Detection

In this guide

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For neurotoxin-producing clostridia in food the detection process is multifaceted. Detection refers to the detection and enumeration of spores (and/or viable organisms), to the detection and typing of toxin and to the detection and identification of toxin-encoding genes. Detection of neurotoxin in a laboratory setting, following a clinical diagnosis and subsequent treatment of illness, is paramount. In addition, the detection of toxin or spores of *C. botulinum*, in food or environmental samples, is a fundamental part of incident investigations and is a quantitative element contributing to scientific research programmes and risk assessments[17]. However, routine monitoring for the presence of spores, cells or toxin in food is not a practical component of food safety.

C. botulinum is a very serious human pathogen and detection involves potentially hazardous materials that require a high level of laboratory biosecurity at all stages. Laboratory work with *C. botulinum* is only pursued at a few specialist locations in the UK (including the UKHSA). Furthermore, because of the serious impacts associated with human botulism, the results of detection always require expert interpretation.

3.1 Healthcare settings

In emergency healthcare settings, a test to detect botulinum neurotoxin in a specimen of serum or faeces etc., immediately follows any clinical diagnosis of human botulism. Detection of toxin is not a requirement prior to the commencement of treatment and timely treatment with antitoxin is known to significantly reduce the severity of outcomes for human botulism cases. In an incident of foodborne botulism in the UK in 2011[18] a clinical diagnosis followed two days after the initial report of symptoms. Neurotoxin was detected in a clinical sample, by Mouse Lethal Bioassay (MLB), approximately four days after the diagnosis and in a sample of food one day later (PCR assay identified spores and vegetative cells of *C. botulinum* in the food sample at approximately the same time as the MLB result).

The *in vivo* MLB is regarded as the gold standard for detection of botulinum neurotoxins in clinical and epidemiological settings. Results from the MLB establish, simultaneously, multiple elements of functionality for neurotoxin, including binding, internalisation and intracellular activity, which together confirm the ability to cause botulism. The MLB is effective for all toxin types and has sensitivity reported as 5-10 pg per ml. The MLB is usually performed with 0.5 ml intraperitoneal injection of an extract from the food or faecal sample; a human oral lethal dose of botulinum neurotoxin is estimated as ~1ng per kg bodyweight. In the clinical setting the MLB is routinely combined with an array of antitoxins (in cross neutralisation reactions) to provide information on toxin type, including complex situations that involve multiple toxin types, and strongly supports the tracking of outbreaks.

Although the MLB is very sensitive, robust and is strongly established, particularly from a public health perspective, it does have several drawbacks; not least a failure to meet the objectives of Reduction, Replacement and Refinement of animal tests (3 Rs) that are supported by many organisations including the European Union and the OECD. A requirement for expensive specialist animal facilities, turnaround times ~4-5 days, and some issues surrounding the interpretation of negative results add to the ethical concerns and have stimulated exploration of improved alternative rapid and validated technologies for detection of botulinum neurotoxins[19].

3.2 Technological solutions

Since 1992 detection for a range of toxin types and with sensitivity at least equal to that obtained by MLB has been achieved using a range of alternative methods [20].

- Modified *in vivo*, and *ex vivo*, methods dominantly employ stem cells or differentiated neurogenic cells to detect toxin activity directly. These methods are used mainly in research settings but do not improve on the slow turnaround time of the MLB and include a level of variability that is inconsistent with the demands of most clinical settings.
- Immunological detection systems, such as ELISA, that use antibodies specific to the botulinum neurotoxin antigens have rapid turnaround times (typically a few hours) and have been combined, effectively, with other techniques, such as mass spectrometry, to achieve good specificity. These methods can be automated, however, they may detect biologically inactive toxin.
- The enzymatic activity of botulinum neurotoxins (such as the ability to cleave the SNAP-25 protein) can be harnessed, often using a combination of specific antibodies, fluorescent dyes or mass spectrometry, to provide automated *in vitro* detection systems. The biological activity is strongly specific to each toxin type so that extension to detect a wide range of known types, and potential new toxins, is not trivial. In clinical settings the MLB is used to confirm biological activity in some scenarios.
- Nucleic acid-based methods[21,22], including PCR, real time PCR, MLST and WGS, can be used for the detection and characterisation of *C. botulinum*, botulinum neurotoxin-encoding genes, but not direct detection of toxin. Generally these methods are readily deployed in multiplex platforms but they do not establish biological activity. A large quantity of gene sequence information for *C. botulinum* is stored in easily accessible databases so that nucleic acid-based methods add powerful tools for use in epidemiological, surveillance and research settings.
- Multiplex PCR methods have been extended to include detection of genes that code for neurotoxins in *C. butyricum* and *C. baratii*.

