12 Clostridium botulinum, Clostridium perfringens, Clostridium difficile

Introduction

The genus *Clostridium* has more than 80 species. *Clostridium* species are Gram-positive, anaerobic, and rod-shaped and form spores. These microorganisms are widely distributed in the environment particularly in soil, water, and decomposing plants and animals. *Clostridium* species are also found in human and animal intestine as part of their gut microbial community. *Clostridium* species cause a variety of diseases including food poisoning, neuroparalytic disease, gas gangrene, myonecrosis, necrotic enteritis, and antibiotic-associated diarrhea. In general, these organisms produce several exotoxins and enzymes, which contribute to the tissue damage and pathogenesis. Some *Clostridium* species are considered zoonotic pathogens (Table 12.1).

Clostridium botulinum is the most important pathogen of the genus, *Clostridium*, and the bacterium produces a potent heat-labile toxin, botulinum neurotoxin (BoNT). The toxin is responsible for a neuroparalytic disease, botulism, which occurs in humans and animals. BoNT has found a therapeutic use to treat dystonia and other muscular disorders. About 15 *C. botulinum* outbreaks are reported each year in the USA, affecting approximately 55 people and 9 deaths. Professor Emile Van Ermengem of Ghent University first isolated *Bacillus botulinus* in 1895, later renamed *Clostridium botulinum*, in the town of Ellezelles (Belgium) from a homemade raw salted ham that was consumed by 34 musicians who developed botulism. Three musicians died in a week. The culture supernatant, when injected into the laboratory animals, produced paralysis confirming the involvement of a deadly neurotoxin.

Clostridium perfringens is another significant microorganism of concern, responsible for foodborne toxicoinfection in humans and gas gangrene and enteritis in humans and animals. It is responsible for about 966,000 illnesses and 26 deaths every year in the USA. William H. Welch isolated a bacterium, *Bacillus aerogenes capsulatus*, in 1891 during a postmortem autopsy of a deceased man at John Hopkins Hospital (Baltimore, MD, USA). Later, Welch and George Nuttall in 1892 reported the organism as *Bacillus welchii*, which afterward was renamed as *Clostridium welchii*, also known as *Clostridium perfringens*.

Clostridium difficile has become a significant public health concern in recent years due to its nosocomial and hospital-acquired infections, *Clostridium difficile* antibiotic-associated diarrhea (CDAD), and life-threatening pseudomembranous colitis. The organism was first reported in 1934, but in 1978, John Bartlett at Tufts University School of Medicine (Boston, MA, USA) established it as the causative agent of CDAD. This microorganism is now routinely isolated from food animals and humans thus likely to have a foodborne implication.

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Table 12.1 Classification of *Clostridium* species

Organism	Disease
Clostridium botulinum	Botulism, infant botulism, wound botulism – affects peripheral nerves – flaccid paralysis
Clostridium baratii	Infant botulism, hidden botulism
Clostridium butyricum	Infant botulism
Clostridium chauvoei	Black leg – cattle and sheep
Clostridium difficile	Antibiotic-associated membranous colitis (diarrhea) in humans
Clostridium histolyticum	Gas gangrene (human)
Clostridium novyi A, B, C, D	Gas gangrene in humans, necrotic hepatitis in sheep
Clostridium perfringens A, B, C, D, E	Gas gangrene, food poisoning, clostridial myonecrosis, enteritis in animals and humans, enterotoxemia in sheep (struck), and pigbel in humans
Clostridium septicum	Gas gangrene
Clostridium sordellii	Gas gangrene and myonecrosis in humans and animals, liver disease in sheep
Clostridium tetani	Tetanus in humans and animals affects CNS causing spastic paralysis
Clostridium sporogenes	Nontoxigenic

Classification of *Clostridium* Species

Clostridium species are classified based on the shape of vegetative cells, cell wall structure, endospore formation, biochemical properties, 16S rRNA gene sequence homology, mol% G + C content of DNA, and PCR amplification of spacer regions of 16S and 23S rRNA genes. Genome sequence of *C. difficile*, *C. perfringens*, *C. tetani*, *C. botulinum*, and *C. acetobutylicum* are available which greatly increase our understanding of the molecular properties of these organisms. *Clostridium* spp. that are involved in human and animal diseases are summarized in Table 12.1.

Clostridium botulinum

Biology

Clostridium botulinum is an obligate anaerobe. It is motile, spore-forming Gram-positive rod, and the spores are located sub-terminally. It grows in the animal intestine, and the spores are found in feces, soil, and plants. *Clostridium botulinum* is a heterogeneous species and produces eight antigenically distinct botulinum toxin (BoNT) types A, B, C, D, E, F, G, and H. Type H is a new toxin discovered in 2014 by Jason Barash and Stephen Arnon. Each toxin is encoded in a toxin gene cluster that includes several nontoxin accessory genes. Two toxin gene clusters are known, hemagglutinin (*ha*) and *orfX*. The *ha* is found in *C. botulinum* types A, B, C, D, and G, while *orfX* is found in types A (different subtypes), E, F, and H (Fig. 12.1). The neurotoxin genes are located either on the chromosome, on the bacteriophage, or on a plasmid. The genome size varies from 3.76 to 4.26 Mb, and the G + C content is 27.4–28.5%. The genes for BoNT types A, B, E, F, and H are on the chromosome, type C and D on bacteriophages, and type G on an 81-MDa plasmid. The genes are regulated by a regulatory element, BotR. Sometimes a strain may produce two toxins. In such cases, the major toxin is designated by the uppercase letter and the minor toxin by the lower case letter, for example, *C. botulinum* types Bh, Ab, Ba, Bf, and so forth.

Toxin types A and B are common in the USA; C and D occur in farm animals; E, F, and G are also produced by non-botulinum species such as *C. argentinese* which produces type G botulinum toxin and *C. baratii* type F toxin. BoNT types A, B, E, H, and, in the rare cases, F cause human botulism, while BoNT types C and D cause animal botulism. Botulinum toxin is produced, when the clostridial cells are lysogenized by the bacteriophages or due to autolysis of the cells late in the growth cycle. Toxin production is influenced by nutrient composition, cultural conditions of the growth medium including the presence of metal ions, certain amino acids, peptides, pH, temperature, and the cell density.

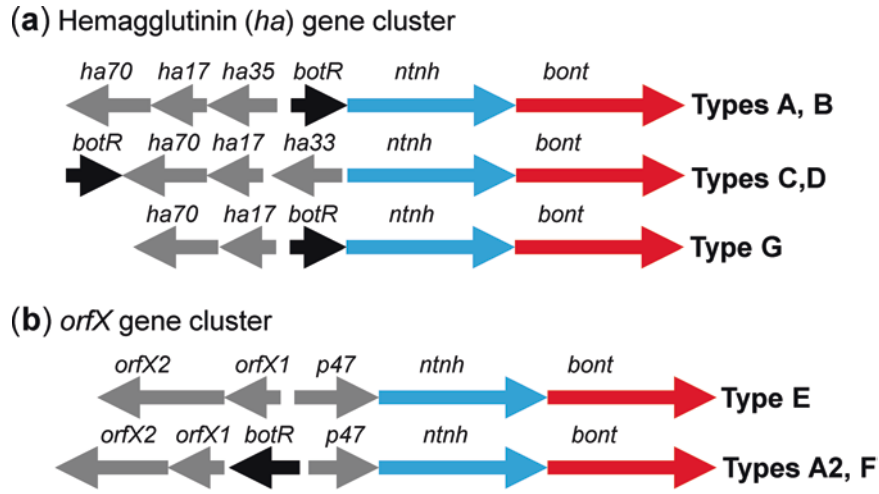

Fig. 12.1 Botulinum neurotoxin gene clusters, (**a**) *hemagglutinin* (ha) and (**b**) *orfX* of *Clostridium Botulinum*. *ha* Hemagglutinin, *ntnh* nontoxic nonhemagglutinin, *bont* BoNT, and *botR* botulinum regulator (Adapted and modified from Johnson and Bradshaw 2001. Toxicon 39, 1703–1722)

