

Laboratory Confirmation of Botulism

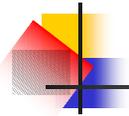
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The number of cases of botulism does not begin to compare with the annual rate of say salmonella or campylobacter infections; however, the fact that *Clostridium botulinum* produces one of the most potent toxins known to man requires that we place it high on our public health prevention lists particularly since it is a real possibility that botulinum toxin can be used as a biothreat agent.



Objectives

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



Botulism Types-

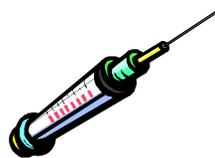
Foodborne



Infant (& adult colonization)



Wound



Classical foodborne botulism is a result of ingestion of preformed botulinum toxin in improperly prepared or abused food products.

Infant botulism, first recognized as a unique syndrome in the early 1970's, results from in vivo production of toxin in the gut of a colonized infant. Now infant botulism has the highest rate of all botulism types. An adult form of this syndrome also occurs.

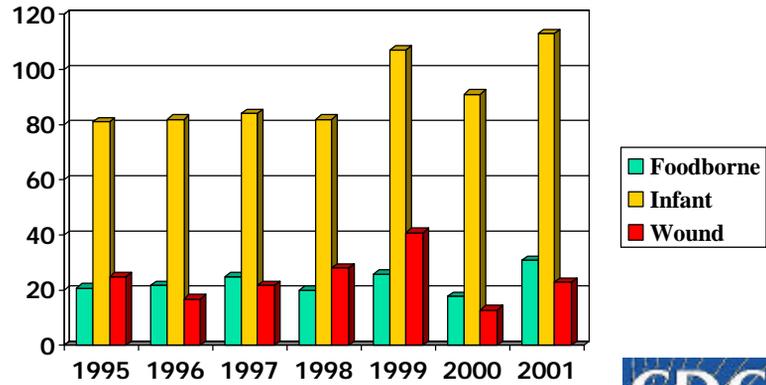
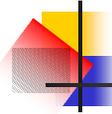
Although rarely seen before 1994, the number of cases of wound botulism surpassed the number of foodborne cases in 1998. These mainly occur in injection drug users and it is suspected that black tar heroin may be a primary source of the infections.

Intentional Contamination

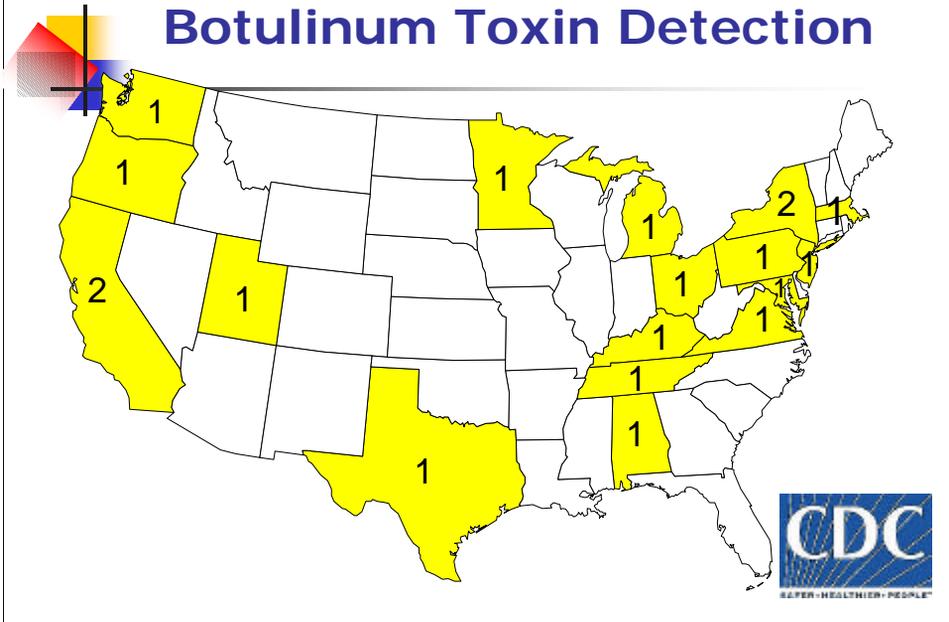


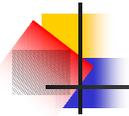
Intentional use of botulinum toxin so far has not occurred in the United States. However the potential risk is real since the toxin is relatively easy to produce in fairly high quantities. It is estimated that 1 gram can kill 1.5 million people. You can even find detailed directions for toxin production on the internet.

