Laboratory Diagnostics of Botulism

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INTRODUCTION

Since the first reported cases of food-borne botulism in the late 18th century, botulism has gained attention not only as a threat to food producers and consumers but also as a potential cause of crib death of small babies, as a deadly trip for intravenous drug abusers, and as an inspiration for bioterrorists. Botulism is caused by botulinum toxins, which are highly potent neurotoxins formed during the growth of the spore-forming bacterium *Clostridium botulinum*.

Based on the serological properties of the toxins they produce, *C. botulinum* strains are divided into toxinotypes A to G. Noteworthy of the bacterial species *C. botulinum* is its old-fashioned taxonomy; the only common denominator of all *C. botulinum* strains is the ability to produce neurotoxins that cause flaccid paralysis. *C. botulinum* strains form four genotypically and phenotypically distinct groups of organisms, designated I to IV (100, 107, 196). Generally, groups I and II cause botulism in humans, while group III is involved with animal botulism, but exceptions occur. Group IV has not generally been associated with illness. Based on its phenotypic and genetic traits, group IV *C. botulinum* has been proposed to be

renamed *Clostridium argentinense* (203). Of the human pathogenic strains, group I cultures produce toxin A, B, or F and group II cultures produce toxin B, E, or F. Dual-toxin-producing strains have also been reported (72, 80, 121), as have those producing only one type of toxin but carrying a silent gene for another (73, 74, 121). Other clostridia, namely, *C. butyricum* and *C. baratii*, are also known to produce type E and F toxins, respectively.

Phenotypically, group I and II *C. botulinum* strains differ from each other significantly. Group I organisms seem to be more of terrestrial origin and are present in temperate climates, whereas group II strains, particularly type E, are frequently found in aquatic environments in the Northern hemisphere (Table 1). Differences in spore heat resistance and growth temperatures are responsible for the safety risks posed by *C. botulinum* groups I and II in the food industry; group I spores, which have a high heat resistance (112, 138, 180, 184, 192, 202), cause problems in canning and home preservation of vegetables and meat, whereas group II spores, with somewhat lower spore heat resistance (135, 140, 168–170), are of great concern in minimally processed packaged foods that have extended shelf lives at refrigerated temperatures (134, 167).

Botulinum neurotoxins are 150-kDa proteins with zinc-endopeptidase activity (155). The toxin molecule is secreted as a progenitor toxin that contains the neurotoxin as well as non-

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TABLE 1. Characteristics of *Clostridium botulinum* groups I and II

Characteristic	*C. botulinum* group I	*C. botulinum* group II	Reference(s)
Toxinotypes	A, AB, B, BF, F	B, E, F	197
Strain origin	Soil	Aquatic environments	34, 98, 106, 123, 126, 151, 194, 195, 208, 220, 222
Genome size (Mbp)	3.9–4.1	3.6–4.1	96, 130
GC content (%) of genome	26–28	26–28	115, 127, 128
Genetic diversity	Narrow	Wide	96, 110, 130, 159, 160
Utilization of proteins as energy source	Yes	No	35
Carbohydrate fermentation	Glucose, fructose, maltose, salicin, sorbitol	Amygdalin, dextrin, fructose, galactose, glucose, glycogen, maltose, mannose, melezitose, ribose, sorbitol, starch, sucrose, trehalose	35, 132, 197
Metabolite production	Acetic, butyric, isobutyric, isovaleric, isocaproic, propionic, and valeric acids; alcohols; hydrogen sulfide	Acetic, butyric, formic, succinic, and lactic acids	197
Bacteriocin production	Boticin	NR[a]	51, 125
Colony morphology on blood agar	2—6 mm (diam); irregular margin; opaque, raised center; narrow hemolysis	1–3 mm (diam); slightly irregular with lobate margins; translucent to semiopaque with matt surface; mosaic structure in oblique light; narrow hemolysis	35, 197
Colony morphology on egg yolk agar (lipase reaction)	Iridescent layer on and around colonies	Iridescent layer on and around colonies	197
Susceptibility to antibiotics[b]	Susceptible to erythromycin, metronidazole, penicillin, rifampin, tetracycline; resistant to cycloserine, gentamicin, nalidixic acid, sulfamethoxazole, trimethoprim	Susceptible to erythromycin, metronidazole, penicillin, rifampin, chloramphenicol, clindamycin, tetracycline, trimethoprim; resistant to gentamicin, nalidixic acid, cephalotin, sulfamethoxazole	204
Minimum/optimum growth temp (°C)	10/35–37	3/26–30	56, 57, 83, 139, 180, 181, 197, 202
Growth-limiting pH	4.3–4.5	5.0–5.1	185, 193
Growth-limiting NaCl in water phase (%)	10	5	197
Heat resistance of spores	High ($D_{121–110°C}$,[c] 0.15–1.8 min)	Moderate ($D_{85°C}$,[c] 1–98 min)	112, 135, 139, 140, 168–170, 184, 192
Typical vehicle foods for botulism	For food-borne botulism, vegetables, meat, and canned foods; for infant botulism, honey	For food-borne botulism, fish, meat, and minimally processed packaged foods; for infant botulism, NR	93a, 134

[a] NR, not reported.
[b] Based on information on *C. botulinum* type A and E strains (204).
[c] D_T, decimal reduction time at temperature T.

toxic components. The nontoxic components protect the neurotoxin from environmental stress and assist in absorption of the neurotoxin into the body (77). The neurotoxin molecule consists of two subunits: a 100-kDa heavy chain, which is responsible for the binding and translocation of the toxin across the synaptic membrane through specific receptors, and a 50-kDa light chain, which cleaves the proteins involved in acetylcholine vesicle docking and fusion to the presynaptic membrane (156). Inhibition of the neurotransmitter release causes paralysis of the corresponding muscle. The maximal potency of the botulinum neurotoxins is achieved only after enzymatic activation of the toxin molecule. This has been suggested to be at least partly related to cleavage of the toxin molecule (45, 165). Generally, in proteolytic group I *C. botulinum* this is done by endogenous enzymes of the organism, but neurotoxins produced by nonproteolytic group II strains may require external proteases, such as trypsin (45, 55), for activation.

Typical of botulism is a descending flaccid paralysis that, unless treated immediately and appropriately, may lead to death when the respiratory musculature fails. Food-borne botulism, the most well-known form of botulism, is an intoxication that follows when food containing preformed botulinum neurotoxin is ingested. Other significant forms of botulism are infections that result from toxin formation from spores in vivo. The most common of these is infant botulism, where botulinal spores germinate, grow, and subsequently produce toxin in the gastrointestinal lumens of small babies, in whom the normal gut microflora is poorly developed (14).

Since botulism is a life-threatening condition, a rapid diagnosis is essential. This challenges not only the awareness of the disease by clinicians but also the laboratory diagnostics. Apart from the ultimate goal of rapid diagnosis and, above all, saving the patient, every attempt to increase our understanding of the epidemiology of botulism should be made. This enables the future development of prevention strategies against the disease. In addition to the detection of botulinum toxin and *C. botulinum* in the patient, the diagnostics should aim at physiological and genetic typing of the disease isolates. In the fol-

lowing we review the current status of the laboratory diagnostics of human botulism.

HUMAN BOTULISM

Botulism is a potentially lethal condition caused by botulinum neurotoxin. The neurotoxin can enter the body via the gastrointestinal tract or through mucous membranes of, for instance, the eyes or the respiratory tract. Toxin production from otherwise harmless *C. botulinum*, *C. butyricum*, or *C. baratii* spores may also occur in vivo. After entering the body, the neurotoxin is absorbed into the blood and lymphatic circulation, which then mediate the toxin to motor nerve endings where it blocks the neurotransmitter release. Typical clinical symptoms of all forms of botulism include cranial muscle paralysis, such as double vision and dilated pupils, slurred speech, dry mouth, difficulty in swallowing and speaking, and facial paralysis. As the disease progresses, paralysis of the limbs and respiratory dysfunction become apparent. Respiratory muscle paralysis can lead to death. Recovery occurs upon the sprouting of transitory nerve endings that reside when the synaptic activity of the original nerve regenerates (148). Recovery time may be several weeks to months and is dependent on the amount of toxin ingested and, to a lesser extent, on the toxin type in question. Type A toxin tends to be more potent than types B and E and causes the longest-lasting disease (71).

