Botulism | *Clostridium botulinum*

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions to inform the diagnosis of key diseases in Australia. This document contains the laboratory case definition (LCD) for Botulism.

Version	Status	Authorisation	Consensus Date
1.1	Update to new template and content to ensure gold standards of practice has been incorporated	PHLN	15 March 2024
1.0	Initial PHLN Laboratory Case Definition	PHLN	27 October 2006

1 PHLN summary laboratory definition

1.1. Condition

Botulism due to infection with *Clostridium botulinum (C. botulinum).*

1.1.1. Definitive criteria

1. Isolation of botulinum toxin gene positive *C. botulinum* from an appropriate clinical specimen (e.g., stool, gastric aspirate, wound swab/tissue) from a patient with clinical features of botulism,
    - OR
2. Detection of the botulinum toxin gene from an appropriate clinical specimen (e.g., stool, gastric aspirate, wound swab/tissue) or from food consumed by a patient with clinical features of botulism[1] [2],
    - OR
3. Detection of botulinum toxin in serum, stool, gastric aspirate, or food consumed by the patient with clinical features of botulism.

1.1.2. Suggestive criteria

- Isolation of botulinum toxin negative or botulinum toxin gene negative *C. botulinum* from food consumed by a patient with clinical features of the disease.

[1] Noting that there are other *Clostridium* species that have uncommonly been reported as carrying the botulinum toxin gene for toxin types (*bont*) linked to human cases, including *C. sporogenes* carrying the *bont/B* gene.(Reference: Differentiating Botulinum Neurotoxin-Producing Clostridia with a Simple, Multiplex PCR Assay - PMC (nih.gov)). Further culture and identification are recommended.

[2] Molecular detection of the toxin gene *bont* does not confirm a functional toxin gene is present or is expressing functional neurotoxin (BoNT). It is recommended that a functional test like the mouse bioassay or sequencing to examine the full gene sequence should be performed to provide additional information.

2 Introduction

Botulism is a paralytic disease in which blurred vision, slurred speech, difficulty swallowing, dry mouth and respiratory insufficiency are important features. The various forms of disease are caused by a neurotoxin and are often associated with food consumption. In developed countries the commonest form of disease is infant botulism in which the immature intestine is colonised by *C. botulinum* after ingestion of the organism. *C. botulinum* is widespread in the natural environment including soils. It can be found in indoor house dust and may be a natural contaminant of ingested foods; honey being implicated as a possible source in the USA in the 1970s.

Neurotoxin (BoNT) released by intestinal *C. botulinum* results in the clinical features of infant botulism. Classical botulism develops in older children and adults who consume toxin-contaminated foods, particularly those that have been inadequately processed or preserved. In developed countries these are often home processed or traditional foodstuffs. Botulism is recognised increasingly in adults and older children with intestinal colonisation by *C. botulinum* following, for example, intestinal surgery. The least common form of botulism is associated with *C. botulinum* contaminated wounds where the neurotoxin is released into the systemic circulation from the infected soft tissues in a manner similar to tetanus toxin. The differential diagnosis of botulism is limited, and includes myasthenia gravis, Eaton-Lambert syndrome, tick paralysis and acute inflammatory polyneuropathy (especially the Miller-Fisher variant). A specialist neurologist opinion will help rule out these possibilities in cases of suspected botulism.

Clostridium species are anaerobic, spore-forming Gram-positive bacilli. Several *Clostridium* species are capable of causing toxin-mediated disease. Botulism is usually caused by neurotoxin-producing strains of *Clostridium botulinum*. Other *Clostridium* species (*C. baratii, C. noyvii, C. sporogenes and C. butyricum*) can produce neurotoxin and may occasionally cause human disease. Serotypes are defined by the toxins produced which are serologically different, with eight exotoxins described (A, B, C (C1,C2), D, E, F, G and H (Barash JR, Arnon SH: A novel strain of *Clostridium botulinum* that produces type B and type H botulinum toxins. J Infect Dis 2013; online publication Oct 7. PMID: 24106296). Four of the seven serotypes (A, B, E, and F) have been linked with human botulism, with the majority of the cases being caused by serotypes A and B. Neurotoxin-positive strains of *C. botulinum* are subdivided into three subgroups, the first two which are associated with human botulism, and the third which only causes disease in non-human animals (Table 1). Group I is so closely related to *C. sporogenes* that it can only be distinguished by its ability to produce neurotoxin. Group III is related to *C. noyvii*. Group I and group II *C. botulinum* strains differ in