Table 12.2 Proteolytic and non-proteolytic *Clostridium botulinum*

Properties	Proteolytic	Non-proteolytic
Neurotoxin type	A, B, F, H	B, E, F
Fermentation of glucose	+	+
Fermentation of fructose, maltose, mannose, sucrose	Variable to negative	+
Motility	+	+
Minimum growth temperature	10–12 °C	2.5–3.0 °C
Optimum growth temperature	37 °C	25 °C
Minimum pH for growth	4.6	5.0
Minimum a_w	0.94	0.97
NaCl conc. to prevent growth	10%	5%
Spore heat resistance	$D_{121\ °C}$ = 0.21 min	$D_{82.2\ °C}$ = 2.4 min

Adapted from Peck et al. (2011). Food Microbiol. 28, 183–191

Based on the enzymatic activity, *C. botulinum* strains are grouped into either proteolytic or non-proteolytic, and these strains are genetically distinct (Table 12.2). Proteolytic *C. botulinum* produces several extracellular enzymes to degrade proteins and carbohydrates in order to harvest nutrients which may result in food spoilage. The non-proteolytic strains do not have similar enzyme activity but can ferment different sugars and are called saccharolytic. Both proteolytic and non-proteolytic *C. botulinum* produce neurotoxins and are responsible for most foodborne botulisms. Besides, *C. baratii* and *C. butyricum* are also involved in foodborne botulism. Proteolytic strains may carry one or two neurotoxin genes, but a majority carry a single gene for neurotoxin types A, B, F, or H, while the majority of non-proteolytic strains produce types B, E, or F.

Proteolytic strains are mesophilic, and the optimum temperature for growth and toxin production is 35–37 °C; however, they can produce neurotoxins at temperatures as low as 12 °C. The non-proteolytic strains are psychrotrophic and can grow and produce neurotoxin at 3.0–3.3 °C, but the optimum temperature is 25 °C. Proteolytic strains do not grow below pH 4.6 and non-proteolytic below pH 5.

Spores are highly resistant to heat, and the decimal reduction time (D value) is 0.15–1.8 min

at 110–121 °C. Spores of proteolytic strains are more resistant to heat than the spores of non-proteolytic strains. A standard heat treatment of 121 °C for 3 min ensures the safety of low-acid canned foods. Spores present in food subjected to heat treatment will germinate under favorable conditions to form vegetative cells. The amino acid, L-alanine alone or in combination with L-lactate, can trigger spore germination. Vegetative cells will grow under an anaerobic environment in food and produce botulinum toxin that would be a cause for concern. The botulinum toxin is sensitive to heat treatment (>85 °C for 15 min); therefore, thorough heating of the food prior to consumption will render food safe. *Clostridium botulinum* spores do not survive well in the healthy adult human gut environment.

Sources

Most *Clostridium* spp. survive in soil and grow in the animal intestine. *Clostridium* spores can be found in poorly or under-processed canned foods such as home-canned foods, spices, sewage, and plants. The spores are heat-resistant and can survive in the canned food when the temperature is used below 120 °C. Low acidity (pH above 4.6), low oxygen, and high water content favor spore germination and toxin production. Spices, herbs, and dehydrated mushrooms may serve as a potential source of spores. Home-canned vegetables – beans, peppers, carrots, corn, asparagus, potatoes, bamboo shoots – and fish are implicated in outbreaks. Foil-wrapped baked potatoes cooled at an inadequate rate and extent and served several hours later are responsible for a restaurant or at-home outbreaks. Yogurt, cream cheese, and jarred peanuts had also caused botulism outbreaks. Blood sausage caused frequent outbreaks in central Europe. Condiments – such as sautéed onions, garlic in oil, hot dog chili sauce, and commercial cheese sauce – were implicated in outbreaks. Bees can carry bacterial spores in addition to pollen and honey, which may contain clostridial spores, and the acceptable limit of clostridial spores in honey is less than 7 per 25 g.

Botulism

Clostridium botulinum produces botulinum toxin, which causes botulism in humans and animals. The name "botulism" came from the Latin word "botulus" meaning sausage. In central Europe, in the eighteenth century, the disease was frequently linked to the consumption of blood sausage. The foodborne botulism is an intoxication disease, not an infection since the preformed toxin causes the disease without the bacterium. The LD_{50} of active botulinum toxin is <0.01 ng in the mouse. In adult humans, intravenous or intramuscular administration of 0.9–0.15 μg or oral administration of 70 μg can cause death. There are five types of botulism: foodborne botulism, infant botulism, hidden botulism, inadvertent botulism, and wound botulism. Foodborne, wound, and infant botulism are caused primarily by *C. botulinum* toxin type A. Toxin B and E types also can cause such botulism but to a lesser frequency or extent.

Foodborne Botulism

Consumption of food contaminated with spores does not generally cause disease in healthy adults. Contaminated food after heating (at least 70 °C) and cooling at a slow rate promotes germination of spores. Spore germination may take place in the colon, but clostridia are unable to survive because of the resident microflora. Heating removes oxygen; thus the anaerobic environment created by the heated food allows germination and growth of the organism and subsequent botulinum toxin production. Ingestion of toxin is lethal, and the incubation period of botulism depends on the rate and the amount of toxin consumed, which can occur as early as 2 h or as long as 8 days, typically 12–72 h. Once consumed, the toxin is absorbed and reaches the blood circulation. The toxin blocks the release of neurotransmitter, acetylcholine, which prevents nerve impulse propagation in the peripheral neurons, affecting parasympathetic and sympathetic nervous system, and the neuromuscular junctions and causes flaccid paralysis (Fig. 12.2). In flaccid paralysis, the peripheral nerves, both sympathetic and parasympathetic nerves, are affected. Another

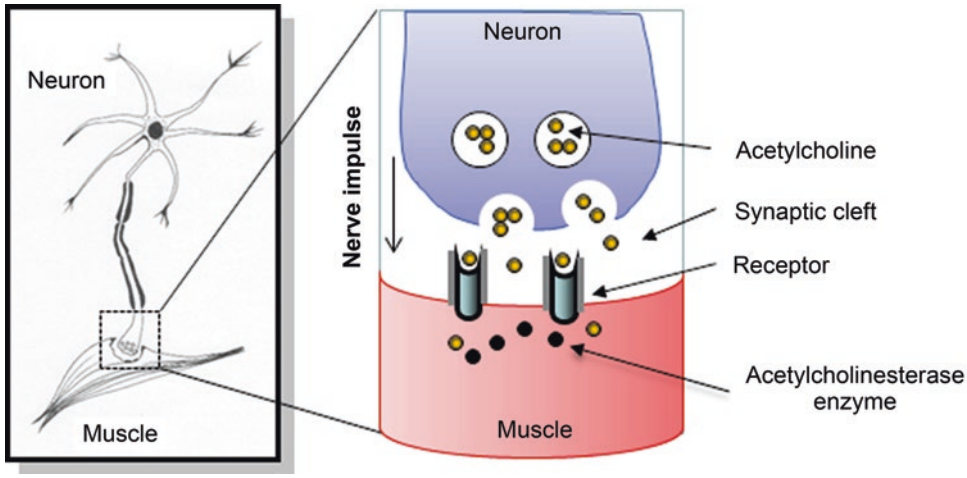

Fig. 12.2 Schematic drawing showing the neuromuscular junction and transmission of nerve impulse

neuroparalytic toxin, tetanus toxin, produced by *Clostridium tetani*, exhibits spastic paralysis, in which the central nervous system is affected. Once the botulinum toxin is bound to a neuron, external intervention has little or no effect.

The symptoms appear 4–36 h after ingestion of the toxin, which include nausea, vomiting, headache, double vision, slurred speech, muscle spasm (dystonia), and muscle weakness. It first affects the upper limbs then the lower limbs and exhibits breathing difficulty because of the pharyngeal and diaphragm muscle weakness. The heart function also weakens, and death follows. Autonomic symptoms include dry mouth, postural hypotension, urinary retention, and pupillary abnormalities. The fatality rate is 5–10%; however, the fatality is much higher for untreated patients.

