New PCR-based testing protocols to detect *C. botulinum* in powdered infant formula

GenomeTrakr Meeting 2026

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Overview

1. A new PCR-based *C. botulinum* detection method was implemented during the 2025 Infant Botulism outbreak to support the traceback investigation
   - Results from this method were the first to identify a contaminated ingredient as the likely source of the powdered infant formula contamination
2. A new PCR-based surrogate test was developed in 2026 to providing industry with a reliable method for monitoring for a risk of *C. botulinum* contamination
   - A pass/fail “grade” is reported within 72 h

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1. PCR-based methods for *C. botulinum* detection

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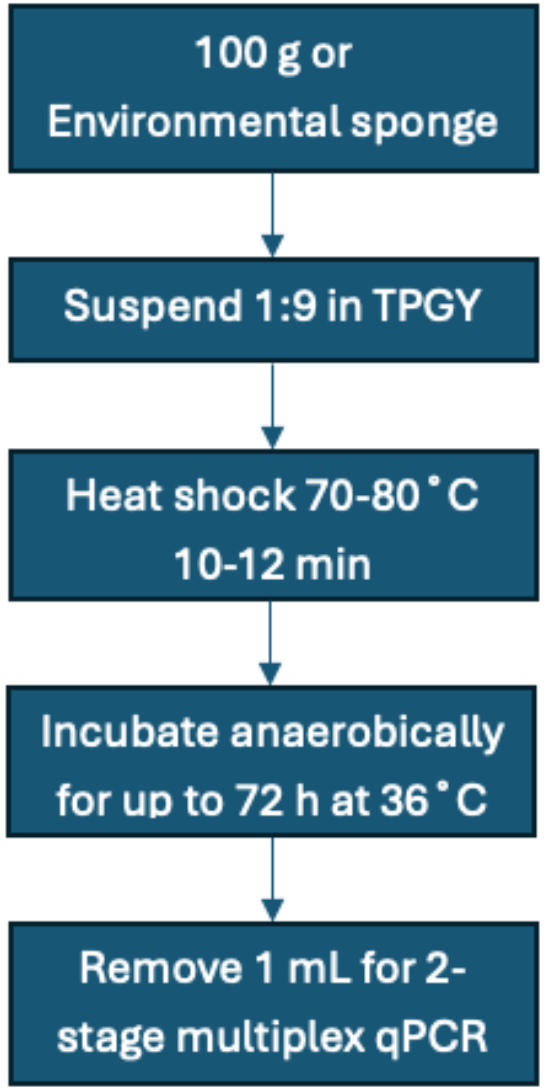
Matrices tested

- **Powdered infant formula (PIF) ingredients** – milk powders (whole and skim), whey protein powder, lacprodan, organic oil blend, lactose, lactalbumin, lactoferrin, corn syrup liquid, etc.
- **Base powder**
- **Finished powder (PIF)**
- **Environmental –** sponges, air samples, filters, water
- **Packing material** – swabs of bulk bags, can ends, plastic lids, cans, can slip sheets

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Sample Preparation & Enrichment



Detection and characterization of *Clostridium botulinum* isolated from powdered infant formula

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C. botulinum PCR detection and isolation

- Detection via a tiered approach
  1. Presumptive positive – multiplex qPCR detects *C. botulinum*
  2. Molecular confirmation – alternative PCR-based confirmation to address isolation challenges
  3. Culture isolation
  4. Strain typing - WGS



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Step 1: Multiplex qPCR

- Two (2) separate multiplex PCR tests
  1. *Clostridium* spp. (16S) + BoNT/A + BoNT/B +IAC
  2. BoNT/E + BoNT/F + IAC

- BoNT PCR according to ISO/TS 17919:2013 Annex C

- BoNT-positive enrichments proceed to molecular confirmation

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Step 2: Molecular confirmation

- Presumptive positive enrichments subject to additional PCR tests targeting different BoNT loci than the primary screen(s)
  - ISO primers used for presumptive detection
  - FDA and/or in-house primers for the appropriate BoNT target utilized for molecular confirmation