Some of these methods have obtained regulatory approval but currently none satisfy the stringent requirements which are necessary to replace the MLB for the detection of botulinum neurotoxins in clinical settings.

3.3 Epidemiology settings

Epidemiological investigations are an essential response to human botulism outbreaks. Investigations include a search for toxins, or spores, in foods and food environments and prioritise detection methods that minimise false negatives, have rapid turnover and high throughput to maximise the potential for source tracking and risk management. *In vitro* immunoassays that can work with complex food matrices, sometimes used in combination with confirmatory MLB,

are cost effective, operate well over a range of toxin concentrations and are used effectively to detect neurotoxin in public health investigations. Multiplex PCR assays have been developed to provide accessible, rapid and cost-effective tools for detecting and screening unknown isolates from environmental samples for genes encoding botulinum neurotoxins (but not necessarily corresponding to toxin-producing capability). PCR assays and WGS can provide additional phylogenetic information that has particular value in outbreak investigations.

In epidemiological settings qualitative detection of neurotoxins, or *C. botulinum* spores, is usually combined with characterisations of toxin type, or genotype, to facilitate source tracking (linking clinical cases with contaminated foods). Increasingly the characterisation step uses sophisticated nucleic technology, with an appropriate amplification step, to establish type matches with high precision.

3.4 Detection of spores and cells

There are no selective culture media for *C. botulinum* so that the detection of spores or vegetative cells involves specialist microbiology. Detection of spores usually involves anaerobic incubation of heated food followed by the detection of toxin (or encoding gene) in the enrichment fluid. ELISA methods with specific sets of antibodies, or multiplex PCR techniques with specialised sets of primers, are used to confirm the detection of spores with toxin types A, B, E and F. Enumeration of spores is achieved by statistical analysis of detection in diluted samples. In research settings this method can detect a few spores per kilogram in food materials[23].

Spores, inactivated toxins, toxin antigens or clostridial DNA do not pose foodborne botulism risks directly (infant botulism, adult infectious botulism and wound botulism are exceptions) so that pre-emptive testing of materials for *C. botulinum* has limited value for ensuring food safety. However, reliable detection of toxin, spores or viable cells is an essential element of challenge tests that are a pillar of assurance during food product developments that involve new formulation or new processes.

C. botulinum and most clostridia reduce sulphite to sulphide under anaerobic conditions and the reaction is easily detected. However, this phenotype is common among many food bacteria, including many food spoilage bacteria and some non-spore forming bacteria, so that detection has minimal relevance for food safety (historically this reaction has been used as an indicator of hygiene for food production; particularly in the dairy sector). Genetic characterisations of

sulphite-reducing spore formers, and sulphite reducing bacteria, indicate the possibilities for the development of a molecular assay, based on sulphite reduction genes, that has increased sensitivity[24].

3.5 Research settings

In research settings the detection and isolation of *C. botulinum* in food materials has helped to inform risk assessments, produced detailed maps of phylogenetic relatedness to support epidemiology and built a picture of diversity, novel toxins, genotypes and horizontal gene transfer possibilities that improves understanding, helps development of therapeutics and aids public health preparedness. Many research activities highlight ongoing searches for sequence specific primers and for high quality, pure, monoclonal antibodies to assist in the development of detection technologies for *C. botulinum*.

In silico detection methods, involving very powerful search techniques across multiple databases, have detected *C. botulinum* neurotoxin sequence homologs in a small number of non-clostridial strains but the biological significance is not, currently, understood.

3.6 Conclusions

Methods for the detection of botulinum neurotoxin in tissues and food materials are well established and provide strong support for clinical, epidemiological and research activities. Technological advances point to emerging test methodologies that maintain sensitivity and can potentially reduce the burden on the use of experimental animals and these developments should be widely supported.

Specialist laboratory techniques effectively detect spores, and viable cells, of *C. botulinum* in food materials and support research activity but do not establish surveillance as a realistic option for improved food safety.