Infant Botulism

Clostridium botulinum, *C. baratii*, and *C. butyricum* are responsible for infant botulism. Honey is the main source of the organism. Infants under 1 year of age are mostly susceptible. Since infants have not developed complete colonic microflora populations, there are no antagonistic effects from resident flora or from bile salts. The spores germinate in the intestinal tract of the infant, and the vegetative cells colonize the gut. The progression of the disease is very slow because of poor absorption of toxin through the colonic cell layers. Symptoms are very similar to the adult botulism; however, nausea and vomiting are absent. Early signs are weak cry, muscle weakness, difficulty in feeding, i.e., poor suckling ability, hypotonia (floppy baby syndrome), and a decrease in spontaneous movement. Constipation, tachycardia, and dry mouth are due to the blockade of parasympathetic nerve impulses. Death occurs in severe cases. Overall mortality rate is 5%. Most recover with adequate supportive therapy and interventions. Infant botulism cases are on the rise since 1990. Both bottle-fed and breast-fed infants are susceptible to infant botulism. Honey has been considered the significant risk factor, at least in 20% cases, for infant botulism; therefore, children should not be given honey in the first year of their lives.

Hidden Botulism

This adult form of botulism is hidden from the clinicians because of the lack of direct evidence for this type of botulism. Hidden botulism is neither food related, wound associated, nor drug use related. This adult form of infant botulism requires bacterial colonization in the gut. Antibiotic treatment due to other illnesses may disturb natural microflora balance in the gut thus allowing *Clostridium* species to colonize and produce toxins. Achlorhydria, prior surgery, or

Crohn's disease may also aid in the development of this form of botulism. *C. botulinum* or *C. baratii* produces toxin type F, which is responsible for hidden botulism in adults. Symptoms are similar to foodborne botulism, i.e., shortness of breath, dizziness, bradycardia, respiratory arrest, decreased voluntary movements, flaccid muscle tone, and so forth. Hidden botulism is diagnosed by isolation and identification of the *Clostridium* cells from feces.

Wound Botulism

Though the wound botulism is rare, it occurs in patients with traumatic and surgical wounds and intravenous drug users. It is also common for soldiers on the battlefield. In recent years, increased numbers of cases have been reported among the intravenous drug users. Wound botulism has also been reported in patients following intranasal cocaine abuse and the laboratory workers from inhalation of toxins. Spores lodge in the deep wound or in the injection sites of the drug users. The anaerobic environment created by tissue destruction and the growth of aerobic bacteria help germination and growth of *Clostridium botulinum*. The incubation period for wound botulism is 4–14 days. Botulinum toxin is produced and absorbed through the mucus membranes, broken skin, or wounds, leading to botulism. Note: *Clostridium tetani* can also enter through wound to cause tetanus in a similar manner.

Inadvertent Botulism

In recent years, botulinum toxin (such as Botox®, Dysport®, NeuroBloc®) is used for the treatment of dystonia and other movement-related disorders, strabismus (hyperactive extraocular muscles), and cosmetic enhancement. The patients treated with intramuscular injection of botulinum toxin have toxin circulating in the blood and can block the neurotransmitter release in adjacent muscle or in the autonomic nervous system. Cosmetic use of BoNT for removal of wrinkles, to improve skin or muscle tones, may serve as a possible risk factor for inadvertent botulism.

Virulence Factors and Mechanism of Pathogenesis

Clostridium botulinum produces three types of toxins: botulinum neurotoxin (BoNT), C2 toxin, and C3 toxin. Botulinum toxin affects neurons, while the C2 and C3 toxins induce epithelial cell damage possibly facilitating the spread of the BoNT to deeper tissues.

Botulinum Toxin

The botulinum toxins are classified as types A, B, C, D, E, F, G, and H and subtypes A1, A2, A3, A4, and A5, which are antigenically distinct. The amino acid sequence of each neurotoxin type differs by up to 70%, while each subtype differs by only 2.6–32%. BoNT types A, B, E, H, and, in rare cases, F cause human botulism, while BoNT types C and D cause animal botulism. Botulinum toxin is produced, when the clostridial cells are lysogenized by the bacteriophage or due to autolysis of cells late in the growth cycle. The BoNT toxin is produced in the culture as a large complex (about 900 kDa) consisting of BoNT, hemagglutinin (HA), and nontoxin nonhemagglutinin (NTNH). The proteolytic *C. botulinum* strains produce BoNT types A, B, F, and H, while the non-proteolytic strains produce types B, E, or F (Table 12.2).

The botulinum toxin is a 150 kDa polypeptide and is derived from a large progenitor inactive form of a toxin called derivative toxin or protoxin (Fig. 12.3). The derivative toxin produced by the proteolytic *C. botulinum* strain is activated by its own protease. In the case of non-proteolytic strains, the toxin is activated by host gastric proteases, since these strains do not produce protease. The active toxin is an A–B-type toxin, consisting of two subunits: B (heavy chain, 100 kDa) and A (light chain, 50 kDa). The heavy and light chains are joined by a disulfide bond (Fig. 12.3). The primary function of BoNT is to block neurotransmitter (acetylcholine) release at the neuromuscular junction, thus affecting nerve impulse propagation leading to flaccid paralysis.

Normally, acetylcholine release from the vesicles at the neuromuscular junction is aided by

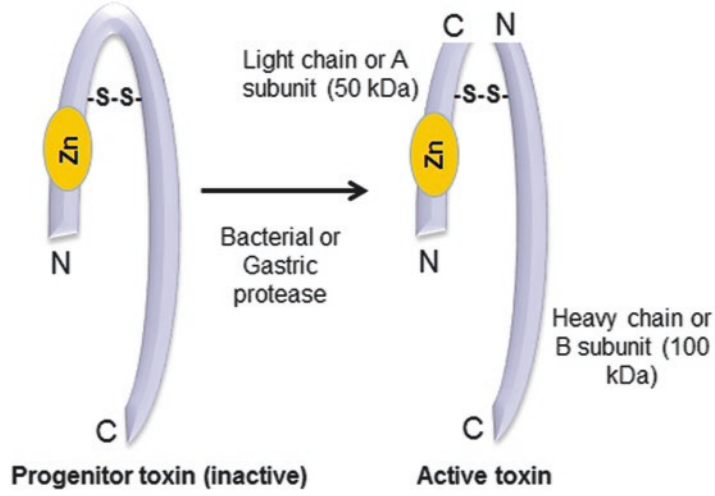

Fig. 12.3 Activation of botulinum toxin by the bacterial or gastric protease

SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins, which consist of synaptobrevin, SNAP-25 (synaptosomal-associated protein-25), and syntaxin. SNAP-25 and syntaxin are integral neuronal membrane bound, whereas the synaptobrevin is a vesicle-associated membrane protein (VAMP). During propagation of the nerve impulse, synaptobrevin associated with the synaptic vesicle fuses with the SNAP-25 and syntaxin to form a synaptic fusion complex. Synaptic vesicle with SNARE complex then fuses with the neuron membrane, and through the exocytosis process, the acetylcholine is released into the synaptic cleft. Acetylcholine binds to the acetylcholine receptor located on the muscle fiber, and acetylcholinesterase enzyme depolarizes the acetylcholine and aids in the propagation of nerve impulse (Figs. 12.2 and 12.4).

In the botulism patient, after absorption through the tissues, the toxin is transported by the blood to the peripheral cholinergic synapses, primarily in the neuromuscular junction. The heavy chain (B subunit) of the neurotoxin has two functional domains. The C-terminal domain facilitates binding of the neurotoxin to a specific receptor on synaptic membrane vesicle proteins at the nerve terminal. The receptor is a sialic acid containing glycoprotein or glycolipid, and it is found only on the neuron. While the N-terminal domain forms a channel in the neuron and delivers the light chain into the nerve cytosol. The light chain (A subunit) has a single zinc molecule and has zinc-dependent endopeptidase activity, which cleaves the SNARE proteins. The protein target may vary depending on the toxin type. The A subunit of BoNT types A and E cleaves SNAP-25; types B, D, F, and G cleave synaptobrevin or VAMP; and type C cleaves syntaxin. As a result, the SNARE complex does not fuse with the synaptic membrane vesicle, and exocytosis of the acetylcholine does not occur, interfering with the nerve impulse propagation (Fig. 12.4).