that group I contains proteolytic strains. Members of Group I are proteolytic and produce BoNTs type A, B, and F. Members of Group II are non-proteolytic and produce BoNTs type B, E, and F. The majority of human cases of botulism are usually associated with members of Groups I and II. In a CDC review of cases of clinically confirmed botulism, only 65% of cases were positive in at least one laboratory test for BoNT *or C. botulinum* culture (https://www.cdc.gov/botulism/pdf/bot-manual.pdf).

Large outbreaks have also been noted where none or only low numbers of clinical specimens are positive in laboratory tests. Early sample collection appears to be beneficial in improving rates of detection. Turnaround times for laboratory testing may require several days and the only specific therapy for botulism is antitoxin, which is most effective if administered early in the course of neurological disease. Therefore, treatment of suspected botulism should not wait for laboratory testing but rather clinical picture and case history.

3 Laboratory diagnosis

Note that *C. botulinum* and botulinum toxin are tier 2 and tier 1 security sensitive biological agents respectively. See 3.2.

3.1. Botulinum neurotoxin detection

A definitive diagnosis of botulism requires detection of *C. botulinum* neurotoxin (BoNT) in an appropriate clinical specimen (e.g., stool, gastric aspirate, wound swab/tissue) from a patient with features of the disease, or in a specimen of food they consumed before the onset of clinical botulism. BoNT detection is by mouse bioassay and is not available in most clinical laboratories and only some jurisdictional public health laboratories with appropriate animal ethics approval. The toxin type is determined by neutralisation of the toxin with specific antitoxins.

Given the ethical considerations associated with the mouse bioassay, the laboratory case definition has been modified to include isolation of *C. botulinum* or the detection of the gene responsible for neurotoxin production. These changes reduce the need for a mouse bioassay to serum only samples. The changes, however, assume the presence of the bacterium in the right clinical specimen or the presence of the gene is sufficient evidence for intoxication.

3.1.1. Specimen collection and transport

Blood and faeces should be collected from the patient as soon after onset of symptoms as possible. The blood is drawn into a blood tube without anticoagulant. Ideally, 15–20 mL serum and 25–50 g faeces should be collected. Two millilitres of serum should be obtained from infants and as much faeces as possible.

3.1.2. Specimen preparation

a) Serum: serum is separated, and a series of 1 mL aliquots dispensed, one for each toxin type and one control. A 0.25 mL aliquot of each antitoxin is then added and incubated for 30–60 minutes at room temperature. 0.4 mL is intraperitoneally injected into 2 mice for the control and 0.5 mL injected for each aliquot containing antitoxin. Antitoxin to the commonest types (A, B and E) are used. Type F neurotoxin is very rarely encountered. Antisera for therapeutic use are unsuitable for the neutralisation test.

b) Faeces: a portion of the faecal specimen is suspended in buffered gelatine (1:1). The supernatant is tested with and without antitoxin as in 3.1.2 (a) above after clarification by centrifugation. Non-specific toxicity of faecal supernatant may be seen at a dilution of less than 1:10.

c) Other clinical specimens: such as tissue or exudate from suspected wound botulism, should be ground up with sterile mortar and pestle then treated as for faeces in (b) above.

d) Food: suspect food sources should be treated in accordance with Australian Standard 1766 and also treated as for faeces in (b) above.

e) Enrichment Culture: Culture supernatant may also be tested as per serum in (a) above.

3.1.3. Specimen inoculation

The 0.4 or 0.5 mL specimen/antitoxin mixtures are each inoculated intraperitoneally into laboratory mice, and the animals observed closely over a 4 day period.

3.1.4. Results and interpretation

Assessment of the effects of mouse inoculation must be carried out by staff with a valid laboratory animal handling license and experience of the mouse BoNT bioassay. A lethal result may take up to 4 days but neurotoxic effects are often evident to experienced staff

after only one day. Low levels of toxin in specimens may produce signs in mice without death for the non-neutralised control. Serum from patients with acute inflammatory polyneuropathy can produce paralysis in mice. The non-specific toxicity produced in mice inoculated with faecal supernatant at lower dilutions may necessitate repetition of the bioassay with supernatant at higher dilution. BoNT toxicity is usually evident in the mouse bioassay at greater than 1:100 dilution. Death in mice without consistent neutralisation patterns is inconclusive.