Food-Borne Botulism

The classical form of botulism is food poisoning, an intoxication that follows the consumption of food containing preformed neurotoxin. Depending on the toxic dose, the incubation period may vary from 12 to 72 h. In addition to the general signs of botulism, the food-borne form may be manifested by gastrointestinal symptoms such as nausea, vomiting, and constipation. The treatment consists mainly of intensive symptomatic care, including respiratory support (24). Intravenous administration of specific trivalent antitoxin towards A, B, and E toxins may be used to neutralize the circulating toxin. However, the success of the antitoxin treatment is strongly dependent on the time of administration; if the toxin has entered the nerve endings and disappeared from blood circulation, the treatment will be irrelevant. Furthermore, due to the risk of severe allergic reactions to the antiserum, it is no longer used in some countries (25).

Typical differential diagnoses include Guillain-Barré syndrome, Miller-Fisher syndrome, chemical intoxication, stroke, and staphylococcal food poisoning (36, 105). Suspected drug and alcohol abuse may occasionally prolong the time for a diagnosis to be made.

Compared with food-borne illness or death due to more common pathogens, such as *Campylobacter*, *Salmonella*, and *Clostridium perfringens*, food-borne botulism outbreaks are relatively rare. However, regions with a high incidence of botulism that causes a considerable public health hazard include the Republic of Georgia, Poland, China, Russia, Kyrgyzstan, and certain ethnic populations in the North, e.g., Alaskan Inuits. Japan, Italy, Portugal, Germany, France, and the former Yugoslavia have also reported high numbers of cases. The foods most frequently involved, if traced, are home-pre-

pared items such as cured meats, canned vegetables, and fermented fish products. These outbreaks are usually sporadic and restricted to a family. Commercial foods have less frequently been involved in food-borne botulism, but such outbreaks may be large, and they cause significant economic losses to the food industry (134).

The case-fatality rate of food-borne botulism in developed countries is 5 to 10% (199). Worldwide, more than half of the cases (52%) were due to type B toxin, with types A and E accounting for 34% and 12%, respectively, in 1995 (91). On rare occasions, type F toxin has been associated with food-borne botulism (90). Apart from the type of toxin causing the outbreaks, no information on the physiologic group of *C. botulinum* type B or F strains involved is usually provided.

Infant Botulism

Unlike food-borne botulism, the other forms of human botulism are actually infections where the toxigenesis occurs in vivo. Infectious botulism is related mainly to group I *C. botulinum*, as its temperature optimum for growth and subsequent toxin production is close to the body temperature, while the growth of group II organisms at the same temperature is limited (197). For infant botulism (150, 172), in addition to group I *C. botulinum* types A and B (92, 93), Bf (21), and F (99), cases due to type E and F toxins produced by *Clostridium butyricum* (18, 92, 144) and *Clostridium baratii* (22, 88, 119), respectively, have been reported. Infant botulism typically affects babies under 1 year of age, with the youngest reported patient being only 54 h old (22, 119). The condition develops as a consequence of ingesting spores of toxin-producing clostridia (9). As the gut microflora of small babies is poorly developed, *C. botulinum* spores may germinate and form a toxin-producing culture in the intestine. The clinical manifestation of infant botulism varies from a subclinical condition to sudden death (12). It often starts with constipation that can last for several days, followed by the distinctive flaccid paralysis manifested by impaired feeding due to muscle weakness in the mouth and throat, facial muscle paralysis, ptosis, and general weakness (10). Infant botulism has been suggested to be a causative agent of crib death (6, 14).

The main treatment for infant botulism is high-quality supportive care, with special attention focused on the patient's nutrition and respiratory functions (13, 116). The use of antitoxin is often not required (13), and the case-fatality rate is less than 2% (39). However, since 2003 a human-derived immune globulin (75) has been available in the United States and has been shown to significantly shorten the hospitalization period and decrease the treatment costs (209). The only foodstuffs that have been associated with infant botulism are honey (19), which may carry high numbers of *C. botulinum* spores (11, 52, 158, 159), and infant milk powder (29). Dust and other materials in the environment also seem to be important sources of spores (11, 52a, 161).

Wound Botulism

Wound botulism is a rare form of botulism, although it is increasingly diagnosed among injecting drug abusers due to the use of contaminated needles or impure heroin (3, 16, 17,

157, 166, 213). Wound botulism follows when *C. botulinum* spores germinate and grow in profound wounds or abscesses that provide anaerobic conditions. The clinical outcome is similar to the food-borne form, albeit with an absence of gastrointestinal signs. Wound botulism is often accompanied by a mixed infection and may be due to more than one botulinum toxin-producing strain (3). The median incubation period is 7 days. In addition to respiratory support, the treatment of wound botulism includes thorough surgical debridement, antibiotics, and the administration of antitoxin. The estimated case-fatality rate is 15% (91).

Infectious Botulism in Adults

The adult form of infectious botulism is rare and resembles infant botulism in its pathogenesis and clinical status. It results from colonization of the intestinal tract by toxin-producing clostridia (40, 64, 143, 145). People with altered intestinal flora due to abdominal surgery (76, 111), prolonged antimicrobial treatment, or gastrointestinal wounds and abscesses are particularly vulnerable (40). Infectious botulism in adults is distinguished from the food-borne form by a different history of food consumption, with a missing association to foods with high risk of botulism (143).

Other Forms of Botulism

Inhalation botulism may result from aerosolization of the neurotoxin. A few human cases have been reported (101). Iatrogenic botulism with local or generalized weakness is rare but can develop as a consequence of therapeutic injection of the neurotoxin (20, 149). A distinctive "outbreak" of iatrogenic botulism due to maladroit botulinum toxin treatment caused botulism in four people in Florida in November 2004 (7).

LABORATORY DIAGNOSTICS OF BOTULISM

As botulism is a life-threatening condition, a rapid diagnosis is required for successful therapy. Apart from the clinical manifestation and patient history, the diagnosis is based on positive laboratory findings. Of these, detection of toxin in the patient's serum and/or feces remains the standard method (118) (Fig. 1). The detection of *C. botulinum* in patient samples, such as feces, gastric and intestinal contents, and wound swabs and tissues, supports the diagnosis (3, 39, 118, 162), but should not exclusively be considered pathognomonic of the disease. Sometimes the presence of several toxin-producing strains may complicate the diagnostics (3, 29, 131). In some cases of infant botulism, the presence of *C. botulinum* alone in the feces or intestinal content of the patient may be sufficient for diagnosis even if the neurotoxin could not be detected in samples from the patient (38).