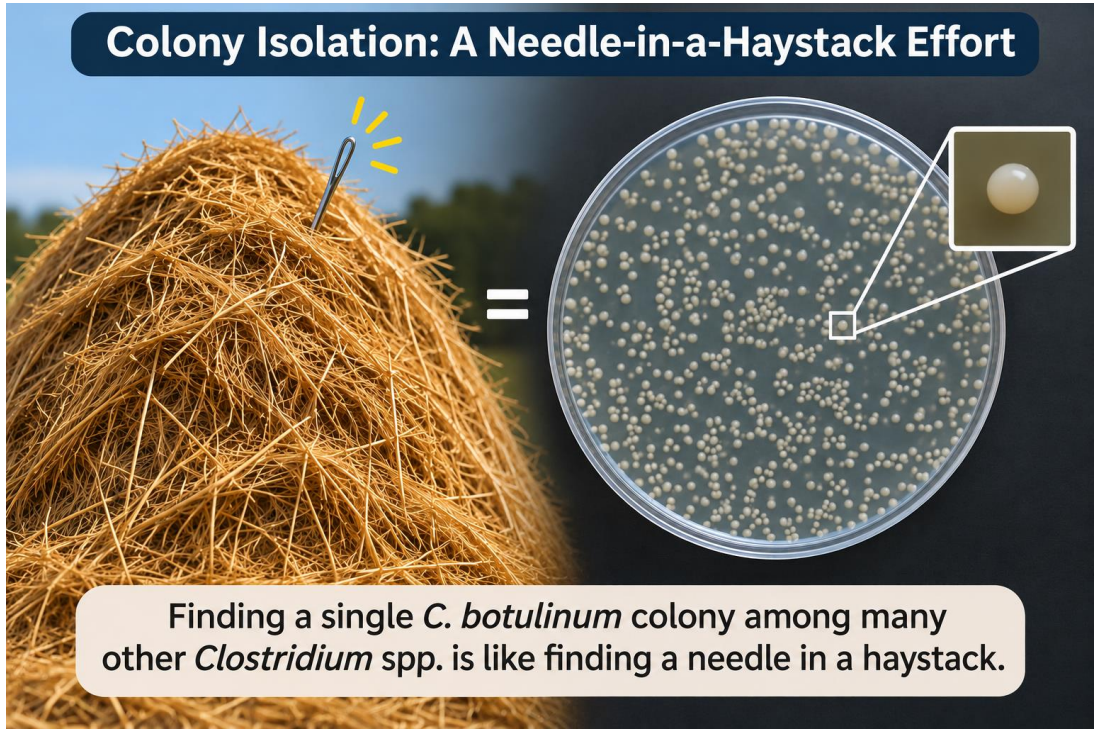
- BoNT PCR product(s) sequenced using Minion (Oxford Nanopore Technologies)
  - Several samples had multiple neurotoxin types (e.g., A and B)

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Step 3: Isolation

- Molecularly confirmed enrichments were streaked to Botulinum Selective Media (BSM)
- Multiple subculture steps using Brain Heart Infusion (BHI) agar w/ antibiotics
- “Needle in a haystack” challenge



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Detection & Isolation: Result Summary

Table 1. *C. botulinum result summary from 2025 outbreak investigation*

Category	N[1]	Presumptive Positive		Molecularly Confirmed		Cultural Isolation	
		Positive	%	Positive	%	Positive	%
Ingredients	1701	15	0.88	4	0.24	3	0.18
Base Powder	83	3	3.6	3	3.6	1	1.2
Finished Product	387	17	4.4	15	3.9	7	1.8
Environmental	468	0	0	-	-	-	-
Packaging materials	1199	0	0	-	-	-	-
Paper slip sheets	850	92	10.8	75	8.8	0	0
Total Samples	4688	127	2.7	97	2.1	11	0.24

