Clostridium botulinum Challenge Testing of Ready-to-Eat Foods

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1. Purpose

The purpose of this document is to recommend experimental design for challenge testing studies to determine if *Clostridium botulinum* can grow and produce toxin in ready-to-eat (RTE) foods.

2. Scope

Experiments conducted according to the recommendations of this document can be used to determine whether *C. botulinum* can grow and produce toxin in RTE food products including (but not limited to) processed plant products such as pasta products; vegetables packed in oil or brine, or salads; cooked, cured and smoked meat and fish products; processed cheese; and modified atmosphere packaged bread. Artisanal food products with physico-chemical characteristics that vary significantly among batches cannot be assessed using this challenge testing method because consistency of physico-chemical characteristics is critical to the interpretation of the results of challenge studies. In addition to providing guidance to food manufacturers, the document can also guide food safety regulators and government inspection staff in their verification activities with respect to the presence of *C. botulinum* in foods.

3. Background

Food poisoning outbreaks caused by ingestion of *C. botulinum* neurotoxins produced in RTE foods are numerous. Implicated ready-to-eat food products include temperature abused mascarpone cheese (Simini, 1996), bean dip (Sobel *et al.*, 2004), clam chowder (Sobel *et al.*, 2004), vacuum-packaged hot-smoked fish (Korkeala *et al.*, 1998), and most recently carrot juice (Sheth *et al*., 2008).

Increased consumer demand for convenient and fresher foods with minimal preservatives and low thermal processing has led to increased sales of RTE foods worldwide. Refrigerated RTE foods are treated with mild heat processes, with maximum temperatures typically reaching 65-95°C, packaged in a vacuum or with modified atmospheres (usually anaerobic), and then refrigerated (Peck, 2006). Refrigerated RTE foods are also known as sous-vide foods, refrigerated processed foods of extended durability, ready meals, cook-chill foods, and minimally heated chilled foods (Peck, 2006). The combination of a heat treatment and refrigerated anaerobic storage is designed to prevent the growth of nonsporeforming pathogens and spoilage organisms, but bacterial spores are not necessarily destroyed or controlled (Lindstrom *et al.*, 2006). Whereas proteolytic *C. botulinum* does not grow at temperatures below 10°C, nonproteolytic strains can grow and produce toxin at 3.0°C, raising particular concern in RTE refrigerated foods with long shelf lives (Lindstrom *et al.*, 2006). Due to the heat treatment-associated destruction or reduction of other organisms, nonproteolytic *C. botulinum* may grow with little competition, as well as proteolytic *C. botulinum* in the case of mild temperature abuse. An extended shelf life exacerbates the problem by providing additional time for toxin production.

In the case of shelf stable RTE foods, both proteolytic and nonproteolytic *C. botulinum* could pose a risk to consumers. For example, bread has not been known to cause botulism, but the use of modified atmosphere packaging in combination with room temperature storage might be sufficient to allow for growth and toxin production by *C. botulinum.* The inhibition of spoilage organisms and the increased shelf life afforded by modified atmosphere packaging can provide a selective advantage for the growth of *C. botulinum*.

Therefore, it is essential that the processor verify that toxin production in such products can be prevented from the time of production to consumption. In the absence of adequate scientific evidence documenting the safety of the product, appropriate challenge studies with *C. botulinum* may be necessary before such products are launched on the market.

The following are recommendations for determining the potential for *C. botulinum* neurotoxin production in RTE foods; the reader is referred to a *C. botulinum* challenge study performed by Austin and colleagues (1998) on fresh-cut packaged vegetables as an example. The following procedures may not be suitable for all types of RTE foods; other protocols that provide assurance of safety are also acceptable.

4. General Information

4.1 Safety Precautions

The Office of Laboratory Security, Public Health Agency of Canada, states that *Clostridium botulinum* should be handled under biosafety level 2, unless the procedures have a high potential for aerosols or produce quantities of toxin, in which case biosafety level 3 should be followed. Personnel must be fully informed about the hazards (refer to Material Safety Data Sheets online at: http://www.phac-aspc.gc.ca/msds-ftss/index-eng.php). Liquid cultures of *C. botulinum* contain high levels of toxin and should be handled only by experienced personnel.