Confirmatory results require observation of clinical signs (ruffling fur, laboured abdominal breathing, weakness of limbs or total paralysis and/or death) in unprotected mice AND protection from clinical signs in antitoxin treated mice (polyvalent or a single monovalent where mice injected with other monovalent antitoxin are expected to show clinical signs).

3.1.5. Timeline

The mouse bioassay will take a minimum of 4 days to produce a negative result from a clinical or food specimen. A need to transport specimens, to obtain type-specific antitoxin, or titrate out non-specific mouse toxicity will lengthen the test period.

3.1.6. Confidence limits

No recent statistical evaluation of sensitivity or specificity of the BoNT mouse bioassay is available. However, it is reported to be very sensitive, with one intraperitoneal mouse 50% lethal dose corresponding to 5 to 10 pg (Smith, L. D. S., and H. Sugiyama. 1988. Botulism. The organism, its toxins, the disease. Charles C. Thomas, Springfield, Ill.). The mouse bioassay has been found by some centres to be more sensitive than developmental toxin immunoassays and is thought to detect toxin in around 70% cases, including a study of clinical wound botulism cases also identified the mouse bioassay as having a sensitivity of 68% (https://academic.oup.com/cid/article/48/12/1669/320221, however other studies report positivity in mouse bioassay of 44% of sera and stool samples in adult botulism cases (https://pubmed.ncbi.nlm.nih.gov/1431246/)). A negative BoNT bioassay does not exclude a diagnosis of botulism in a patient with clinical features of the disease. Although theoretically very specific, the bioassay can produce mouse paralysis when sera from patients with acute inflammatory polyneuropathy have been inoculated or false positives may be caused by endotoxins from gram-negative bacteria or tetanus toxin, which is why the inclusion of neutralisation in this test is important (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1471988/).

3.2. *C. botulinum isolation*

Detection of *C. botulinum* in faeces from a patient with clinical features of botulism is strongly suggestive of the diagnosis even if tests for neurotoxin are negative. BoNT is rarely detected in faeces of patients who have botulism and who are *C. botulinum* positive. The isolation of *C. botulinum* from samples with high levels of competitive bacterial flora, such as faecal and environmental samples, can be challenging. Several broth cultures and platings may be required to isolate a pure culture, and repeated attempts may not successfully yield an isolate. It is thought that the presence of nontoxigenic *C. botulinum*-like strains can disturb the culture of BoNT positive *C. botulinum* strain. Isolation of *C. botulinum* from toxin positive specimens can provide useful confirmatory evidence that can be kept indefinitely for subsequent investigation. However, isolation of *C. botulinum* from toxin negative food specimens is of questionable value given the widespread dissemination of *C. botulinum* spores in the inanimate environment. Sera should not be considered for *C. botulinum* culture.

C. botulinum bacteria are a Tier 2 Security Sensitive Biological Agent in Australia and facilities handling cultures should be compliant with the SSBA Regulatory Scheme. BoNT is a Tier 1 reportable agent at 0.5 mg and above – any broth cultures of *C. botulinum* should be assumed to be producing reportable levels of toxin and therefore should only be handled by a registered entity.

3.2.1. Specimen collection

a) Faeces: a portion of the faecal specimen collected for BoNT bioassay can be used for culture.

b) Exudate or tissue should be sought from suspected wound botulism cases.

3.2.2. Culture media

Two tubes of cooked meat/glucose/starch broth should be inoculated. One should be heated to 80 °C to kill vegetative bacteria, then both should be incubated anaerobically at 30 °C for 4 days.

3.2.3. Neurotoxin screen

The culture medium can be screened for BoNT using the mouse bioassay as described in 3.1 above.

3.2.4. Isolation of *C. botulinum*

C. botulinum is isolated from enrichment media and directly from specimens by streaking onto selective egg yolk media (e.g., BSM) and incubating the egg yolk plates at 37 °C anaerobically. Typical colonies are small and demonstrate lipase but not lecithinase activity.