The impaired nerve impulse in both sympathetic and parasympathetic nerves results in flaccid paralysis, where the muscle is tensed and relaxed showing characteristic symptoms. In comparison, tetanus toxin prevents the release of γ-aminobutyric acid thus inhibits neuronal transmission. Muscle is tensed and not relaxed and results in spastic paralysis.

C2 Toxin

The C2 toxin is a binary toxin similar to A–B--type toxin produced by some *C. botulinum* strains, in particular, types C and D. C2 toxin is an ADP-ribosyltransferase. It causes depolymerization of the actin cytoskeleton in the host cell. C2 consists of two non-linked proteins, the enzyme component C2I (50 kDa) and the binding and translocation component C2II (90 kDa),

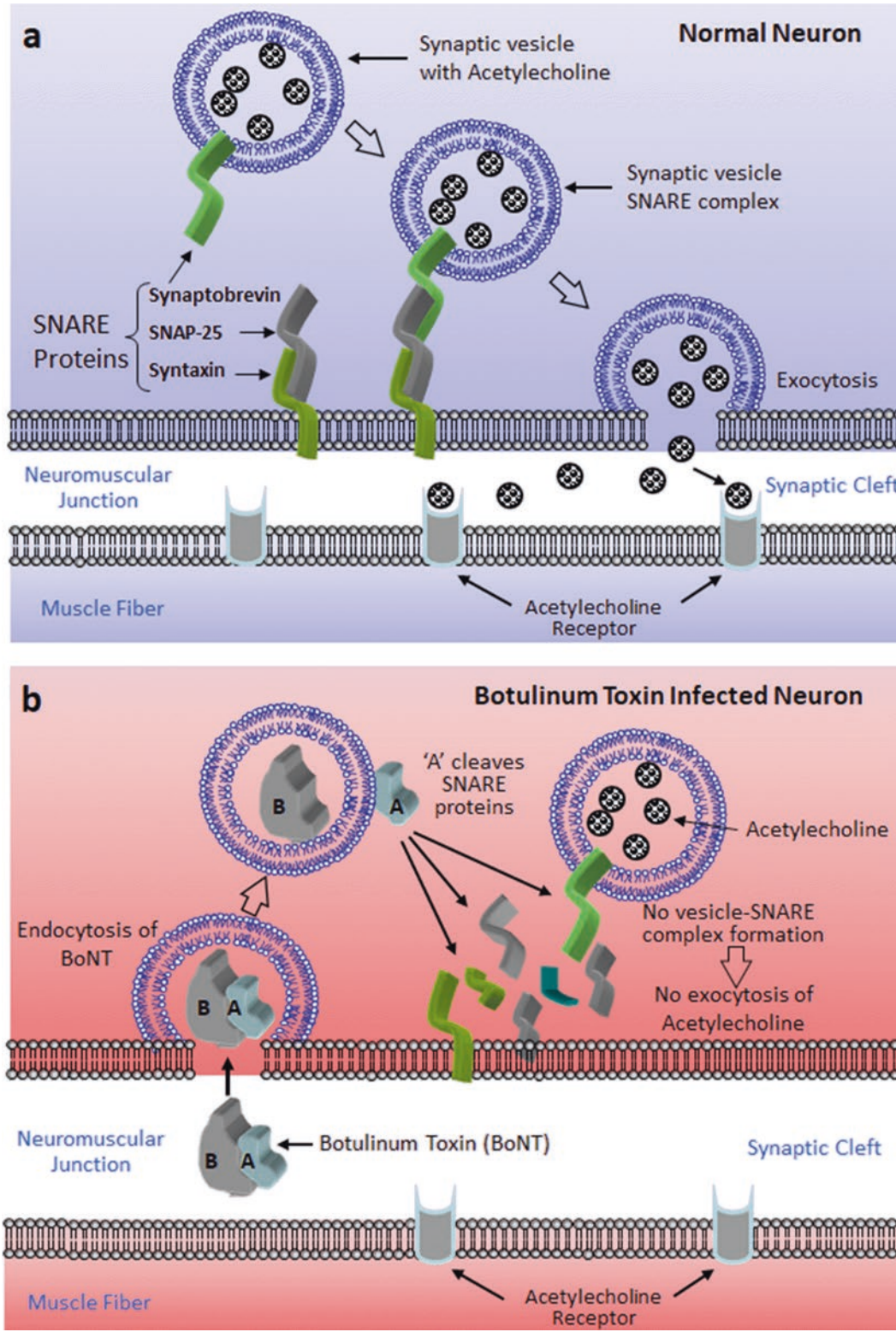

Fig. 12.4 Diagram showing the mechanism of action of botulinum neurotoxin (BoNT) (Arnon et al. 2001. J. Am. Med. Assoc. 285, 1059–1070)

which delivers C2I into the host cell. C2II binds to a variety of cells, and receptor-mediated endocytosis follows. The C2I with enzymatic activity translocates across the membrane and catalyzes the ADP-ribosylation of intracellular monomeric G-actin. C2I cleaves ADP-ribose from nicotinamide adenine dinucleotide (NAD^+) and attaches it to the actin protein, thus interferes with the microfilament formation. It is proposed that C2 toxin induces the formation of long microtubule-based protrusions forming a dense meshwork on the surface of intestinal epithelial cells providing a favorable environment for *C. botulinum* colonization and toxin production. C2 also disrupts the cytoskeleton structure leading to cell damage and increased vascular permeability and thus probably helps in dissemination of the botulinum toxin. C2 toxin also induces necrosis of the epithelial cells and edema in the lamina propria.

C3 Toxin

Clostridium botulinum C3 toxin is a 25 kDa protein. There are two isoforms, C3bot1 and C3bot2, with 60% sequence identity. The C3 proteins are also produced by *Clostridium limosum*, *Bacillus cereus*, and *Staphylococcus aureus*. C3 toxin ADP-ribosylates Rho, a GTP-binding signal transduction protein, which controls the actin polymerization. This toxin alters the cell morphology and leads to cell death. The pathological significance of C3 in the disease process is unclear; however, it has been preferentially internalized by monocytes and macrophages.

Symptoms

The clinical symptoms vary depending on the botulism type, i.e., foodborne, infant, wound, adult, or inadvertent form of botulism. Symptoms of foodborne botulism, which is most common, are visible 4–36 h after ingestion of the toxin, which include nausea, vomiting, headache, double vision, slurred speech, muscle spasm (dystonia), and muscle weakness. It first affects the upper limbs followed by the lower limbs and exhibits breathing difficulty because of pharyngeal and diaphragm muscle weakness. The heart function also weakens, and death follows. Other symptoms include dry mouth, postural hypotension, urinary retention, and pupillary abnormalities. The fatality rate is 5–10%; however, fatality is much higher in the untreated patients.

Prevention and Treatment

Prevention strategies are most important to avoid botulism, which include proper canning of food products, boiling of homemade canned foods since homemade canned foods present serious dangers, restraining from feeding infants with honey, and early diagnosis. The botulinum toxin is heat-labile; therefore, heating of suspected products for at least 85 °C for 15 min before consumption can prevent botulism. Botulinum toxin formation in foods can be prevented by various methods. Store food at (1) <3.0 °C, (2) a pH ≤ 5.0 and a chilled storage, (3) a NaCl concentration ≥ 3.5% plus storage at chilled temperature, and (4) an $a_w \leq 0.97$ plus chilled storage. (5) A 10-day rule should be followed, i.e., storage at ≤ 8 °C and a shelf life of <10 days, and (6) a heat treatment of 90 °C for 10 min or equivalent lethality (e.g., 80 °C for 129 min, 85 °C for 36 min for 6D process lethality) combined with the storage at chill temperature can be adopted.