Whereas a rapid diagnosis of botulism is a prerequisite for the patient to survive and recover, a more thorough investigation of botulism outbreaks is needed to provide epidemiological information about the disease (Fig. 1). This includes the isolation of *C. botulinum* from the patient and from vehicle foods or other sources of spores or toxin and genotypic analysis (see below) of the disease isolates. Furthermore, characterization of the physiological group of these isolates is fundamental to improve our knowledge of the risk factors for and prevention strategies against different forms of botulism.
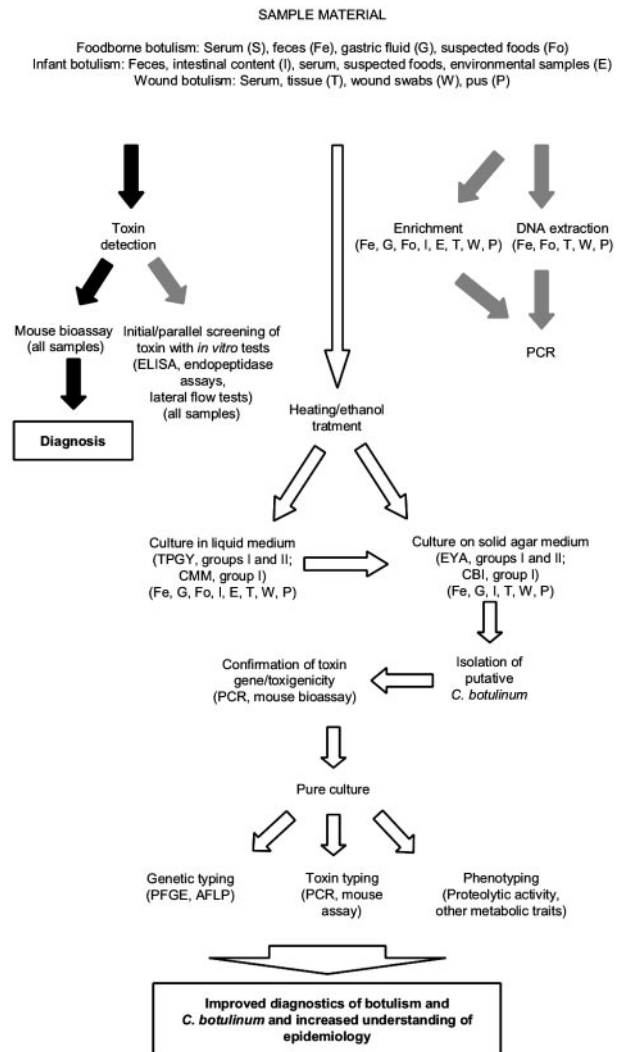

FIG. 1. Laboratory diagnostics of botulism. The standard methods are marked with black arrows, and methods aiming at rapid initial screening are marked with gray arrows. Measures aiming at the characterization of the disease isolates and providing complete epidemiological information are marked with white arrows. TPGY, tryptone-peptone-glucose-yeast extract medium; CMM, cooked-meat medium; CBI, *C. botulinum* isolation medium.

In the following we discuss the problems and developments in the laboratory diagnostic tools and procedures established for investigation of human botulism.

Laboratory Safety

The extreme potency of botulinum neurotoxin necessitates rigid requirements to ensure the safety of laboratory workers. Despite the potency of its neurotoxin, the noninvasive and noncontagious *C. botulinum* has been graded as a class II pathogen. Appropriate biosafety level 2 containment facilities and trained personnel are therefore a minimum requirement for work with *C. botulinum*. Additional contingencies should be considered whenever aerosol or droplet formation from toxic materials is expected. As the neurotoxins form a potential

threat for bioterrorism, restricted entrance to the laboratory facilities containing toxic cultures is a necessity. The definition of additional contingency measures should be based on a thorough risk assessment of activities in each laboratory handling *C. botulinum* and its toxin. The efficacy of the pentavalent toxoid formerly used to immunize laboratory staff worldwide has been shown to be lower than expected (Centers for Disease Control and Prevention, unpublished data). Therefore, careful attention needs to be paid to the everyday safety of the laboratory staff. Attempts to develop new vaccines against botulism are being made both in the United States and in Europe (24).

Detection of Botulinum Neurotoxin

While the mouse lethality assay has remained the standard test for the detection of botulinum neurotoxins, there has been tremendous progress in the development of alternative tests during the past decade (Table 2) (188). The latest advances in assay development have successfully aimed at rapidity and sensitivity; the fastest tests are now performed in 20 min, and the test sensitivities have surpassed that of the mouse bioassay. However, further research is warranted to gain speed and sensitivity with a single test that would optimally detect all seven neurotoxin types simultaneously. Furthermore, many of the recently developed test systems have been targeted to the medical industry for testing the potencies of therapeutic preparations of purified botulinum toxins, and validations on foods or clinical materials have not been done. Components of complex matrices, such as feces, blood, pus, and foods, may interfere with test reactions, and high concentrations of competitive microbes in many sample materials can retard the growth of and toxin production by *C. botulinum*. Fecal proteinases may degrade botulinum toxin, causing false-negative results. Therefore, there is a need for systematic validation of botulinum toxin detection tests in clinical sample materials and foods.

Mouse lethality assay. The standard procedure for the detection of botulinum toxin is the mouse lethality assay (162, 201). The test is based on an intraperitoneal injection into laboratory mice of sample diluted in phosphate buffer. If the sample contains toxin, the mice develop typical signs of botulism, including fuzzy hair, muscle weakness, and respiratory failure that manifests as a wasp-like narrowed waist. These symptoms usually develop within a day postinjection (39) but may take several days to appear. The nascent toxin becomes active only when cleaved (55), so toxins from strains of group II *C. botulinum* require trypsin activation before analysis, as these strains lack the required proteolytic activity.

The toxin type is determined by neutralization of the toxin with specific antitoxins (37). Briefly, mice injected with the neutralizing antitoxin survive, while the others develop botulism. Alternatively, the sample eluate may be treated with antitoxins before administration to mice. While the method is very specific, any of the seven toxin types can be identified. The mouse bioassay is very sensitive, with one intraperitoneal mouse 50% lethal dose corresponding to 5 to 10 pg (197) and the detection limit being 0.01 ng/ml of sample eluate (216). In clinical microbiological laboratories, the assay has been used with fecal, serum, gastric, wound, and food samples as well as supernatants from bacterial cultures. However, the assay is

laborious and expensive and naturally presents an ethical dilemma due to the use of laboratory animals. Only a limited number of laboratories worldwide perform the mouse bioassay. In suspected clinical cases of botulism where immediate treatment is required, the mouse assay may also be too slow to make a diagnosis. False-positive test results due to in vivo toxin formation from a high number (10^7) of *C. botulinum* spores (154), endotoxins from gram-negative bacteria (200), and tetanus toxin (197) have been reported.

Nonlethal mouse assay. Another type of mouse assay with a more humane endpoint of local muscle paralysis as a result of subcutaneous injection of botulinum toxin type A has been explored for testing the potency of botulinum neurotoxins for therapeutic use (186). The nonlethal mouse assay is equal to the conventional bioassay in sensitivity and specificity, but it does not cause signs of distress or impaired movements in the animals (186). However, the assay has been targeted to potency testing of purified neurotoxins, and thus it has not been validated for microbiological laboratories investigating complex sample matrices.

Immunological methods. A wealth of immunoassay formats for the detection of botulinum neurotoxins have been reported. Compared with the mouse test, the immunoassays are technically simple and fast to perform and interpret (58). Although many of the earliest assays, such as radioimmunoassay (15, 23), gel diffusion assay (69, 152, 210), passive hemagglutination assay (114), and the early applications of enzyme-linked immunosorbent assay (ELISA) (47, 164, 177) have poor sensitivities or specificities, the recent developments in signal amplification have enabled sensitivities equal to that of the mouse bioassay (Table 2). A drawback with immunological tests is that high-quality antibodies are not generally available. In addition to biologically active neurotoxins, inactivated toxin (e.g., after a heat treatment) may cause false-positive results. Furthermore, genetic variation within the different serotypes of the neurotoxin may result in decreased affinity to monoclonal antibodies, causing false-negative results (198).