Some of the components of selective egg yolk media (e.g., BSM) may suppress the growth of group II *C. botulinum* (E and F) so including non-selective plates in adult botulism work-ups is recommended (https://journals.asm.org/doi/10.1128/CMR.19.2.298-314.2006#T1).

3.2.5. Confirmation of *C. botulinum*

Definitive confirmation of the identity of suspected *C. botulinum* colonies requires demonstration of BoNT production, since group I is biochemically and genetically identical to strains of *C. sporogenes* and group III closely resembles *C. noyvii*. The following phenotypic features would be taken as suggestive but not conclusive evidence of *C. botulinum*: Gram positive bacilli, with oval subterminal spores, anaerobic growth, and lipase activity. Current IVD MALDI-TOF MS libraries are unable to correctly identify *C. botulinum* and may return an identification of *C. sporogenes* (reference https://pubmed.ncbi.nlm.nih.gov/29021156/). Therefore, laboratories should not rely on MALDI-TOF as part of their identification algorithm for *C. botulinum.*

3.2.6. Confidence limits

The taxonomic complexity of *C. botulinum* and related neurotoxin-positive Clostridia dictates that presumptive identification of *Clostridium* species from patients with suspected botulism should not be used as the sole justification for a diagnosis. The phenotypic and genotypic characteristics of *C. botulinum* are not specific to this species and only common denominator of all *C. botulinum* strains is the ability to produce neurotoxins that cause flaccid paralysis. Without further testing such as demonstration of BoNT activity, the presence of the *bont* gene or genomic sequencing of the isolate, an isolate cannot be considered to be confirmed *C. botulinum.* Moreover, variations in expression of BoNT activity may render some strains negative by mouse bioassay. Cultures were positive for 51% of stool specimens and toxin testing was positive for 37% of sera and 23% of stool specimens collected from 309 persons with clinically diagnosed botulism reported to CDC from 1975 to 1988 (https://www.cdc.gov/botulism/pdf/bot-manual.pdf).

3.3. PCR

Molecular detection of the toxin gene *bont* does not confirm a functional gene is present or expressing as toxin. Ideally, a functional test like the mouse bioassay or genomic

sequencing to examine the full gene sequence should be performed to provide additional information.

3.3.1. Specimens

PCR can be performed on direct clinical extractions from faeces, gastric aspirate or tissue or enrichments of such specimens. PCR of the toxin gene is a highly useful tool for screening bacterial colonies and sample enrichments of clinical specimens and food to detect the presence of toxin gene positive *C. botulinum.* Sera should not be screened by PCR as there is no *C. botulinum* toxin gene expected in patient sera.

3.3.2. BioFire gene testing

C. botulinum toxin A gene is included in the BioFire Defense BioThreat panel. This test is intended for use on swabs, liquids, cultures and powders and is not listed on the Australian Register of Therapeutic Goods for use on clinical specimens.

3.4. Alternative testing approaches

Immunoassays for detecting BoNT may be commercially available overseas but are not readily available in Australia. In house testing can be developed using polyclonal or monoclonal antibodies but validation can be challenging. Alternative assays including endopeptidase mass spectrometry and colorimetric assays have been developed but are not readily available to Australian public health laboratories at this point in time,

3.5. Quality control

At present there is no external quality control programme for BoNT detection or *C. botulinum* isolation from clinical specimens, however the Royal College of Pathologists of Australasia (RCPA) Quality Assurance Program for Biosecurity does offer some simulated clinical samples containing inactivated or attenuated *C. botulinum* or related organisms.

3.5.1. Special considerations

The sporadic nature of botulism and, the technically demanding procedures required for its confirmation mean that there are very few Australian laboratories are able to provide a test service. Performance of the mouse bioassay requires approved animal handling facilities, ethical approval and staff with the appropriate animal handling certification. Supplies of type-specific diagnostic antisera can be sporadic. Competent laboratories may therefore be unable to offer a test service for an unspecified period depending on availability of antisera.

3.5.2. Timeline

Clinical testing may produce a positive mouse bioassay within 48 hours and culture and PCR may detect toxin gene positive *C. botulinum* in 48–72 hours. However typically testing requires 405 days to return a result on a clinical specimen. Completion of a set of *C. botulinum* neurotoxin and culture tests on matched clinical and food samples can take between 10 and 14 days. This period includes assembly of reagents, lengthy anaerobic culture and mouse bioassay procedures. Transportation of specimens, acquisition of type-specific antisera and repetition of mouse bioassay at higher dilutions can prolong the time taken to produce a reliable result.