Botulinum toxins are detected in patients sera, wound, or stool specimens. Sixty percent of botulism patients show a positive stool sample for *C. botulinum*. The suspected food should be tested by a mouse bioassay, which can detect as low as 0.03 ng of toxin, and results are obtained within 1–2 days. Antisera are used to type the toxin obtained from blood serum or other sources.

Treatment is unsuccessful if toxin has already entered the blood circulation and has bound to the receptors on the neuron. A successful intervention depends on the concentration of toxins being ingested and for how long. If the botulism is detected early, an antitoxin can be injected to neutralize the toxin that is still circulating in the blood. Toxins that are already bound to the receptor cannot be neutralized by the antitoxin. Mechanical respirators and life support are required to facilitate breathing, to counter neuro-

logical damage, and to regenerate the nerve endings. The recovering patients can suffer from a permanent neurological disorder (damage). The recovery period is generally very long. Dry mouth and general fatigue can last for weeks or months. An anticholinesterase drug such as edrophonium chloride may be beneficial to some patients. Guanidine and 4-aminopyridine (4-AP) have been reported to improve the ocular and limb muscle strength, but those compounds have a little or limited effect on the respiratory paralysis. In the case of the infant, wound, and hidden botulism, antibiotics are needed to clear the bacteria from the system.

Detection of *C. botulinum* or toxin

Culture Method

Blood, stool, or food samples can be tested for the presence of the toxin or the *C. botulinum* cells. Food samples are first enriched in the cooked meat medium or trypticase–peptone–glucose–yeast extract (TPGY), which is steamed for 10–15 min to remove dissolved oxygen and incubated at 35 or 28 °C for 5 days under a strict anaerobic condition. Gram staining or observations under a bright field microscopy should reveal the presence of typical cells with "tennis racket" appearance. For selective isolation of *C. botulinum*, enriched culture is either mixed with alcohol or heat-treated at 80 °C for 10–15 min, then streaked onto egg yolk agar or liver–veal–egg yolk agar, and incubated at 35 °C for 48 h under anaerobic conditions. The typical *C. botulinum* colonies appear as raised or flat, smooth or rough with some spreading, and irregular edges. On egg yolk agar, colonies show a luster zone (referred to also as a pearly zone), when observed under an oblique light. Often another subculture in TPGY or chopped liver broth under anaerobic conditions for 5 days followed by streaking onto egg yolk agar is needed to isolate the pure cultures of *C. botulinum*.

Immunoassays

Immunoassays are widely used for detection of the toxins. In a sandwich assay format, polyclonal antibody to the toxin is first bound to the solid surface, and then the sample containing the toxin is added. A second antibody labeled with an enzyme (alkaline phosphatase or horseradish peroxidase) is added. The addition of an appropriate substrate will produce color. This assay is 10–100-fold less sensitive than that of the mouse bioassay, and negative results have to be reconfirmed by other assays. Lateral flow immunoassay has been developed which provides results in less than 30 min; however, the assay is less sensitive; thus the negative results have to be confirmed by the mouse bioassay.

Mouse Bioassay

Mouse bioassay is considered the gold standard but very expensive. The assay requires a large number of mice. Bacterial cell-free culture supernatants are treated with trypsin at 200 μg ml^{-1}, and 0.5 ml of each toxin preparation is injected intraperitoneally into mice. Typical symptoms include labored breathing, pinching of the waist, and paralysis, which develop in 1–4 days.

BoNT Enzyme Activity Assay

The BoNT has zinc endoprotease activity, and it degrades neuronal proteins that regulate the release of the acetylcholine. BoNT from type A strain cleaves SNAP-25, while type B cleaves VAMP or synaptobrevin. A synthetic VAMP peptide is used as a substrate to assay for the presence of BoNT enzyme activity colorimetrically. This assay is more sensitive and much faster than the mouse bioassay.

PCR and Oligonucleotide Microarray

A highly specific multiplex PCR assay has been developed for detection of *C. botulinum* types A, B, E, and F from food and fecal materials. The assay is able to detect types A, E, and F at 10^2 cells, while type B was detected at 10 cells per reaction mixture from naturally contaminated meat, vegetable, and fish. An oligonucleotide array has been developed to detect multiple foodborne pathogens including *C. botulinum*, *E. coli*, *Listeria monocytogenes*, *Shigella dysenteriae*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Proteus vulgaris*, and *Bacillus cereus*.

Clostridium perfringens

Biology

Clostridium perfringens is a Gram-positive, rod-shaped, nonmotile, and spore-forming anaerobic but somewhat an aerotolerant bacterium. The genome size is 3.6 Mb, and all virulence genes are located either on the chromosome or on a plasmid. The bacterium can grow at a temperature between 15 °C and 50 °C with an optimal growth temperature of 43–45 °C. The growth rate is very fast, and the generation time is below 8 min at temperatures 33–49 °C. *C. perfringens* does not grow below 15 °C, but some rare strains can grow at 6 °C. The pH range for growth is 5–9.0. *C. perfringens* can survive and grow in the presence of curing salts consisted of 300 ppm of sodium nitrite and 4–6% NaCl. *C. perfringens* is a fastidious organism and requires more than 12 different amino acids and vitamins for their growth; thus it grows very well in the meat products. Meat and poultry are generally implicated in most outbreaks. Beef products are responsible for about 40% of *C. perfringens* outbreaks. Roast beef is a major vehicle of the outbreak because of improper handling, temperature abuse, and inadequate cooling after cooking.

C. perfringens is classified into five types, A, B, C, D, and E, based on the production of four types of extracellular toxins, alpha (α), beta (β), epsilon (ε), and iota (ι) (Table 12.3). Altogether, *C. perfringens* produces at least 20 different toxins, and the list is growing. In addition to the above four toxins, it produces several hydrolytic enzymes and toxins including lecithinase, hyaluronidase, collagenase (κ-toxin), DNAse, sialidases (affects sialic acid in the host cell membrane), amylase, *Clostridium perfringens* enterotoxin (CPE), hemolysin (perfringolysin O, PFO or theta toxin), necrotic enteritis toxin B (NetB), and so forth. The genes encoding the toxins are located either on the chromosome or on a plasmid. *C. perfringens* produces a double zone of hemolysis on blood agar plate. The clear inner zone is produced by PFO and the hazy outer zone by phospholipase C (α-toxin). *C. perfringens* type A is responsible for food poisoning (gastroenteritis), while type C and certain strains of type A cause necrotic enteritis. *C. perfringens* type A is also responsible for about 5–15% of all cases of antibiotic-associated diarrhea in humans and sudden infant death syndrome (SIDS).

Sources

Clostridium perfringens is found in soil, water, sludge, spices, dust, sewage, raw and processed foods, and contaminated equipment. It is present in low numbers (10^3–10^6 spores per gram) in human/animal intestine and feces. Ubiquitous distribution of the organism (spore), heat resistance of spore, and rapid growth and enterotoxin production made this organism a successful foodborne pathogen. It grows well in meat, especially in ground beef. During heating and cooling, if food is allowed to stand at room temperature – it will grow rapidly and produce toxin.

From 1992 to 1997, 248,520 human illnesses due to *C. perfringens* were documented by the Centers for Disease Control and Prevention (CDC). In the year 1993, at least 10,000 cases with 100 deaths were recorded. It is estimated that about 965,958 illnesses, 438 hospitalizations, and 26 deaths are associated with *C. perfringens* infection in the USA each year.