11 culture confirmed positives out of 4688 samples tested
3-ingredient (organic whole milk powder)
8-finished product (base powder, PIF)

Only 11% of the molecularly confirmed enrichments yielded a positive isolate

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Cq values predicted the probability of obtaining an isolate

- Majority of *C. botulinum*-positive samples were co-contaminated with other *Clostridium* spp.
- Non-*botulinum* *Clostridium* (e.g., *C. sporogenes*) are typically present at much higher levels
- The differences in Cq (16S vs. BoNT) were used to determine the expected log-level population difference
  - Q: How many colonies need to be selected for identification?
  - A: Often >1000 colonies OR isolation is not likely



Cultural isolation alone would have likely missed several *C. botulinum* positive samples from the outbreak investigation.

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Example: Relative Abundance of *C. botulinum* vs. *Clostridium* spp. as Estimated by Real-Time PCR

Cq. 1 (*Clostridium* spp.)	Cq. 2 (*C. botulinum*)	Δ Cq (=Cq 2-Cq1)	*Clostridium* spp. (%)	*C. botulinum* (%)
11	24	13	99.988	0.012



≈ 1 colony per 10,000 total colonies

Cq (quantification cycle):

PCR value indicating the amount of target organism

- Lower Cq = more target
- Higher Cq = less target
- 1-Cq difference ≈ **2× change** in target number

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- **Differences in Cq** predicted the ratio of *C. botulinum* to other *Clostridium.*
- *C. botulinum* isolates were only obtained when the **ratio was <2000**.
- Isolation of *C. botulinum* required extensive colony screening **(up to 3500 colonies**)

Table 2. Cq data from select molecularly confirmed *C. botulinum*-positive samples

Sample description	Real-time PCR Cq			ΔCq	Log difference	Clost. / C. bot. ratio	Colony isolation
	16S	bont A	bont B				
Base powder-1	16.1	20.8	-	4.7	1.4	25	Yes*
Organic whole milk-1	13.5	21.87	20.18	8.4	2.5	329	Yes
Finished product-1	20.8	21.5	-	0.7	0.2	2	Yes
Finished product-2	12.7	15.7	-	3.0	0.9	8	Yes
Finished product-3	15.3	-	19.9	4.6	1.4	23	Yes
Finished product-4	12.6	18.6	-	5.9	1.8	61	Yes
Finished product-5	12.2	20.0	-	7.8	2.4	229	Yes
Finished product-6	12.4	21.7	-	9.3	2.8	639	Yes
Finished product-7	19.2	29.9	-	10.7	3.2	1,663	Yes
Finished product-8	12.1	27.9	-	15.8	4.8	57,052	No
Finished product-9	11.9	29.9	28.6	18.0	5.4	262,144	No
Finished product-10	10.3	28.7	-	18.4	5.5	345,901	No
Finished product-11	11.4	31.63	-	20.2	6.1	1,229,807	No
Base powder-2	10.6	25.5	-	14.8	4.5	28,725	No
Organic whole milk-2	13.5	28.1	-	14.6	4.4	24,834	No
Organic whole milk-3	15.9	32.4	-	16.5	5.0	92,682	No
Organic whole milk-4	15.7	34.6	-	18.9	5.7	489,178	No
Whey protein-1	13.1	-	30.6	17.5	5.3	185,364	No