ELISA is by far the most commonly applied immunoassay format in the detection of botulinum neurotoxins. Briefly, the neurotoxin in a sample binds to a solid test matrix that is usually precoated with polyclonal or monoclonal capture antibodies against one or more toxins (sandwich ELISA) (47, 164). A second toxin antibody is then used to bind the toxin. Finally, an antiantitoxin molecule, carrying an enzyme such as alkaline phosphatase or horseradish peroxidase, is used to produce a signal through enzymatic cleavage of a chromogenic substrate. The sensitivity of the conventional ELISAs for botulinum neurotoxin detection is some 10- to 100-fold lower than that of the mouse bioassay (47, 164, 174, 177) (Table 2). Signal amplification using a chromogenic diaphorase system (189), biotinylated antibodies and avidin-enzyme conjugate (66, 68), or enzyme-linked coagulation assay (ELCA) (53, 54, 178) has enabled increased assay sensitivity.

ELISAs have been extensively tested with purified botulinum toxin (60, 173, 206), toxic *C. botulinum* cultures (47, 48, 60, 70, 164, 189), and foods related to botulism outbreaks (67, 68) or artificially contaminated with botulinum toxin or *C. botulinum* (174, 177, 189). A collaborative study in 11 laboratories showed high reproducibility of an amplified ELISA with foods containing botulinum toxins (70). Food components

TABLE 2. Diagnostic assays applied in the detection of botulinum neurotoxins

Assay	Time to perform	Type of toxins	Detection limit	Application(s)	Special feature(s)[a]	Reference(s)
Mouse lethality assay	1–4 days[b]	A, B, C, D, E, F, G	20–30 pg/ml, 1 MLD/ml	Bacterial cultures, serum, feces, gastric contents, foods, environmental samples	Standard method	39, 162
ELISA	1–2 days	A	10 MLD_{50}	Inoculated beans and mushrooms	Polyclonal (horse) Ab as CA	177
	8 h	A	4–8 pg/ml, 1–2 MLD/ml	Toxin potency testing in the medical industry	Monoclonal (BA93) and polyclonal (rabbit) Ab as CA	60
	8 h	A, B	NR[c]	Fecal samples related to infant botulism cases	Polyvalent, polyclonal (burro) Ab as CA	49
	8 h	A, B	0.2 ng/ml	Human serum	Polyclonal (horse) Ab as CA	206
	8 h	E, F	0.5–2 ng/ml	Human serum	Polyclonal (horse) Ab as CA	173
	8 h	A, B, E	9–45 pg	Inoculated ground turkey meat	Polyvalent, polyclonal (horse) Ab as CA	174
Amplified ELISA	8 h	A	5–10 MLD_{50}	Inoculated canned salmon and corned beef	Monoclonal antibody (BA11), diaphorase-based amplification system	189
	8 h	A	10 MLD/ml	Bacterial culture from cheese related to botulism outbreak	Polyclonal (goat) Ab, diaphorase-based amplification system	66
	8 h	A, B, E, F	1–10 MLD, 0.2–1 ng/ml	Chili and potato linked to botulism outbreak	Polyclonal (goat/rabbit) Ab as CA, biotin-streptavidin-based amplification system	67, 68
ELISA-ELCA	5 h	E	<1 MLD	Inoculated fish	Polyclonal (chicken) Ab as CA	178
	1–2 days	A, B, E	5–10 pg/ml, 1 MLD	Bacterial cultures	Polyclonal (chicken/horse serum) Ab as CA	53, 54
Immuno-PCR	8 h	A	5 pg	Purified toxin	Monoclonal antibody (BT57-1), PCR amplification of reporter DNA attached to antibody	219
Chemiluminescent slot blot immunoassay	6 h	E	4 MLD	Bacterial cultures, inoculated fish, naturally contaminated soil	Polyclonal (rabbit) Ab, chemiluminescent detection of slot-blotted culture supernatant	32
Electro chemiluminescence	1–3 h	B	<1–2 ng/ml	Purified toxin	Monoclonal (B 2/3) and polyclonal (horse) Ab, immunomagnetic concentration	87
Radioimmunoassay	8 h	A	100 MLD_{50}	Purified toxin	Polyclonal (rabbit) Ab	23
Lateral flow immunoassays	15–30 min	A, B, E	15 pg–10 ng/ml	Inoculated milk products	Immunochromatographic detection[d]	187
	20 min	A	15–150 pg/ml	Vegetables and seafood	Ganglioside-liposome (GTb1) as CA, immunological detection	1
Endopeptidase assay	8 h	A	0.1–0.8 MLD_{50}	Toxin potency testing in the medical industry	Peptide substrate specific for SNAP-25[e], immunological detection	59
	5–6 h	B	5–10 pg/ml	Inoculated meat pate, cheese, cod, mince, sausages	Immunoconcentration of toxin with monoclonal antibodies, peptide substrate specific for VAMP[f], immunological detection	215, 216
	8 h	A, B	0.1–4.5 ng/ml	Bacterial cultures	Peptide substrates specific for VAMP/SNAP-25, immunological detection	89
	8 h	A, B, D, F	2 ng/ml	Toxin potency testing in the medical industry	Fluorigenic peptide substrates specific for VAMP/SNAP-25	182
	8 h	A, B, E, F	0.04–0.6 MLD_{50}/ml	Purified toxins	Peptide substrates specific for VAMP/SNAP-25, multiplex detection with mass spectrometry	26

[a] Ab, antibody; CA, capture antigen.
[b] In 98% of cases a positive result is observed in 24 h (39).
[c] NR, not reported.
[d] The type of antibody was not reported.
[e] SNAP-25, 25-kDa synaptosomal-associated protein.
[f] VAMP, vesicle-associated membrane protein.

tend to interfere with the ELISA, decreasing the test sensitivity, but successful ELISA detection of botulinum toxins at least in fish fillets (178), canned salmon and corned beef (189), turkey meat (174), mascarpone cheese (66), pasta products (46), potatoes (68), chili (67), and canned beans and mushrooms (177) has been reported. The limitations of each assay should be borne in mind; for example, the ELISA-ELCA that employs chicken and biotinylated antibodies is not suitable for investigation of foods containing chicken meat, egg yolk, egg white, and milk (54, 188).

Reports on the performance of ELISAs on clinical specimens such as serum (173) and feces (49) are limited (Table 2). Fecal extracts have been shown to drastically interfere with the ELISA reaction, decreasing its sensitivity (49). In a study on 22 cases of infant botulism, the performance of the ELISA was improved using extended incubation times and fetal bovine serum to block the interfering fecal substances, and the test proved to be even more reliable than the mouse assay, which produced some false-positive results (49). An ELISA-ELCA was suggested to perform well with complex clinical matrices such as blood and excreta (54), but to our knowledge, no reports on the systematic validation of any ELISA procedures for the detection of botulinum toxin in clinical materials have been published. However, as the ELISA procedures can be finished within a working day, they would be suitable for initial screening in suspected cases of botulism (Fig. 1).

A chemiluminescence immunoassay for type E toxin, combined with a slot blot transfer of culture supernatants into a polyvinylidene fluoride membrane, was reported to be even faster to perform than ELISA and possessed a relatively high sensitivity (32). However, false-positive results were obtained with inoculated fish samples (32). The detection limit of an electrochemiluminescence assay employing an immunomagnetic concentration of target antigen is similar to that of the mouse bioassay (87). The electrochemiluminescence technology seems to be a promising alternative for the detection of botulinum toxins, but like many of the recent advances in toxin detection, it requires further testing with complex matrices.

Commercial lateral flow assays in a dipstick form would provide convenience in toxin testing, as they are easy and rapid (<30 min) to perform and no additional equipment is required. However, the speed backfires in terms of the sensitivity of many lateral flow tests, and their usefulness may thus prove to be limited (104, 187) (Table 2). An assay employing the receptor for botulinum neurotoxins, trisialoganglioside GT1b, as a capture agent was shown to provide a sensitivity similar to that of the mouse assay with type A toxin, but fatty foods were reported to interfere with the test (1). Lateral flow assays may serve as efficient and valuable initial screening tools in suspected cases of botulism, but negative results particularly should be confirmed with the mouse bioassay. Attention should also be paid to the optimization of sample pretreatment to avoid false-negative results due to interference by the sample matrix (187).