Contaminated sealed packages may be under pressure and must be opened in a fume hood or safety cabinet for protection from aerosols. Goggles must be worn when handling liquids that may contain toxin.

All materials to be autoclaved should be contained in stainless-steel boxes with handles. Used glassware and other supplies in contact with toxin are placed in a sturdy, heat-resistant container which should be placed in the autoclave by the investigator. Disposable material such as gloves, cotton or tissue paper is collected as biohazardous waste and autoclaved.

Potentially toxic materials should always be contained in unbreakable, leak-proof trays or boxes. This is particularly important in the incubation or manipulation of botulinum cultures which may contain in excess of 10^5 mouse LD/mL. If accidental spills occur, the toxin may be inactivated with saturated or dry sodium bicarbonate or 0.1 N NaOH. Spores can be inactivated by either

0.1% sodium hypochlorite or commercially available products meant for inactivation of spores.

Therapeutic antisera must be available in case of accidental intoxication.

4.2 Equipment/ Materials

Equipment and materials may vary depending upon the foods tested and type of packaging used.

Anaerobic jars (GasPack)
Biological safety cabinet
Bunsen burner
Centrifuge, refrigerated, high-speed
Centrifuge tubes
Clean dry towels
Culture tube racks
Disposable syringes (1 and/or 3 ml)
Filters, disposable, 0.45 μm pore size
Freezer
Gloves
Incubators
Mice, 16-24 g
Micropipettors
Microscope slides
Microscope, phase-contrast or bright-field
Mouse cages, feed, water bottles
Refrigerator
Sterile can opener
Sterile cotton-plugged pipettes
Sterile forceps
Sterile mortar and pestle
Sterile petri dishes, 100 mm
Sterile reserve sample jars
Sterile screw-cap culture tubes
Stomacher
Transfer loops
Trypsin (1:250; Difco Labs, Detroit, MI)
Water Bath

4.3 Media / Reagents

The following media and reagents are recommended; they may vary depending upon the foods tested:

- Absolute ethanol
- Cooked meat medium
- Distilled water
- 1 N Hydrochloric acid solution
- McClung-Toabe egg yolk agar
- Monovalent antitoxin preparations, types A-F
- Saturated solution of $NaHCO_3$ or 0.1 N sodium hydroxide solution for cleaning of spills
- 0.05 M sodium phosphate-gel buffer (pH 6.2) (if necessary)
- Sterile gelatine phosphate buffer, pH 6.2
- Trypticase-peptone-glucose-yeast extract (TPGY) broth
- 1% (w/v) trypsin solution

5. Suggested Experimental Design

Technical factors that require consideration in the design of a challenge study to determine whether *C. botulinum* can grow and produce neurotoxin in foods are outlined in this section.

5.1 C. botulinum *Strains*

Ideally, *C. botulinum* strains that have been previously isolated from similar food products should be used in challenge studies. Additional strains from known foodborne outbreaks might be included to ensure the formulation being tested would not support their growth. Strains representing different toxin types (A, B, E, and F) should be selected. Pre-screening strains could assist in selecting those that grow the best in the formulation being assessed. Strain selection should parallel the risk and storage conditions associated with the product. For example, refrigerated foods pose a greater risk from nonproteolytic *C. botulinum*. Strain information provided should include strain history, characterization, and culture collection identification number.

Cocktails containing multiple proteolytic strains and cocktails containing nonproteolytic strains should be tested separately to account for potential strain variation. A minimum of five proteolytic or five nonproteolytic strains should be used for cocktail preparation. Each strain should be assayed annually for toxin production by the mouse bioassay to ensure a minimum production level of 1,000 mouse lethal doses (MLD)/mL. Strains producing less should be replaced with a productive culture of the same strain or a different strain. The strains used in cocktails should be screened before use for possible mutual antagonism due to bacteriocin-like compounds or bacteriophages (Eklund *et al*., 2004).

It should be noted that *C. botulinum* strains are no longer available for purchase from culture collections for reasons of biosecurity. The lack of availability of strains and appropriate expertise may necessitate contracting the services of a laboratory that can perform *C. botulinum* challenge studies. The use of *Clostridium sporogenes* strains as surrogate organisms for proteolytic *C. botulinum* under some circumstances (*e.g.*, in a sterile product) may be acceptable if sufficient rationale and references are provided to validate that growth of the surrogate would accurately predict toxin production by *C. botulinum*. Currently, there is insufficient data comparing growth characteristics of *C. botulinum* and *C. sporogenes* to validate use of a surrogate.

