# Laboratory Confirmation of Botulism

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## Objectives

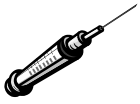
- Methods for toxin detection
- Culture characteristics of toxin producing *Clostridia*, spp
- Safe handling practices

## Botulism Types-

**Foodborne**

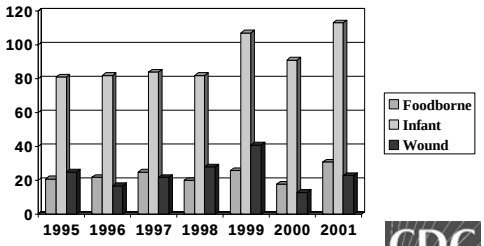
**Infant**
**(& adult colonization)**

**Wound**



## Intentional Contamination

## Laboratory-confirmed Botulism 1995-2001




## United States Capacity for Botulinum Toxin Detection

## Botulinum toxin types

- Seven known toxins:A,B,C,D,E,F,G
  - Human botulism
    - A, B, E, F (C & D were described in late 1950's)
  - Animal
    - B, C, D, E
  - Natural disease unidentified
    - Type G

## Human Case Types

- Food
  - Type E most common in Alaska
  - Type A most common in non-Alaska cases
  - Type B, <15% of cases
  - Type F rare
- Infant
  - Equal distribution of A and B cases;rare F
  - Geographically distributed

## Human Case Types

- Wound
  - Type A, >90%
  - Type B, <10%
- Adult colonization
  - Majority type F
  - Type A also reported

## Neurotoxigenic *Clostridia*, sp

- *Clostridium botulinum*
- *C. baratii*
- *C. butyricum*

## Physiological Groups

| | I | II | III | IV | butyricum | baratii |
|---|---|---|---|---|---|---|
| Toxin | A,B,F | B,E,F | C,D | G | E | F |
| Proteolysis | + | - | - | + | - | - |
| Lipase | + | + | + | - | - | - |
| Lecithinase | - | - | - | - | - | + |
| Opt temp | 35-40 | 18-25 | 40 | 37 | 30-37 | 30-45 |
| Min temp | 10 | 3.3 | 15 | | 10 | |

Note: Non-toxigenic simulates exist

## Egg Yolk Agar Plate Preliminary Identification

- Lipase positive-pearly luster film around the colonies on the surface of the agar
- Lecithinase- opaque precipitate within the agar, diameter of precipitate varies among strains
  - *C. perfringens*, a lecithinase producer is commonly found in human stool

## Non-neurotoxigenic Similates

- *C. botulinum* group I: *C. sporogenes*
- *C. botulinum* group II: no name assigned
- *C. botulinum* group III: *C. novyi*
- *C. botulinum* group IV: *C subterminale*
- *C. baratii*: all typical strains
- *C. butyricum*: all typical strains

## Differentiation from Similates

The only definitive method to differentiate botulinum producing strains from non-neurotoxigenic simulates is through toxin identification

## Culture characteristics of *C. botulinum*

- Anaerobic
- Gram positive (>24 hr culture may be negative)
- Spore former (not always present)
  - Spores resistant to heat
- Sensitive to high salt or sugar
- Inhibited by low pH (<4.6)

## Acceptable Specimens (from patients exhibiting symptoms consistent with the diagnosis of botulism, only)

| Foodborne | Infant | Wound |
|---|---|---|
| serum, gastric, vomitus, stool, sterile water enema, food samples | serum, stool, rectal swabs, potential sources | serum, stool (in case not wound), tissue |

All specimens should be maintained at 4 C, not frozen, until tests are performed.

## Current approved test

- Mouse bioassay is the only currently approved test for the laboratory confirmation of botulism
- An ELISA (FDA/CDC) was recently validated for toxin detection in cultures
  - Currently under evaluation at CDC for utility in the clinical diagnostic laboratory

## Mouse Bioassay

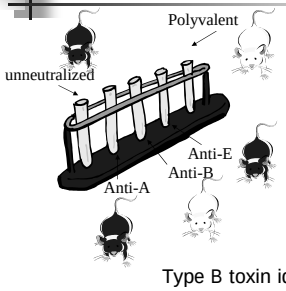
**Advantages**

- Exquisitely sensitive ($10 \times 10^{-12}$ grams= 0.000000000010 g)

**Disadvantages**

- Hazardous
- Extensive animal use
- Only 20 test sites around the country (17 state, 2 local, CDC)

## Basis for Botulinum Toxin Detection




- Preparation of extract
- IP injection into 2 mice
  - Unneutralized
  - Neutralized with specific antitoxin
- 4 day monitor of mice for symptoms

Type B toxin identified

## Sample processing

- Wipe outer surface of container with isopropyl alcohol
- Open containers in a biosafety cabinet

## Sample processing

- Weigh specimen
- Add 1 ml cold gelatin buffer to each gram of specimen (food, stool, etc) up to ~25 grams.
  - Very dry material may require additional buffer
  - Large pieces of material must be cut and/or pulverized before adding buffer.
- Hold sample for 30 minutes at room temperature (several hours or several days at 4 C is acceptable).
- Centrifuge 20 minutes at 27,000 X g to prepare for direct toxin tests