Endopeptidase assays. The property of the botulinum neurotoxin of having a highly specific zinc-endopeptidase activity with selected targets in the synaptic cleft has inspired the development of in vitro assays for toxin detection. The endopeptidase assays are based on specific cleavage of synaptic proteins (SNARE complex proteins) by different botulinum neurotoxins, combined with immunological detection of the cleaved peptide (89, 216), or detection of fluorescence released when a peptide labeled with a quenched chromophore is cleaved (182).

Endopeptidase assay formats for types A (59, 89, 182), B (89, 182, 215, 216), D (182), E (T. Ekong, J. W. Austin, J. P. Smith, I. Dufresne, and M. Brett, Abstr. Interagency Botulism Res. Coord. Committee Meet., Orlando, Fla., p. 36, 1999), and F (182) have been described, but commercial solutions are available only for type A toxin detection. The endopeptidase assays have potential to replace the mouse lethality assay, as they detect only biologically active neurotoxin and are generally more sensitive than the mouse assay (26, 65, 216). Detection of the cleaved peptide by mass spectrometry further increases the sensitivity (26), but due to the requirement of expensive equipment and specialized skills, the technique is not suitable for every laboratory. The highest sensitivity, 200-fold compared to that of the mouse assay, has been obtained with surface plasmon resonance spectroscopy detection of the cleavage of native SNARE complex proteins by botulinum toxin types B and F in rat brain synaptic vesicles (65). This method was also assumed to be compatible with complex matrices such as serum and foods; however, its use for this purpose was not reported (65).

The endopeptidase assays are highly specific, and no cross-reactivity between different botulinum toxins or with tetanus toxin has been reported. Functional assays based on immunological detection have been successfully used to detect botulinum toxin type B in various foods, such as paté, cod, and cheese (215, 216). The interference of the assay by fatty foods was avoided by immunological concentration of the neurotoxin using monoclonal antibodies against the type B toxin prior to the endopeptidase assay. However, false-negative results were obtained due to the inability of the monoclonal antibodies to recognize all type B toxins produced by different *C. botulinum* strains (215).

More research is needed to validate these assays for all toxin types and, particularly, with complex matrices such as feces and foods that may contain endogenous proteinases. Also, substances such as EDTA, which is used as a common plasma anticoagulant in blood sample tubes, have been shown to inhibit the assay for type A toxin (59).

Culture Methods for *Clostridium botulinum*

C. botulinum requires strict anaerobic conditions for growth. This creates challenges for laboratory work with the organism. All culture media must be deoxygenated by heating in a boiling water bath or by a continuous flow of an anaerobic gas mixture. Reducing agents, such as thioglycolate, may be added in media to maintain anaerobiosis during culture and incubation. The use of appropriate oxygen indicators in culture media as well as in anaerobic jars and anaerobic workstations is part of the daily routine in a *C. botulinum* laboratory. All glassware and plastic supplies, such as containers, tubes, and pipette tips, should be deoxygenated before contact with *C. botulinum* cultures. Continuous work in an anaerobic workstation is a necessity for successful diagnostics.

The species *C. botulinum* consists of physiologically distinct

organisms (Table 1), which hampers the development of diagnostics. Conventional detection and isolation of *C. botulinum* are based on culturing in liquid medium and subsequent detection of botulinum toxin in the culture supernatant by the mouse bioassay (118). Positive samples are streaked on solid media, and the toxin formation by colonies is traditionally further confirmed by the mouse test. Clinical samples, such as serum and feces, can be cultivated directly or pretreated with ethanol to eliminate vegetative bacteria while recovering bacterial spores (163). Heating may alternatively be used to eliminate nonsporeformers, but care should be taken in selecting the temperature; 80°C for 10 min has been recommended for group I spores, but this temperature may injure group II spores (197). Heating at 60°C for 10 to 20 min is thus a safer choice for group II spores. The addition of a heat-resistant lytic enzyme, such as lysozyme (5 µg/ml), to the culture medium may enhance germination of heat-stressed spores (169, 170).

Routine liquid media include chopped-meat–glucose–starch medium (39); cooked-meat medium (175, 176); broths containing various combinations of tryptone, peptone, glucose, yeast extract, and trypsin (e.g., tryptone-peptone-glucose-yeast extract medium) (129); reinforced clostridial medium (79); and fastidious anaerobe broth (179). All of these media are nonselective and thus allow the growth of a range of other bacteria.

Blood agar and egg yolk agar (EYA) (94) serve as the most common unselective plating media, with EYA enabling the lipase reaction typical of *C. botulinum* (Table 1). However, many other clostridia produce lipase and may therefore confuse the identification (35). EYA medium alone does not contain any inhibitory compounds, but when supplemented with cycloserine, sulfamethoxazole, and trimethoprim, EYA-based selective media (e.g., *C. botulinum* isolation medium) have been reported to select group I *C. botulinum* (50, 153, 190). These media have been successfully used in the investigation of fecal samples in cases of infant botulism (48, 50, 82). However, as these media may suppress the growth of group II *C. botulinum* (214), their value in the diagnostics of food-borne botulism is limited.

The identification of botulinum toxin in and around *C. botulinum* colonies grown on agar plates would facilitate the identification and isolation of *C. botulinum* and neurotoxin-producing *C. butyricum* and *C. baratii* among competitive microflora. Procedures based on immunodiffusion (69, 152, 190, 210) or immunoblotting (83) have been published. As a single *C. botulinum* colony may produce as much as 10^5 minimal lethal doses (MLD) of toxin within 24 h (69), the colony immunoblot assay, which detects 10 to 25 50% MLD (MLD_{50}) of toxin per spot (83), would be sufficiently sensitive for the identification of *C. botulinum* on an agar plate. No reports on the performance of these tests with naturally contaminated clinical or food samples or mixed bacterial populations have been published. Fluorescent antibodies against the cell walls of vegetative *C. botulinum* have also been used to identify *C. botulinum* in a culture (81), but this method suffers from cross-reactivity between *C. botulinum* and its nontoxingenic counterparts.

A dilemma arising from the different physiologies of group I and II strains is the selection of the correct incubation temperature (Table 1). Group I strains grow optimally at 35 to 37°C, whereas group II strains favor lower temperatures of 25 to 30°C (197). If both groups are being sought in a single

sample, a compromise of 30°C has been proposed (197). However, investigating replicate samples at 26 to 30°C and at 35 to 37°C is preferable to ensure optimal growth for both groups. The optimal incubation time varies with the sample material and the detection method selected to identify the presence of *C. botulinum* in the culture tube. When the mouse bioassay is used to identify toxin formation in culture tubes, an incubation period of 5 to 7 days has been recommended (197). Shorter incubations may become relevant if molecular biological techniques, such as PCR (see below), are employed (133).

As the prevalence of *C. botulinum* in naturally contaminated samples is generally relatively low (10 to 1,000 spores/kg) and as there are no proper selective media available, quantification of *C. botulinum* in samples containing other bacteria by use of plate count procedures is difficult, if not impossible. Therefore procedures for enrichment in liquid media are often needed. In such cases the quantification of the organism is obtained with a most-probable-number technique. The most-probable-number estimate is based on the amount of sample material containing the target organism and the total amount of sample material investigated. In general, the greater the number of culture tubes that are used, the more accurate is the estimate obtained. The presence of *C. botulinum* in the culture tubes can be determined, e.g., with the mouse bioassay or by PCR (98).

As the group I and II strains differ in their epidemiologies, discrimination between the two groups should be done whenever an outbreak strain is isolated (Fig. 1). To be able to distinguish between the two groups, the proteolytic activity of group I strains can be determined on a casein-containing agar, e.g., reinforced clostridial medium supplemented with milk (169). Alternatively, molecular biological techniques such as probe hybridization or ribotyping (see below) can be employed. Although group I and II *C. botulinum* strains are dissimilar in many respects, both are able to grow at an incubation temperature range of 30 to 37°C, which is often used. Therefore, the ability to grow at a certain temperature should not be used as a feature determining the physiological group of a *C. botulinum* isolate.