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

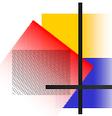




Human Case Types

- 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

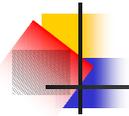




Human Case Types

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





Neurotoxicogenic *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-toxicogenic simulates exist

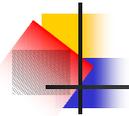




Egg Yolk Agar Plate Preliminary Identification

- 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

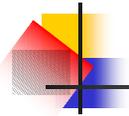




Non-neurotoxigenic Similates

- *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-neurotoxicogenic 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)



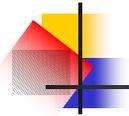


Acceptable Specimens (from patients exhibiting symptoms consistent with the diagnosis of botulism, only)

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.

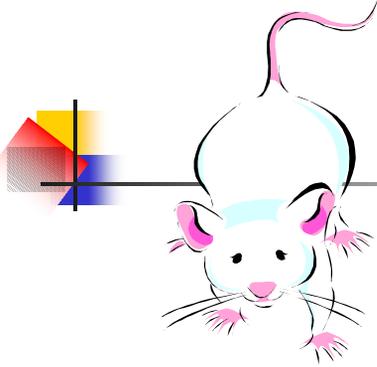




Current approved test

- 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 X 10⁻¹² grams= 0.000000000010 g)

Disadvantages

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

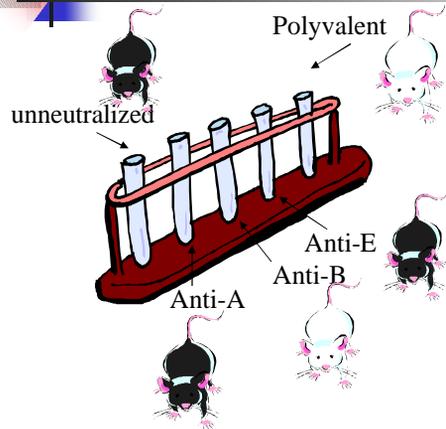


The identity of *Clostridium botulinum* is defined by the ability to produce botulinum toxin.

The mouse test can detect as low as 10 pg of toxin. So it is a daunting task to develop a replacement.

However there are a number of disadvantages. Number 1 is the hazard. The mouse bioassay is the Hazardous Critical Control Point for all our tests. Loading syringes with high levels of active toxin, particularly from culture supernatants, and then injecting into a mouse puts the laboratorian at risk. In addition, we use 15 to 20,000 mice per year for our laboratory activities. Many state health departments are unwilling to do the testing because of the mice and safety issues.

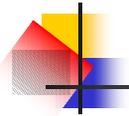
Basis for Botulinum Toxin Detection



Type B toxin identified

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

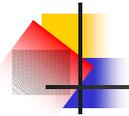




Sample processing

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

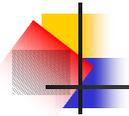




Sample processing

- 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

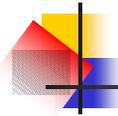




Specimen culture

- 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

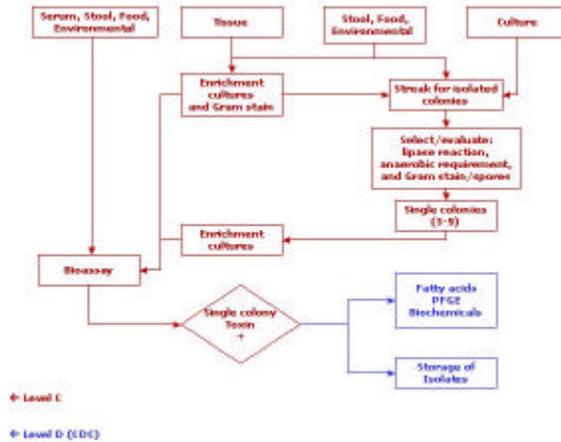


Figure 1: *C. fetus* procedure flowchart

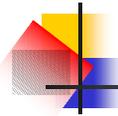




Laboratory Confirmation Criteria

- 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



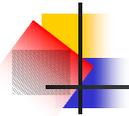


Acceptable Source Specimens

- 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**

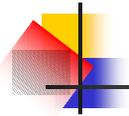




Non-Alaska Native Implicated Foods

- 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)**



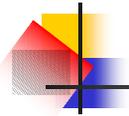


Unlikely Foodborne Botulism Sources

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**

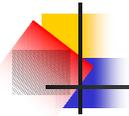




Safe Work Practices

- 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

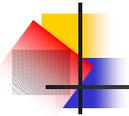




Safe Work Practices

- 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)

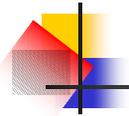




Safe Work Practices

- 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)

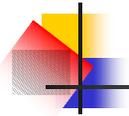




Safe Work Practices

- 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

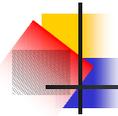




Safe Work Practices

- 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

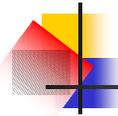




Botulinum Toxoid Vaccine

- “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-neurotoxicogenics exist.
- Good safe work practices are essential for handling *C. botulinum* and its associated neurotoxin.





For Additional Information

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