Virulence Factors and Mechanism of Pathogenesis

Clostridium perfringens causes food poisoning in humans and gas gangrene, myonecrosis, and enteritis in humans, animals, and birds. Food poisoning disease is characterized as toxicoinfection, not intoxication. Preformed *Clostridium perfringens* enterotoxin (CPE) in food is not responsible for the disease, since the toxin may be destroyed during passage through the stomach. CPE is heat-labile and destroyed at temperature 60 °C for 10 min. The infectious dose is about 10^7–10^9 *C. perfringens* cells. Raw foods or ingredients may be contaminated with spores. Spores are heat resistant and some will survive

Table 12.3 *Clostridium perfringens* classification and their toxin production profile

Type	Disease	Enterotoxin	α-toxin	β-toxin	ε-toxin	ι-toxin
A	Gas gangrene, food poisoning, myonecrosis	+	+	−	−	−
B	Dysentery in lambs; enteritis in calves, goats and foals; enterotoxemia in sheep	−	+	+	+	−
C	Necrotic enteritis in humans and animals (pigbel disease)	+	+	+	−	−
D	Pulpy kidney disease	+	+	−	+	−
E	Enteritis in animals	Variant	+	−	−	+
Mol. WT		35 kDa	43 kDa	35 kDa	33 kDa	48 and 74 kDa
Gene		*cpe*	*plc*	*cpb*	*etx*	*ia*, *ib*
Gene location		Plasmid/ chromosome	Chromosome	Plasmid	Plasmid	Plasmid

Adapted from Brynestad and Granum (2002). Int J Food Microbiol 74, 195–202

cooking. Slow cooling after cooking allows the spores to germinate since heating removes oxygen and creates an anaerobic environment. In addition, room temperature storage after cooking allows the vegetative cells to grow and multiply rapidly to reach an infective dose of $>10^7$ cells. Upon consumption, many vegetative cells die when exposed to stomach acid, but some survive and begin to form spores. After completion of the sporulation step, the mother cell undergoes lysis and releases the toxin and spores in the small intestinal lumen. The toxin causes epithelial cell damage, and pathology described below. *C. perfringens* spores with high heat resistance tend to produce increased levels of enterotoxin.

Clostridium perfringens Enterotoxin

Food Poisoning

Clostridium perfringens produces CPE, which is synthesized during sporulation in the intestine. CPE is a 319 amino acid containing the polypeptide of 35 kDa. The CPE toxin is sensitive to acidic pH but resistant to digestive enzymes, trypsin, and chymotrypsin, which remove about 25 and 37 amino acids, respectively, from the N-terminal end, thus making the toxin highly active. The *cpe* gene encoding the toxin is located either on the chromosome or on plasmids. About 5% of all *C. perfringens* isolates produce CPE. *C. perfringens* type A strains are the primary producer of CPE; however, a majority of type C and D strain also produces CPE. Type E strain carries silent *cpe* or variant *cpe* gene. The CPE production by the type B strain has not been confirmed yet. CPE-positive type A strains are responsible for the great majority of human food poisoning cases.

CPE is a β-pore-forming toxin and has two important domains, (i) the C-terminal domain (amino acids 290–319) which interacts with the cellular receptor protein, claudin on enterocytes, and (ii) the N-terminal domain (amino acids 26–171) which oligomerizes in the membrane to form a pore. Claudin proteins are the member of a large family consisting of 27 proteins (20–27 kDa in size), which are integral to the tight junction (TJ) of epithelial and endothelial cells. Claudin-3, claudin-4, claudin-6, claudin-8, and claudin-14 have a high affinity for CPE and serve as receptors for CPE.

In the small intestine, the cytotoxic effect of CPE starts with binding of the toxin to the claudin proteins forming a complex of ~90 kDa referred to as "CPE small complex." This small complex then oligomerizes to form a hexamer of ~450 kDa pre-pore (CH-1). The CH-1 inserts into the membrane forming a complex with occludin designated CH-2 (~600 kDa), which forms an active pore in the membrane (Fig. 12.5) allowing an influx of calcium ions. The calcium ion influx

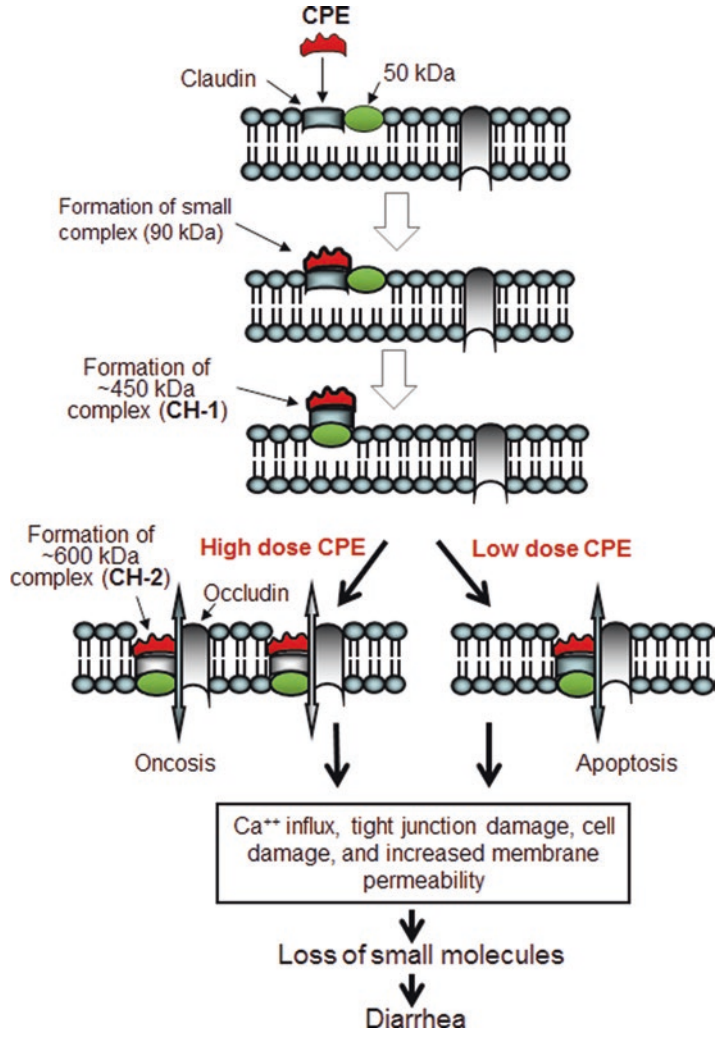

Fig. 12.5 Diagram showing the mechanism of action of *Clostridium perfringens* enterotoxin (CPE)

triggers apoptosis or oncosis, depending on the CPE concentration. CPE at low concentration (1 μg ml^{-1}) induces apoptosis through activation of caspase 3 and caspase 7; thus there is very little intestinal inflammation. The CPE at high concentration (10 μg ml^{-1}) induces necrosis (oncosis), which may contribute to the inflammatory process that is evident during infection. CPE modulates epithelial tight junction architecture, affects the membrane barrier function, and alters the paracellular membrane permeability, resulting in the loss of fluid and ions (Na^+, Cl^-) and the onset of diarrhea. Histopathological changes include desquamation of epithelial cells, damage to the villi tips, and shortening of villi. These changes affect the fluid and electrolyte transport, decreased intestinal absorptive capacity, and increased stool mass.

Antibiotic-Associated Diarrhea

The CPE-positive *C. perfringens* type A strain can cause non-foodborne diseases, such as antibiotic-associated diarrhea (AAD), which occurs as a consequence of the antibiotic treatment, such as penicillin, cephalosporins, trimethoprim, or co-trimoxazole. Antibiotic treatment inhibits resident microflora creating a favorable environment for *C. perfringens* bloom and CPE production in the intestine. The infectious dose is much lower than the food poisoning, and it is suggested that *C. perfringens* strain carrying the plasmid-borne *cpe* is involved in the development of AAD.

Sudden Infant Death Syndrome

The CPE-positive *C. perfringens* type A strains are also attributed to causing sudden infant death syndrome (SIDS). Infants under the age of 1 succumb to unexpected death possibly due to high numbers of type A strain in their intestine and consequent elevated CPE production. CPE can cause local damage in the intestine and can be absorbed into the blood circulation causing enterotoxemia. The toxin possibly exhibits superantigenic function (*see* Chap. 4) triggering the release of massive amounts of IL-2 by T lymphocytes. Toxemia can also affect the liver and kidney.

Enteritis Necroticans

The CPE-positive type C strain contributes to human enteritis necroticans (EN), though the β-toxin produced by these strains is essential for the disease, which is discussed under the section on β-toxin (see below).