Commercial test systems based on series of biochemical reactions have been developed for the identification of anaerobic bacteria. Contradictory reports on the ability of these tests to identify *Clostridium* spp. are available; different tests have been able to correctly identify 54% to 96% of the clostridial strains studied to the species level (31, 85, 95, 141). Due to the diverse physiology of *C. botulinum*, not all strains can be identified to the species level (28, 132). The biochemical test systems are also susceptible to technical bias, and factors such as incubation environment (171), incubation time (85), and concentration of the cell suspension (28) have been reported to drastically affect the success of identification.

For the reasons discussed above, the identification and isolation of *C. botulinum* from samples with high levels of competitive bacterial flora, such as fecal and environmental samples, are laborious and time-consuming. Several subsequent broth cultures and platings are required to isolate a pure culture, and often the isolation simply fails. The presence of nontoxigenic *C. botulinum*-like strains (30, 127, 132) disturbs the culture of *C. botulinum*. Whether these strains are completely different bacterial species from *C. botulinum* or whether

TABLE 3. PCR and probe hybridization protocols applied for the detection and identification of *Clostridium botulinum* groups I and II in different types of samples

Sample type and pretreatment	Assay type	Toxin types detected	Sensitivity	Reference(s)
Extracted DNA from pure cultures	PCR + gel electrophoresis	A, B, E, F	0.3 ng	42
	PCR + gel electrophoresis	A, B, C, D, E, F	2.5 pg	207
	PCR + probe hybridization	A	12.5 fg	62
	Real-time PCR	A, B, E	42–195 fg	2
	Real-time PCR	A	0.1 ng	223
	Multiplex PCR + hybridization onto array	A, B, E, F	0.015–0.5 pg	78
Extracted DNA from spores inoculated in foods	Real-time PCR	A	10^2–10^3 spores/ml	223
Extracted DNA from enriched foods	PCR + probe hybridization	A, B, E, F	0.1 cell/g	8
	PCR + probe hybridization	A, B, E, F, G	10 cells/g	61
	PCR + probe hybridization	A, B, E	0.1–21 spores/g	27
	PCR + capillary electrophoresis	E	10 cells	183
Extracted DNA from enriched foods, environmental samples, and clinical samples	PCR + gel electrophoresis	A, B, E	10^2 spores/g	205
Extracted DNA from enriched feces	Nested PCR + gel electrophoresis	B	NR[a]	117
Extracted DNA from enriched foods and environmental samples	PCR-ELISA	A, B, E, F	10^{-1} cell/g	63
Extracted DNA from heated and enriched feces	Nested PCR + gel electrophoresis	B, E, F	10–10^3 spores/g	43, 44
Extracted DNA from foods and feces	Real-time PCR	A, B, E	NR	2
Extracted DNA from foods	Multiplex PCR + gel electrophoresis	A, B, E, F	NR	Wyatt et al., Abstr. Rapid Methods 2005
Extracted DNA from wound swabs, tissues, pus	Real-time PCR	A, B, E	10–10^2 cells	3
Crude cell lysate from enrichment broth	PCR + probe hybridization	A, B, E	NR	73
	Multiplex PCR + gel electrophoresis	A, B, E, F	10–10^2 cells	133
	Multiplex PCR + hybridization onto array	A, B, E, F	4×10^2 cells	78
Crude cell lysate from enriched foods	PCR + probe hybridization	A	10–10^3 cells/g	62
Crude cell lysate from enriched foods and feces	Multiplex PCR + gel electrophoresis	A, B, E, F	Food, 10^{-2}–10^{-1} spore/g; feces, 10^{-1}–10^3 spores/g	133

[a] NR, not reported.

they derive from toxigenic *C. botulinum* (221) by complete or partial deletion of the toxin gene cluster during the exhaustive subculture procedures warrants further investigation. In botulism cases due to type E and F toxins, the possibility of toxin-producing *C. butyricum* or *C. baratii* causing botulism should not be ignored unless *C. botulinum* can be identified. These bacteria differ physiologically from *C. botulinum* strains (35), and the laboratory routines established for *C. botulinum* are not directly applicable for the isolation and identification of these species.

Molecular Detection of *Clostridium botulinum*

DNA-based detection methods have overtaken conventional techniques, with numerous molecular detection protocols published for *C. botulinum* (Table 3). Ascendant techniques include PCR and Southern hybridization, both of which are sensitive and specific and are rapid compared to culture techniques and the mouse bioassay. Many laboratories have already invested in PCR thermocyclers and electrophoresis equipment, and their staff are experienced with molecular

techniques. In the case of *C. botulinum*, molecular detection techniques are based merely on the detection of the botulinum neurotoxin gene, *bot*, in the sample, and thus they do not detect the activity of the gene nor the toxin. Naturally this is a disadvantage, but, apart from the advantages mentioned above, molecular techniques do not require the use of laboratory animals. This makes them a suitable tool for screening bacterial colonies, pure liquid cultures, and sample enrichments for the presence of the neurotoxin genes, indicating the presence of *C. botulinum* and other toxin-producing clostridia.

Most reports on PCR detection of *C. botulinum* are based on a single detection of one of the seven types of toxin genes in a reaction (Table 3). An improvement has been the development of a multiplex PCR assay that enables the simultaneous detection of the *botA*, *botB*, *botE*, and *botF* genes in a single reaction (133). This significantly decreases the assay time and labor and naturally the reagent costs. The multiplex PCR products are detected using conventional gel electrophoresis (133) or by further hybridization onto a membrane coated with cDNA probes to the PCR products (78). The two methods provide similar sensitivities (Table 3).

As both the *botB* and *botF* genes of group I and II *C. botulinum* are highly similar, the molecular detection methods based on these neurotoxin genes do not reveal the physiological group of *C. botulinum*. An oligonucleotide probe specific for the 23S rRNA gene specifically detects group II strains (33), but as this probe also recognizes the nontoxigenic *C. botulinum*-like strains within group II, its value as the only diagnostic tool is limited. A HindIII fragment-based DNA probe specific for group I *C. botulinum* would better suit the purpose, as it does not hybridize to *C. sporogenes* (147). Some molecular typing methods such as ribotyping and amplified fragment length polymorphism (AFLP) (see below) are also suitable for differentiation between *C. botulinum* groups I and II (97, 120).

The sensitivities of the PCR and probe hybridization assays reported for the detection of *C. botulinum* in feces, serum, and food samples vary markedly (Table 3). Greater sensitivity is generally obtained when extracted DNA, as opposed to crude cell lysates, is used as a template. The DNA extraction procedures applied for *C. botulinum*, however, may be laborious and time-consuming (109, 120, 142), while a cell lysate is easily prepared in an hour (73).

The sensitivity of PCR can also be increased by a nested design, where two primer sets targeted to the same gene are used in subsequent reactions. Nested PCR protocols have been published for the *botB*, *botE*, and *botF* genes (43, 117). The nested PCR format may allow the shortening (43) or complete exclusion of enrichment steps that are often required with normal PCR. *C. botulinum* type B was successfully detected directly from feces of an infant botulism case when using a nested PCR format (117). However, the detection limit of the assay was not determined, and this should ideally be done before the method can be reliably applied in diagnostics.