5.2 Preparation and Enumeration of Spores

Spore crops can be produced using any available method, including those that use a variety of liquid and agar media, or use liquid media over agar in a biphasic method. Since sporulation characteristics vary by strain, multiple media types may be necessary to prepare spores from the strains to be used.

Media should be incubated at 35°C and 26 - 30°C for spore production for proteolytic and nonproteolytic strains, respectively. The incubation time required to achieve sporulation depends on the cultural conditions and strain characteristics; cultures should be periodically examined microscopically to determine the degree of sporulation.

For harvest, spore crops should be washed ten times with sterile distilled water, resuspended in sterile distilled water, and stored at 4°C, -20°C, or -80°C. Spore suspensions are heat shocked prior to use to ensure the absence of vegetation cells and toxin.

Spore suspensions can be enumerated by direct plating. Spore suspensions are heat-shocked, diluted, and then inoculated onto plates or into tubes of TPGY medium. Tubes are incubated anaerobically for 7 days at 35°C for proteolytic strains and 30°C for nonproteolytic strains.

Heat shocking spore suspensions

Proteolytic Strains	Nonproteolytic Strains
75°C for 20 minutes	60°C for 20 minutes

Austin et al., *1998*

The spore suspensions should be checked for purity by plating onto duplicate McClung-Toabe egg yolk agar plates, incubating one plate aerobically and one plate anaerobically, and ensuring that all resulting growth is *C. botulinum.*

If desired, spores can be enumerated in food products using a most probable number (MPN) procedure because there are too few spores to be detected by direct plating.

5.3 Preparation of Food Product

The characteristics of the food product that serve to control *C. botulinum* require consideration. These might include salt concentration, water activity, pH, preservatives used, packaging conditions, storage temperature. If the specifications of the food product to be tested include an acceptable range for a given characteristic (*e.g.*, pH range 5.0 to 5.4), the product should be tested at the level which is the most permissive to growth and toxin production in order to provide a margin of safety. Characteristics with permitted variability in the formulation may require testing singly as well as in combination (FDA, 2001).

5.4 Inoculation of Food Products

Multi-strain spore cocktails should be prepared that contain an approximately equivalent number of spores of each type of strain (containing proteolytic *or* nonproteolytic strains). Spores should be heat shocked immediately before inoculation.

The characteristics of the formulation that serve to control microbial growth require further consideration for designing the inoculation of the food product. When liquid inocula are used, a minimum volume must always be used. Where the volume of the inoculum might affect the intrinsic characteristics of the product, the properties of the diluent used for the inoculum (pH, water activity, salt concentration) could be adjusted to match those of the food product. Since testing is destructive, the number of samples to be inoculated should be planned based upon the sampling considerations outlined in section 5.7.

Foods that are fluid or can be mixed can be inoculated with a minimum volume of liquid spore suspensions targeting an inoculum level of 1000 to 5000 spores/g, followed by mixing to ensure uniform distribution. For multi-component foods, spores should be inoculated into the component most permissive for growth (e.g. vegetable pieces that have a higher a_w than the surrounding brine). Solid foods or those in which inocula cannot be uniformly distributed can use surface inoculation, with a target inoculum level of 1000 spores/cm^2 (Doyle, 1991). Spore suspensions are added dropwise and then spread with sterile utensils, or added to the product in packaging followed by manual massaging through the packaging material. Preliminary tests in which the same volume of food dye is applied to the food can be used to ensure the mixing procedure achieves homogeneity.

5.5 Special Product Packaging Conditions

Inoculation should not alter special conditions used in product packaging, such as controlled or modified atmospheres. Inoculation should be performed in such a way as not to interfere with the normal progression of the gas mixture within the package. Alternative packaging may be used provided that the conditions of the commercially packaged product are reproduced.