α-Toxin

Gas Gangrene

Clostridium perfringens alpha-toxin (CPA), also known as phospholipase C, is produced by *C. perfringens* type A, B, C, D, and E strains. It is a 43 kDa toxin and is responsible for gas gangrene in humans and animals resulting in high fever, pain, edema, and myonecrosis. CPA and perfringolysin O (PFO) are essential for causing gas gangrene. The CPA polypeptide has two domains: the N-terminal zinc-binding domain has phospholipase C (PLC) and sphingomyelinase (SMase) activities, while the C-terminal calcium-binding domain binds to the eukaryotic cell membrane to exert a cytotoxic effect. The substrates for PLC and SMase are phosphatidylcholine (PC) and sphingomyelin (SM), respectively. This toxin has significant homology with PLC from *Listeria monocytogenes* and *Bacillus cereus*. At high concentration, the α-toxin causes massive degradation of PC and SM in membranes leading to membrane disruption, and at low concentration, the damage is less severe.

PFO is a cholesterol-dependent cytolysin (CDC), and it binds to cholesterol on the membrane. The PFO monomers insert into the membrane, oligomerize, and form pore size of about 15 nm and cause cell lysis. The α-toxin induces production of IL-8, TNF-α, platelet activation factors, and the endothelial leukocyte adhesion molecules, which possibly contribute to the increased vascular permeability and swelling.

β-Toxin

Enteritis Necroticans or Pigbel

Clostridium perfringens β-toxin (CPB) is a 35 kDa toxin, which is produced by type C and B strains, and contributes to enteritis necroticans or pigbel in humans. The *cpb* gene is located on large virulence plasmids of ~ 65–110 kb, which also carry other toxin genes. CPB toxin is produced as a 336 amino acid-long protoxin, and cleavage of 27 amino acid leader sequence yields the active 35 kDa toxin. The toxin oligomerizes to form a cation-dependent channel in the host cell membrane. It forms channels of 12 Å in diameter in planar bilayers consisting of phosphatidylcholine and cholesterol. The channel is selective for transport of monovalent cations such as sodium and potassium.

The CPB-producing *C. perfringens* type C strain can cause fulminant disease in both humans and animals (piglet, chicken, calf, lamb, and goats). The necrotizing β-toxin acts on the autonomic nervous system causing arterial constriction leading to mucosal necrosis. The toxin also forms multimeric transmembrane pores and facilitates the release of cellular arachidonic acid and inositol. Additionally, the α-toxin with the phospholipase and sphingomyelinase activity is responsible for necrotizing effects. The enteritis necroticans or pigbel in humans is characterized by vomiting, abdominal pain, bloody stool, and, in severe cases, toxemia leading to rapid death. The disease is endemic in Southeast Asia. In animals, necrotic enteritis is characterized by hemorrhagic diarrhea and enterotoxemia similar to humans.

Epsilon Toxin

Dysentery, Enteritis, and Enterotoxemia

Epsilon toxin (ETX) is produced by *C. perfringens* type B and D strains and is responsible for dysentery, enteritis, and enterotoxemia in lambs, goats, calves, and foals. The *etx* gene encoding the toxin is located on a plasmid. The ETX toxin is produced as an inactive protoxin of 32.9 kDa and is activated by the host protease enzyme, trypsin, and chymotrypsin or *C. perfringens* λ-protease. Proteolytic cleavage removes 10–13 residues from the N-terminal end and 22–29 residues from the C-terminal end and makes the toxin highly active. The toxin affects brain and kidney cells and forms heptameric pores in the cell membrane. Edema in the brain increases the intracerebral pressure and occasionally causes the parenchymal brain necrosis. The toxin is similar to the aerolysin from *Aeromonas hydrophila* and parasporin-2 from *Bacillus thuringiensis*.

Iota Toxin

Enterotoxemia and Enteritis

C. perfringens type E strain produces iota toxin (ITX) and α-toxin, which are responsible for enterotoxemia and enteritis in cattle, sheep, and rabbits. The ITX is a binary toxin, Ia and Ib, similar to a classic A–B-type toxin; however, the genes encoding each toxin on the chromosome are separated by a noncoding sequence in between. ITX is similar to *C. botulinum* C2 toxin (C2I and C2II) and *C. difficile* ADP-ribosyltransferase (CdtA and CdtB). Ia is a 48 kDa enzyme, while the Ib is 74 kDa. Ib binds to the cellular receptor and helps in the translocation of Ia into the cytosol of the cell. Ia has enzymatic activity and catalyzes ADP-ribosylation of globular actin by inhibiting its synthesis resulting in cell rounding and cell death.

NetB Toxin

Avian Necrotic Enteritis

C. perfringens type A also produces necrotic enteritis toxin B (NetB), which is a β-pore-forming 33 kDa toxin that causes cell lysis. The NetB toxin production is controlled by a two-component VirS and VirR regulatory system. NetB is responsible for necrotic enteritis in chickens, primarily in broilers. The disease is of two types: acute and subclinical. Acute form kills broilers at the age of 5–6 weeks, the time of harvest, while the subclinical form lowers nutrient intake and thus decreases body weight. The intestinal lesions are characterized by a massive infiltration of granulocytes. The disease has a significant economic impact due to low productivity.

Genetic Regulation of Virulence

In *C. perfringens*, a majority of virulence genes are located on plasmids; however, the CPE production is both plasmid or chromosomal-linked. About 5% of *C. perfringens* isolates that are responsible for food poisoning carry *cpe* gene, which usually is located on the chromosome. CPE production is linked to sporulation. Inorganic phosphate or the bile activates master sporulation regulator Spo0A, which in turn activates SigF, a global transcription factor. SigF is essential for sporulation and CPA production. The two-component *virR* and *virS* locus also regulates many virulence genes in *C. perfringens* including phospholipase C (α-toxin), perfringolysin O (θ-toxin), and collagenase (κ-toxin) as well as many nontoxic proteins.

Animal and Mammalian Cell Culture Models

CPE action has been studied using rodents (mice, rats, rabbits), intestinal loop, and cell culture (Vero, Caco-2, and MDDK (Madin-Darby canine kidney)) models. Caco-2 is the most appropriate model since it is an enterocyte-like cell, representing cells of intestinal origin. Loss of membrane integrity due to the CPE action has been assayed by the release of ^{86}Rb. Toxic effects of β-toxin have been studied using a human umbilical vein endothelial cell line (HUVEC), in which the toxin affects the membrane permeability resulting in the loss of essential cellular materials.

Symptoms

The CPE-mediated gastroenteritis symptoms in humans are manifested by abdominal cramp and pronounced diarrhea, appearing within 8–12 h and in most cases resolve within 12–24 h. In healthy individuals, the disease is self-limiting; however, in malnourished persons and debilitated, elderly, and very young patients, the organism may colonize, invade, and cause severe ulceration and death. Death may occur due to severe dehydration. Sudden infant death or SID is associated with this pathogen due to high levels of toxins in the blood resulting in shock and sudden death.

Necrotic enteritis or enteritis necroticans is a rare but very serious disease in humans caused by *C. perfringens* type C and type A strains. The symptoms include diarrhea, abdominal cramps, vomiting, fever, and severe bowel necrosis, which can be fatal.

Prevention and Control

In food poisoning cases, treatment and prevention include bed rest and fluid supplement. In these patients, antibiotic therapy is discouraged, since the antibiotic will inhibit resident microflora and promote *Clostridium* bloom and toxin production in the colon. However, antibiotic is recommended for another form of the diseases including enteritis necroticans, gas gangrene, and myonecrosis in humans and animals.

A majority of *C. perfringens* outbreak is associated with meat from beef or poultry. Therefore, to prevent food poisoning, care should be given in food preparation, handling, and storage. Foods should not be allowed to stand at room temperature for an extended period. Adequate cooking, holding at hot temperatures (≥60 °C), or rapid cooling can avoid *C. perfringens*-related food poisoning. Organic acid salts, such as 1% sodium lactate, 1% sodium acetate, or 1% buffered sodium citrate (with or without sodium diacetate), can inhibit the germination and outgrowth of *C. perfringens* during the chilling process.