Step 4: Strain typing using WGS

- Isolates are using Illumina MiSeq or NextSeq

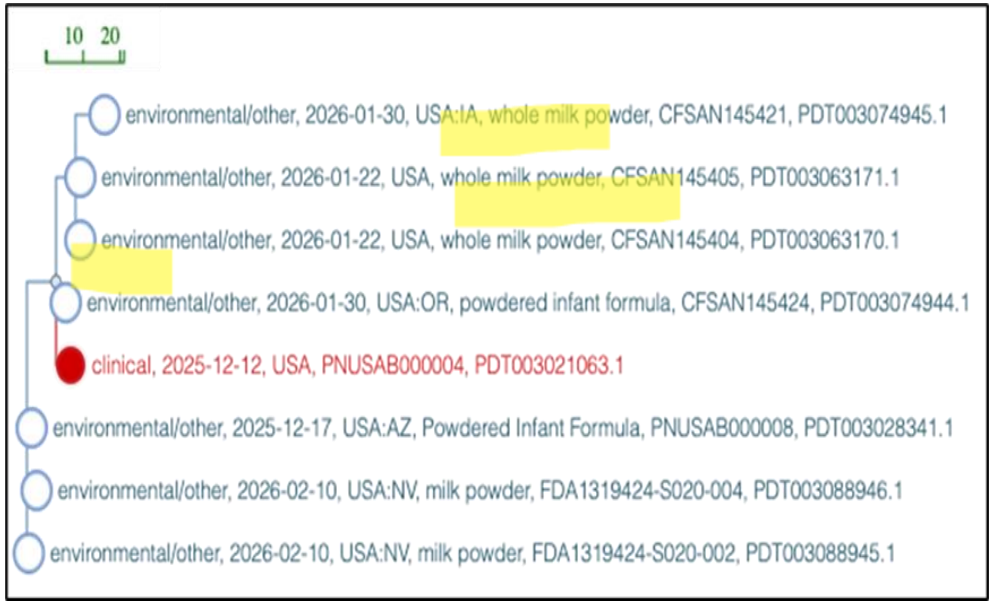


Table 3. Cluster analysis from the *C. botulinum* 2025 outbreak investigation

Sample Type	MLST	Type A	Type B	NCBI Cluster
Finished product	ST1	+	-	PDS000040580.23
Organic whole milk	ST97	+	+	PDS000250219.4
Finished product	ST97	+	+	
Finished product	ST1	+	-	PDS000252757.3
Base powder	ST1	+	-	
Finished product	ST148	-	+	PDS000252986.1
Finished product	ST1	+	-	PDS000253174.2
Finished product	ST1	+	-	Unique
Finished product	ST1	+	-	Unique

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2. PCR-based surrogate method for monitoring for the risk of *C. botulinum*

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The IEH 0-12 PIF Grading Test – A PCR-based *C. botulinum* surrogate method

- PIF ingredients containing *C. botulinum* spores are unsafe for infants age 0-12 months
- The ingredients are safe for consumers > 1yr
- *C. botulinum* is a select agent, a confirmed positive, regardless of intended use will have regulatory consequences
- There is an industry need for a reliable indicator test
- Sulfite Reducing Clostridial (SRC) lacks sensitivity and specificity

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IEH 0-12 PIF Grading Multiplex PCR Test

- Assessing the suitability of PIF or PIF ingredients for infants ages 0-12 months
- Enrichment procedure is harmonized with the *C. botulinum* PCR method
- Results are “graded” and reported as pass/fail

Gene	Target	PASS	PASS	PASS	FAIL
C. botulinum 16S rDNA	*Clostridium* spp.	-	**2**	**2**	**2**
fldB	Proteolytic *Clostridium*	-	-	**2**	**2**
ntnh	Co-localized with *bont*	-	-	-	**10**
Interpretation		0 – All targets absent	2 – *Clostridium* spp.	4 – Proteolytic *Clostridium* spp.	14 – Likely BoNT-producing *Clostridium*

- A grade ≥ 10 is not suitable for infants 0-12 months

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Key Takeaways

- Multiplex qPCR provided key insights into the ratio of *C. botulinum* to other *Clostridium* spp.
- Non-*botulinum Clostridium* dominated the enrichment population by up to 6 orders of magnitude
- Differences in Cq values predicted how challenging it would be to isolate *C. botulinum*
- 90% of samples that molecularly confirmed did not yield an isolate
- Using only a stand-alone cultural method would likely not have detected the outbreak strain
- The IEH 0-12 PIF Grading Test provides a suitable surrogate method for monitoring *C. botulinum* spore contamination

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Thank you!

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