4 SNOMED CT Terms

SNOMED CT code	Term name	Description
406614006	Botulism	*Disorder*
13080008	Clostridium botulinum	*Organism*

5 References

1. Anon. 1998. Botulism in the United Sates, 1899-1996. Handbook for Epidemiologists, Clinicians and Laboratory Workers. Centers for Disease Control and Prevention, Atlanta, GA.

2. Balows A, Duerden BI. Systematic Bacteriology. 1998. Topley & Wilson's Microbiology & Microbial Infections. Vol. 2. Collier L, Balows A, Sussman M. Arnold, London, 1998.

3. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. 1999. Manual of Clinical Microbiology, ASM Press, Washington, D.C., USA.

4. Mandell GL, Bennett JE, Dolin R. 2000. Principles and Practice of Infectious Diseases. 5th Edition. Vol.2. Churchill Livingstone, Edinburgh, UK.

6 Glossary

Ag/Ab – Antigen/Antibody

AMR – Antimicrobial resistance

ARTG – Australian Register of Therapeutic Goods

BA – Blood agar

Biotype – Strain distinguished from other microorganisms of the same species by its physiological properties or a group of organisms with the same genotype

CCNA – Cell cytotoxicity neutralisation assay

(US) CDC – Centers for Disease Control and Prevention

CDNA – Communicable Diseases Network Australia

CDS – Calibrated dichotomous susceptibility

CIA – Chemiluminescent immunoassay

Clade – Group of organisms composed of a common ancestor and all its lineal descendants

CLSI – Clinical and Laboratory Standards Institute

CSF – Cerebrospinal fluid

Ct – Cycle threshold

DFA – Direct fluorescent antibody

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

EIA – Enzyme immunoassay

ELISA – Enzyme linked immunosorbent assay

EUCAST – European Committee on Antimicrobial Susceptibility Testing

HI – Haemagglutination inhibition

ICT – Immunochromatographic test

IFA – Immunofluorescent antibody

IgA – Immunoglobulin A

IgG – Immunoglobulin G

IgM – Immunoglobulin M

IVD (device) – In vitro diagnostic medical device

In vitro – performed in a test tube, culture dish, or elsewhere outside a living organism

In vivo – performed or taking place in a living organism

ITS – Inter-genic spacer region

LAMP – Loop-mediated isothermal amplification

LPS – Lipopolysaccharide

MALDI-TOF – Matrix-assisted laser desorption ionisation-time of flight

MAT – Microscopic agglutination test

MDST – Molecular drug susceptibility testing

MDR – Multidrug resistant

MIA – Microsphere immunoassay

MLST – Multilocus sequence typing

NAAT – Nucleic acid amplification test/ing

NATA - National Association of Testing Authorities, Australia

NGS – Next generation sequencing

NPAAC – National Pathology Accreditation Advisory Council

NRL – National Serology Reference Laboratory

PCR – Polymerase chain reaction

PC2 laboratory – Physical containment level 2 laboratory

PC3 laboratory – Physical containment level 3 laboratory

PC4 laboratory – Physical containment level 4 laboratory

PFGE – Pulsed field gel electrophoresis

POC – Point-of-care

QAP – Quality assurance program

QC – Quality control

RAPD – Random amplified polymorphic DNA

RCPA – Royal College of Pathologists of Australasia

RFLP – Restriction fragment length polymorphism

RNA – Ribonucleic acid

RT – Reverse transcriptase

RT-PCR – Reverse transcription polymerase chain reaction

SBT – Sequence based typing

Serotype – Pathogens of the same species that are antigenically different

SNT – Serum neutralisation

SSBA – Security sensitive biological agent

STI – Sexually transmitted infection

Strain – Variant that possesses unique and stable phenotypic characteristics

SQAP – Serology quality assurance program

Test sensitivity – Ability of a test to correctly identify patients with a disease

Test specificity – Ability of a test to correctly identify people without the disease

TGA – Therapeutic Goods Administration

UTM – Universal transport medium

VTM – Viral transport media

WGS – Whole genome sequencing

WHO – World Health Organization

WHO CC – WHO Collaborating Centre

XDR – Extensively drug resistant