Australia and Argentina (*6*). BoNT is encoded by the *bont* gene cluster, which is located on either the chromosome or plasmid of *Clostridium* species. The *bont* gene clusters are classified into two types: orfX+ and ha+. The *bont/A3* gene belongs to the orfX+ gene cluster, which comprises the botulinum neurotoxin-encoding gene (*bont*) and several accessory genes, including *orfX1*, *orfX2*, *orfX3*, *botR*, *p47*, and *ntnh* (*7*). Obtaining the complete *C. botulinum* genomes in this study allowed us to identify the locations and arrangements of *bont* gene clusters within the isolates. This is important for strain typing and virulence gene detection.

This investigation revealed that foodborne botulism remains a public health issue in China. Therefore, strengthening education regarding the health risks of consuming homemade, traditionally pickled foods is critical to safeguarding public health.

Conflicts of interest: No conflicts of interest.

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