Some components in clinical and food samples, such as bile salts or competitive microflora in feces, immunoglobulins, and other components in blood and high protein and fat contents in foods, may inhibit PCR or at least drastically decrease its sensitivity (4, 5, 124). For example, the PCR detection limit for *C. botulinum* type E in feces was shown to be 4 log units higher than that in a fish or meat matrix (133). This should be borne in mind when setting up PCR protocols for clinical specimens. Each type of sample material requires thorough optimization of PCR as well as prior sample preparation. Sample pretreatment should focus on concentrating target cells and eliminating competitive flora and PCR-inhibitory substances. Internal amplification controls as well as other appropriate control setups should be routinely used in diagnostic PCR to control inhibition by the sample material or contamination (102, 103). Reports on their use in the molecular diagnostics of *C. botulinum* are so far scarce (63; G. M. Wyatt, J. Plowman, C. F. Aldus, M. W. Peck, and W. Penaloza, Abstr. Rapid Methods 2005, Noordwijk, The Netherlands, p. 8, 2005).

As the number of *C. botulinum* organisms in naturally contaminated samples is often very low and the target organism forms resistant spores, PCR detection directly from sample materials may often fail due to insufficient sensitivity of the assay. Enrichment is thus required to germinate spores and increase the target cell concentration in the sample (Table 3). Again, when investigating samples where both group I and group II *C. botulinum* may be present, careful consideration of enrichment conditions is urged, and optimally replicate samples will be incubated both at 35 to 37°C (for group I) and at 26 to 30°C (for group II). The duration of the enrichment step for optimal performance of PCR is also an important issue and should always be determined for each sample material (133) and enrichment medium. Too short an enrichment may result in competitive bacteria overtaking *C. botulinum*, whereas extensive incubation may result in lysis or sporulation of *C. botulinum* cells. Ideally PCR is done from a culture on several subsequent days to determine the optimal length of enrichment for each type of sample (133).

Another problem of direct PCR analysis from sample material is the possible detection of dead cells due to intact DNA after cell lysis (218). Enrichment procedures partly solve the problem by increasing the number of live cells in proportion to dead cells (98). Another approach to ensure that only live cells are detected is reverse-transcription PCR (RT-PCR), where gene expression rather than the gene itself is detected. Reverse transcription-PCR protocols for the *botB* and *botE* genes have been described (136, 137, 146). In a case of a human botulism outbreak, however, laborious procedures to obtain high-quality RNA may prove to be too time-consuming.

The different chemistry (intercalating fluorescent dyes or probes with a quencher dye) of real-time PCR from that of conventional PCR allows real-time monitoring of the amplification of the target gene after each PCR cycle, enabling rapid interpretation of results. In some experimental settings the melting temperature of the amplicons also can be calculated, which is essential to differentiate the specific PCR product from unspecific amplicons. Real-time PCR has been applied in the qualitative detection of *C. botulinum* types A, B, and E in foods and feces from food-borne and infant botulism cases (2). The same PCR protocol was recently employed in investigation of tissue, wound swab, pus, and aspirate samples from cases of wound botulism (3). When subjected directly to DNA extraction and PCR, only a small number of tissue samples from two patients were positive in the real-time PCR analysis. PCR inhibition was suggested to explain some of the negative results; however, after a 1- to 5-day

enrichment in liquid medium, most samples from patients with toxin detected in their sera were shown to carry one or more neurotoxin genes (3).

Apart from rapidity, another advantage of real-time PCR is its ability to quantify the target sequence, reflecting the level of the organism. To obtain reliable quantification, the copy number of the target gene in the genome of the organism in question must be known. Furthermore, the requirements for selection of reagents, reaction standardization, and sample pretreatment are higher than those for conventional PCR. Quantitative detection of the *botB* gene has been employed for research purposes (136, 137), whereas a recent application allowed the quantitative detection of *C. botulinum* type A spores spiked in canned corn and sausage (223) (Table 3). In this method, DNA is extracted directly from spores and no enrichment steps are employed. This allows the whole analysis to be finished within a working day. However, the speed is achieved at the cost of sensitivity; no fewer than 100 spores are detected (223). As the natural contamination levels of *C. botulinum* in food, clinical, and environmental samples may be 10 to 1,000 spores/kg, it is obvious that enrichment steps are needed for successful detection.

As discussed above, *C. botulinum* strains carrying silent toxin genes may confuse PCR detection by causing false-positive results (73). Conversely, point mutations or natural genetic variation in the template at the site of one or both PCR primers may impair primer annealing, thus causing false-negative results even if the gene is present and fully functional (Wyatt et al., Abstr. Rapid Methods 2005). Hence, although PCR can be a powerful screening tool in the laboratory diagnostics of botulism outbreaks, the results should ideally be confirmed by other methods. The specificity of PCR should be confirmed by combining PCR with *bot* gene-specific probe hybridization or by DNA sequencing of the PCR product instead of or in addition to plain fragment size-based gel electrophoresis analysis. Relying merely on PCR results is somewhat risky and should ideally be avoided if possible. It should also be borne in mind that PCR does not detect botulinum neurotoxin.

Genetic Characterization of *Clostridium botulinum*

A number of molecular typing tools have been used for the genetic characterization of *C. botulinum*. These include DNA sequencing, pulsed-field gel electrophoresis (PFGE), and ribotyping, as well as PCR-based techniques such as AFLP, randomly amplified polymorphic DNA analysis (RAPD), and repetitive element sequence-based PCR (Rep-PCR) (Table 4). International molecular typing libraries with genetic fingerprints, preferably obtained by use of more than one technique, of *C. botulinum* strains isolated in botulism epidemics would serve as a valuable source of information when investigating new outbreaks. As the modern food industry focuses on long shelf lives and refrigerated storage, product delivery routes may be long and the same product may be transported to many different countries. It is therefore likely that botulism outbreaks, instead of being sporadic and restricted to a limited geographic area, will be distributed over a wide area and thus be difficult to solve (122).

TABLE 4. Genetic typing tools applied for *Clostridium botulinum* groups I and II

Method	Discrimination of groups I and II (level)	Effort and time required to completion from pure colony	Reproducibility	Special feature	Application(s)	Reference(s)
PFGE	Strain	Laborious, 5–7 days	Excellent	DNA degradation by clostridial endonucleases may disturb analysis	Epidemiological studies, comparison of clinical isolates	96, 110, 130, 131, 160, 161
rRNA gene sequencing	Group	Moderate, 3–4 days	Excellent		Taxonomy	33, 41, 107, 108
Ribotyping	Group	Laborious, 3–5 days (for automated ribotyping, easy, 1 day)	Moderate	Can be automated (RiboPrinter)	Phylogeny, species/group identification	97, 191
AFLP	Strain and group	Easy, 3 days	Excellent		Epidemiological studies, comparison of clinical isolates, phylogeny	120
RAPD	Strain	Easy, 1–2 days	Low	Can be performed with crude cell lysate (although this further reduces reproducibility)	Rapid comparison of clinical isolates, not suited to interlaboratory comparison of strains	109
Rep-PCR	Group I, toxinotype; group II, strain	Easy, 2 days	Moderate	Not well suited to gram-positive bacteria	Rapid comparison of clinical isolates	109

Pulsed-field gel electrophoresis. Macrorestriction pattern analysis by PFGE has become one of the most powerful genetic typing tools in the diagnostics of food-borne pathogenic bacteria. The method is based on the electrophoresis of genomic DNA digested with rare-cutting restriction enzymes, yielding a distinctive fingerprint pattern for each bacterial strain. These fingerprints consist of 5 to 15 fragments in the size range of 10 to 1,000 kbp. In the diagnostics of botulism, PFGE can be used to trace the source of outbreaks by genetic comparison of *C. botulinum* isolates originating from patients and suspected food items (113, 131, 161). PFGE has also been used to study the biodiversity of *C. botulinum* (96, 110, 160).

The first report on PFGE for genotyping of *C. botulinum* was targeted to group I A toxin-producing strains and used MluI, RsrII, and SmaI restriction enzymes (130). Three different strains gave a distinctive PFGE pattern, whereas different isolates of the same strain gave similar PFGE patterns. A recent, more extensive study on PFGE typing of *C. botulinum* group I recommended the use of SacII alone or in combination with SmaI and XhoI (160). Analysis of 55 group I type A, B, AB, and F organisms revealed a high similarity among types AB and B and among type F organisms (160). For *C. botulinum* group II, PFGE using XhoI and SmaI proved to be highly discriminative, yielding a large genetic diversity among strains isolated from aquatic environments (96, 110).