## Specimen culture

- Streak processed specimen on to Egg yolk agar plates for isolation
  - Incubate plates at 35 to 37 C for 4 to 6 days in an anaerobic atmosphere
  - Check plates at 48 hours and then daily
  - Pick suspect colonies and inoculate into broth media
    - Incubate 4 to 10 days
    - Test supernatant for botulinum toxin

## Specimen culture

- Inoculate broth media (2 X chopped meat glucose starch and 1 X Trypticase-peptone-glucose-yeast with trypsin) with ~ 0.5 ml of specimen/buffer suspension.
  - Heat 1 chopped meat tube at 80 C for 10 min; cool to room temperature
  - Incubate all 3 broth cultures at 35 to 37 C for 4 to 10 days
  - Test supernatant for botulinum toxin
  - Streak broth cultures on to Egg yolk agar plates
    - Incubate, select suspect colonies
    - Inoculate into broth media
    - Test for toxin

## Laboratory Tests Flow Chart

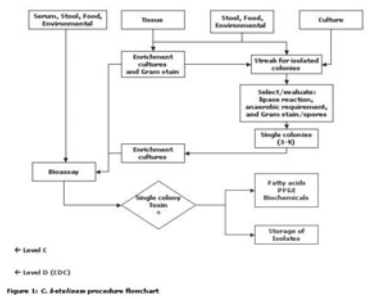


Figure 1: C. botulinum procedure flowchart

## Laboratory Confirmation Criteria

- Clinical specimens
  - Direct toxin test positive and/or
  - Culture positive
    - Isolation of *C. botulinum* from normal humans is rare.
- Source specimens (food, etc)
  - Direct toxin test positive
    - Even in absence of positive clinical samples, positive direct toxin test on consumed food provides laboratory confirmation
  - Culture of *C. botulinum* from source specimens only is NOT confirmatory

## Acceptable Source Specimens

- Food cases
  - Remnants of consumed food (commercial or home-prepared)
  - Unopened home-prepared from same batch as consumed by patient
- Infant cases
  - Honey
  - Opened formula
  - Other foods/liquids given to infant
  - Environmental sampling is discouraged

**Note: Do not test unopened commercial products. Contact FDA if commercial product involved: (301) 443-1240**

## Non-Alaska Native Implicated Foods

Home-canned vegetables of any kind
**Pickled material- pigs feet, artichoke hearts, eggs**
**Herbs in oil (garlic, etc)**
**Smoked fish**
**Potato salad**
**Home prepared soup**
**Temperature abused commercial products**
**Clam chowder**
**Tiramisu-low sugar**
**Baked potato (foil wrapped, room temperature)**
**Cheese sauce (contaminated from potato)**

## Unlikely Foodborne Botulism Sources

**Dried foods**
**"Fresh" vegetables**
**Foods with high sugar content- jams, etc**

**Commercial foods are less likely, but should be considered if they do not fall in the categories listed above**


## Safe Work Practices

- Critical control points
  - Unpackaging specimens
  - Extraction of specimens
    - Mixing buffer with stool
    - Cutting, grinding, mixing of food
  - Centrifugation of extracts and culture supernatants
  - Preparation of syringes
  - Injection of mice


## Safe Work Practices

- Personal protective equipment
  - Gloves
  - Gown
  - Eye protection
  - Facial or desktop plexiglass shield
  - Biological safety cabinet during procedures that could produce aerosols (cutting, grinding, etc of food/environmental specimens)

## Safe Work Practices

- Minute quantities of toxin are hazardous
- Decontamination
  - 0.1N sodium hydroxide
    - Will inactivate toxin
  - 10% household bleach (prepared fresh daily)
    - Will inactivate toxin
    - Will kill vegetative cells and spores
  - Treat spills sequentially (15 to 20 minutes each) with sodium hydroxide, bleach solution, and finally isopropyl alcohol (to reduce caustic effects of decontamination procedure)

## Safe Work Practices

- Waste handling
  - All material with potential contact with botulinum toxin and/or *C. botulinum* must be autoclaved for 60 minutes, 121C, at 15 to 20 PSI
  - Since small quantities of toxin may cause illness, all material in the laboratory should be considered contaminated

## Safe Work Practices

- Response to potential exposure
  - Workers should be made aware of early symptoms of botulism
    - Blurred or double vision
    - Dry mouth
    - Slurred speech
    - Peripheral muscle weakness
  - Self-monitor 2 to 4 days for symptoms
  - Report to emergency care facility if symptoms develop
  - Prophylactic antitoxin is not administered in the absence of symptoms

## Botulinum Toxoid Vaccine

- "Investigational New Drug"- for the past 30+ years
- Available for laboratory workers through your health clinic after enrollment with CDC Drug Services (404 639-3356)
- Initial series: 0, 2 weeks, 12 weeks, 1 year
- Booster provided every 2 years following proof of need (serum submission to CDC for residual antitoxin test prior to boost)
- May eliminate future treatment options of therapeutic toxin preparations.


## Summary

- The mouse bioassay is the only currently accepted method for laboratory confirmation of botulism.
- Detection of toxin directly in clinical specimens or source specimens provides laboratory confirmation; production of toxin in cultures of clinical specimens also provides confirmation.
- Most botulinum producing cultures produce lipase on egg yolk agar plates and some rare strains produce lecithinase; however non-neurotoxigenic simulates exist.
- Good safe work practices are essential for handling *C. botulinum* and it's associated neurotoxin.


## For Additional Information

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