5.6 Incubation of Inoculated Food Products

Recommended incubation conditions depend upon the food product. For refrigerated foods, nonproteolytic strains should be examined at 4, 10, and 25°C for one and a half times the product's shelf life, or until it is overtly spoiled. For shelf stable foods, both proteolytic and nonproteolytic strains should be examined at 25°C for one and a half times the product's shelf life, or until it is overtly spoiled. A margin of safety is provided by examining refrigerated products under conditions of temperature abuse and shelf stable products for longer than their shelf life. For products containing multiple components, incubation should continue for 1 day after overt spoilage of the last component of the product (Doyle, 1991).

Time and Temperatures for Incubation of Inoculated Product

Refrigerated RTE Foods:	**Shelf Stable RTE Foods:**
4, 10, and 25°C	25°C
1.5 X shelf life or overtly spoiled	1.5 X shelf life or overtly spoiled
Nonproteolytic strains at all temperatures	Proteolytic and nonproteolytic strains
Proteolytic strains in addition at 25°C	

If time-temperature integrators (TTIs) are used as indicators of temperature abuse for the product, time-to-toxicity studies should be done to ensure that TTIs will change prior to the production of toxin in the product.

5.7 Sampling the Inoculated Food Product

An initial sampling timepoint for botulinum toxin assay should be planned on the day of inoculation (time 0). At least four additional sampling timepoints should be planned; the timing of sampling will vary according to the expected shelf life of the product. At least three sampling timepoints should be planned between the final sampling timepoint and the halfway point. The final sampling timepoint in any experiment is the time at which the formulation becomes overtly spoiled. The degree of spoilage of the product should be assessed and recorded at each sampling time by documenting the product's visual appearance (*e.g.* spoilage, drying, mold growth, swelling).

For botulinum neurotoxin testing, it is recommended that the entire sample be homogenized or extracted followed by withdrawal of a 50 g sample. The 50 g sample should be used to perform five replicates per sampling timepoint. Botulinum toxin should be detected in two successive samples, after which no further testing is required.

Positive controls could be included in the experimental design to ensure that toxin, produced in the food product, can be detected in analysis of the food product. Depending upon the food product, key safety parameters may need to be altered to enable production of toxin in the food product. For example, unacidified shrimp could serve as the positive control for acidified shrimp.

5.8 Botulinum Neurotoxin Assay

Refer to MFHPB-16 of the Compendium of Analytical Methods (Austin and Sanders, 2009) for the mouse bioassay procedure for botulinum neurotoxin testing.

Toxin analyses should proceed on the day of sampling wherever possible. Alternatively, samples can be homogenized or extracted in 0.05 M sodium phospate-gelatin buffer (pH 6.2), centrifuged (27,000 X g, 20 min, 4°C), and the supernatant fluids removed (at least 10 mL, adjusted to pH 6.2 if necessary) and stored at -20°C (Doyle, 1991).

An alternative procedure (such as an ELISA method or endopeptidase assay) for the mouse bioassay test may be used, providing that the alternative method has been validated to be of equal or greater sensitivity than the mouse bioassay. Such methods would include those published in Health Canada's *Compendium of Analytical Methods*, FDA's *Bacteriological Analytical Manual*, AOAC methods, or other similarly validated methods. The limitations of each assay should be borne in mind given that some of them may yield false negatives due to matrix interference.

5.9 Additional Product Analyses

Additional product analyses should be performed on duplicate samples to allow evaluation of how changes in intrinsic characteristics would be expected to affect the growth and toxin production of *C. botulinum.* Analyses should include water activity, pH, salt content, preservative level, aerobic plate count, and gas analysis for MAP products. Depending on the type of product, other analyses could include protein content, fat content, titratable acidity, moisture content, lactic acid bacteria count, psychrotroph count, spore count, and anaerobe count.

5.10 Measures to Take if Food Product Supports Toxin Production

Food products that allow toxin formation within the time frame described in section 5.7 may present a risk of botulism to consumers. It may be possible to reformulate the food product to prevent toxin production by increasing salt content, decreasing water activity with humectants, or adding approved preservatives. Alternatively, mechanisms can be used to identify those products which have been temperature abused; these mechanisms must display an endpoint before the point at which botulinum neurotoxin can be produced. If an indicator of temperature abuse such as a TTI (e.g. TTIs based on the Skinner-Larkin relationship; Skinner and Larkin, 1998) is to be used, studies must be done to correlate safety with temperature abuse and the response of the integrator.

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