Although reproducible and discriminative, PFGE analysis is laborious and takes several days to complete. A further disadvantage is that the method is sensitive to DNA degradation by extracellular DNases, and not all strains, particularly group II strains, are typeable with standard procedures. Cell fixation with formaldehyde treatment has solved this problem in most cases (95) (Table 4).

Ribotyping. The conservative ribosomal genes (rRNA genes) have become the most widely researched targets for phylogenetic analysis of organisms. One application is bacterial rRNA gene restriction pattern analysis (ribotyping), which is based on a genomic restriction pattern hybridized with labeled cDNA probes targeted to *Escherichia coli* rRNA genes (86). Possessing a lower discriminatory power than PFGE in bacterial strain-to-strain differentiation but a sufficiently high power to discriminate between bacterial species makes ribotyping an ideal tool for phylogenetic analysis. The method has effectively discriminated between 8 *C. botulinum* group I and 19 group II strains with EcoRI (97), confirming that the two groups belong to distinct phylogenetic lineages (41). Ribotyping is highly reproducible, but as with PFGE, degradation of DNA may hinder the typing of some strains (97) (Table 4). A good indication of the strong position of ribotyping in today's phylogenetic analysis is RiboPrinter, an automated ribotyping system (Qualicon, Wilmington, DE) that has also been used for *C. botulinum* (191).

Amplified fragment length polymorphism. The AFLP technique is a relatively new genetic typing tool that is based on digestion of genomic DNA with two restriction enzymes, followed by ligation of restriction site-specific adapters and amplification of a subset of fragments by PCR (212). An AFLP analysis of 33 group I and 37 group II *C. botulinum* strains with HindIII and HpyCH4IV revealed that the method is fast, highly reproducible and discriminative, and an excellent typing tool for *C. botulinum* (120). The same study also showed that

AFLP, like ribotyping, is well suited to phylogenetic analysis and satisfactorily discriminates between group I and group II strains. Furthermore, AFLP is not disturbed by DNA degradation, so it provides 100% typeability (Table 4).

Randomly amplified polymorphic DNA analysis. Also referred to as arbitrarily primed PCR, RAPD is based on PCR with randomly annealing universal primers under low-stringency conditions (217). It is fast and easy to perform, and the protocol applied for group II *C. botulinum* has been reported to be even more discriminatory than PFGE (109). The discriminatory power was lower with group I strains. All strains are typeable with RAPD. However, due to the random annealing of unspecific primers, the method lacks sufficient reproducibility and is thus not well suited for interlaboratory comparison of bacterial strains (Table 4).

Repetitive element sequence-based PCR. Bacterial genomes harbor conservative repetitive extragenic elements that can be used as targets for PCR with single or multiple consensus primers (211). The number and size of amplification products define a species-specific fingerprint; however, differentiation to the strain level is limited, restricting the applicability of the method in epidemiological and clinical research. A Rep-PCR protocol established for *C. botulinum* allowed the group II type B and E toxin-producing strains to be differentiated to the strain level, whereas the group II type F toxin-producing strains and all group I strains were differentiated only to the toxinotype level (109). A group-specific amplification fragment was obtained for both groups, suggesting that the method could be used for determining the physiological group of disease isolates in botulism cases. Like RAPD, Rep-PCR is rapid to perform; the advantage of Rep-PCR over RAPD is its higher reproducibility due to the use of specific primers (Table 4).

The findings obtained with the different genetic typing tools described above suggest that group I and II strains may have different epidemiological behavior in the environment, resulting in a narrower diversity of group I strains than of group II. This might be due to different bacterial life cycles in soil and water; aquatic environments may provide more optimal conditions for frequent spore germination than soil, leading to greater genetic variation in organisms of aquatic origin, such as group II *C. botulinum*. Whether this is true or whether the current typing tools are just insufficiently discriminatory with group I strains warrants further investigation.

FUTURE PERSPECTIVES

While there has been tremendous progress in the development of in vitro tests to potentially replace the mouse assay in the detection of botulinum neurotoxins and molecular biological tools for the detection and characterization of *C. botulinum*, far more needs to be done. The in vitro toxin tests need further rapidity and sensitivity in a single test, and all seven toxin types should ideally be identified simultaneously. Furthermore, the tests should be validated with a range of complex matrices, such as feces, foods, and environmental samples, in addition to testing with purified toxins.

Although the molecular biological methods for the detection and identification of *C. botulinum* are promising, the conventional culture techniques should not be overlooked. Selective

media suitable for both group I and II *C. botulinum* and other toxin-producing clostridia are needed to harvest all possible toxin-producing strains from sample materials containing competitive bacteria. As the distinct physiologies of the different groups of *C. botulinum* apparently complicate the detection and isolation procedures, the taxonomy of the species should be reconsidered to correspond to the true genetic variation among the botulinum neurotoxin-producing strains.

Whereas the main goal of the laboratory diagnostics of botulism is to achieve a rapid diagnosis and to save the patient, appropriate laboratory procedures should aim at a more thorough understanding of the epidemiology of, risk factors for, and prevention of botulism due to either of the two groups of *C. botulinum*. The isolation and genetic characterization of *C. botulinum* originally from the patient and the source should be done whenever possible (Fig. 1).

Bacterial genome sequencing has created new possibilities in genetic typing as well as in researching the genetic mechanisms behind different metabolic traits such as neurotoxin formation and its regulation. DNA microarrays enable genome-wide analysis of microorganisms and will probably overtake the more conventional genetic typing tools within a few years. While bacterial genomes are being sequenced at an increasing speed, more genomic information about closely related species and bacterial strains is needed. The genome of a group I *C. botulinum* strain (type A, ATCC 3502) has been elucidated (Sanger Institute, Cambridge, United Kingdom), enabling the development of a *C. botulinum* microarray (http://pfgrc.tigr.org /organisms.shtml). However, due to differences between group I and II organisms, the applicability of the type A array in genomic analysis of group II organisms may prove to be limited. Attempts to sequence a group II *C. botulinum* genome are therefore necessary. Naturally, genomic information on a single strain will not provide a good overall understanding of the genetic diversity within the species. Thus, additional research funding allocated to *C. botulinum* genome sequencing projects is needed to shed light on the genetic diversity and the mechanisms underlying different metabolic phenomena among botulinum neurotoxin-producing clostridia.

CONCLUSIONS

As botulism is a potentially lethal disease, a rapid diagnosis is essential. Active research on assay development is thus required. In vitro assays for rapid and sensitive detection of all types of botulinum toxin are needed, as are thorough validations of these tests with a range of complex matrices. In the meantime, molecular biological detection and identification tools should be used in screening and surveillance whenever possible to diminish the use of the mouse bioassay. More sophisticated and efficient tools to isolate group I and II strains from clinical, food, and environmental sample materials are needed. To enhance the development of culture methods for group I and II (and III) *C. botulinum*, the taxonomy of the organism should be reconsidered to correspond to the true genetic variation among the botulinum neurotoxin-producing bacteria.

Whereas the rapid laboratory diagnosis of botulism is based on detection of toxin in the patient, a thorough epidemiological investigation of botulism outbreaks and the causative or-

ganism is needed to increase our understanding of the different forms of the disease and their prevention. The laboratory diagnostics of each case of human botulism should include every attempt to isolate *C. botulinum* from the patient as well as from the source. In addition to the identification of the toxin type that they produce, these isolates should be typed to reveal the physiological group of *C. botulinum*. Genetic characterization of the isolates should be included in routine diagnostics and epidemiological investigation (Fig. 1).

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