Detection of *C. perfringens* or Toxins

Culture and PCR Methods

Conventional culturing methods are used to isolate *C. perfringens* from stool or food samples. Samples are first suspended in fluid thioglycolate medium and then heat shocked for 70–75 °C for 15–20 min and then enriched at 37 °C for 18–24 h. The liquid culture is then streaked onto tryptose–sulfite–cycloserine agar containing egg yolk (10%) or brain heart infusion agar with 10% sheep blood and incubated under an anaerobic condition (such as an anaerobic jar) at 37 °C for 18–24 h. Isolates of pure colonies can be obtained from the plate.

Multiplex PCR methods have been developed to detect the presence of toxin genes, enterotoxin (*cpe*), alpha (*cpa*), beta (*cpb*), epsilon (*etx*), and iota (*itx*), in *C. perfringens* isolates. The toxin typing using PCR also allows the typing of isolates based on the presence of specific toxin-encoding genes.

Immunoassays

There are two antibody-based assay methods available commercially, reverse passive latex agglutination test from Oxoid and CPE receptor-based enzyme immunoassay from Tech Lab.

Clostridium difficile

Biology

Clostridium difficile is a Gram-positive, rod-shaped, spore-forming obligate anaerobe. *C. difficile* produces two major toxins called toxin A (TcdA, toxin clostridium difficile A) and toxin B (TcdB) that are encoded by *tcdA* and *tcdB* genes, respectively, located on a 19.6 kb pathogenicity island. *C. difficile* also produces a binary toxin, CDT (*C. difficile* toxin), and the toxin has high sequence similarity to the iota toxin produced by *C. perfringens*. *C. difficile* also produces adhesion and motility factors. The genome size of *C. difficile* is about 4.3 Mb.

Clostridium difficile causes toxicoinfection. It is traditionally considered a nosocomial

(hospital-acquired) opportunistic human pathogen and is responsible for *Clostridium difficile* antibiotic-associated diarrhea (CDAD) and pseudomembranous colitis. *C. difficile* infection (CDI) is also associated with young, elderly, immunocompromised, and organ transplant patients and increasingly found in patients with inflammatory bowel diseases (IBD), ulcerative colitis (UC), and Crohn's disease (CD). Antibiotic therapy for treatment of other ailments is the predisposing risk factor for CDI in addition to immunosuppressed conditions. Broad-spectrum antibiotics, such as fluoroquinolones and cephalosporins, cause greater disruption of the gut microflora and are thus more likely to promote CDI.

The CDI cases have risen dramatically since 2003, and the CDI has caused numerous fatalities in both North America and Europe. In 2007, 3875 deaths due to CDI have been reported in the USA. The CDC reported that in a single year (2011), *C. difficile* infected almost half a million American patients and approximately 29,000 deaths. Of these 15,000 deaths were directly attributed to the *C. difficile* infection.

Source

Clostridium difficile colonizes human and animal intestines that are a major source of this pathogen. About 3% of adult humans and 50% of neonates are asymptomatic carriers of *C. difficile*. *C. difficile* has been isolated from many domestic and wild animals including pigs, calves, poultry, horses, donkeys, dogs, cats, seals, snakes, rabbits, and ostrich. The organism has been also isolated from ground meat (pork, beef, and poultry) at a prevalence rate of 3–12% or higher and less frequently from raw vegetables (cucumber, onion, radish, carrot, mushrooms, and ready-to-eat salads) and milk. *C. difficile* produces spores, which can be distributed widely due to fecal contamination of soil, food processing and packaging environment, and the food. *C. difficile* association with meat animals and routine isolation of this organism from meats provide a strong evidence for its probable involvement as a foodborne pathogen.

Virulence Factors and Pathogenesis

Clostridium difficile spores are transmitted by fecal–oral route or through contaminated foods. In the small intestine, the bile acid (taurocholate) helps germination of spores and the vegetative cells colonize the intestine. *C. difficile* produces TcdA, TcdB, CDT, and adhesion and motility factors.

Flagella help in bacterial motility and also adhesion and colonization. Suppression of flagella synthesis also affects TcdA and TcdB production and bacterial virulence. In addition, fibronectin-binding protein A (FbpA) and cell wall proteins such as Cwp66, S-layer protein A, and Spo0A contribute to the bacterial adhesion and in the biofilm formation.

The TcdA and TcdB are the two major toxins responsible for pathology. TcdA is an enterotoxin, disrupts the intestinal epithelial lining, and allows the passage of TcdB to induce cytotoxicity. TcdB causes glycosylation of RhoGTPase, disrupts tight junctions and cellular architecture, and induces cell death. TcdB also activates a cascade of proinflammatory cytokines and leukotrienes production, such as TNF, IL-6, IL-8, IL-1β, leukotrienes B4, and IFN-γ, resulting in inflammation and enhanced epithelial permeability, epithelial apoptosis, ulceration, and diarrhea.

Some strains, especially the hypervirulent ones, may express the binary toxin, CDT. CDT gene is not encoded in the pathogenicity locus. CDT is composed of two proteins, CDTa and CDTb. CDTa (53 kDa) has enzymatic activity, while the CDTb (98.8 kDa) has the adhesion function and binds to a receptor. CdtB binds to LSR (lipolysis-stimulated lipoprotein receptor), forms pores in the endosome, and facilitates the transfer of CDTa to the cytosol. CDTa is an ADP-ribosyltransferase that ribosylates actin protein in the eukaryotic cells, affecting the cell structure. Actin depolymerization helps the formation of

microtubule-like structures that facilitate further bacterial adherence.

Symptoms

The clinical symptoms and severity of the disease vary among the patients, which may include (1) *C. difficile* diarrhea, (2) *C. difficile* colitis, (3) pseudomembranous colitis, and (4) fulminant colitis.

(1) *C. difficile* diarrhea is mild self-limiting and non-bloody. Symptoms begin to appear shortly after initiation of the antibiotic therapy and cease when the antibiotic therapy is stopped. (2) *C. difficile* colitis presents a severe form of CDI without pseudomembrane formation. The symptoms include nausea, anorexia, severe abdominal cramp, fever, malaise, and high-volume watery diarrhea with traces of blood in the stool. (3) The pseudomembranous colitis presents a severe form of CDI, associated with bloody diarrhea, fever, and abdominal pain. About 50% of the CDI patients show pseudomembranous colitis, due to the disruption of cytoskeletal structure, ulceration, serum proteins leakage, inflammatory cell accumulation, and mucus forming plaques on the mucosa. (4) Fulminant colitis is associated with life-threatening toxic megacolon and intestinal perforation. About 3% of the patients account for the most serious form of CDI, i.e., the fulminant colitis characterized by systemic inflammatory syndromes including diffuse abdominal pain, with or without diarrhea, high fever, chills, hypotension, tachypnea, and marked leukocytosis.

Diagnosis and Detection of *C. difficile*

The endoscopy (colonoscopy) is used for examination of pseudomembrane formation, but it is difficult to distinguish from IBD-induced colitis. Stool culture for isolation of *C. difficile* cells followed by analysis of toxin production is the most reliable method for diagnosis. Enzyme immunoassay specific for toxins A (TcdA) and B (TcdB) and PCR methods are used as diagnosis and detection tools. For food sample testing, culture methods employing a suitable enrichment broth and the solid agar media supplemented with antibiotics, cefoxitin and cycloserine or moxalactam and norfloxacin are used for pathogen isolation.

Treatment and Prevention

Metronidazole (500 mg three times daily orally for 10–14 days) is recommended for treatment of mild-to-moderate cases of CDI. Fidaxomicin at 200 mg twice daily in adults is also approved for CDI treatment. Vancomycin (125 mg, four times a day orally) is recommended for more severe cases. Vancomycin followed by rifaximin (400 mg, twice daily for 10–14 days) is successful in treating recurrent CDI. Oral administration of probiotics such as *Saccharomyces boulardii* and *Lactobacillus* has been found to be effective, but results are inconsistent. Fecal bacteriotherapy to repopulate the patient gut with healthy microbiota through nasogastric or rectal route is highly efficacious for recurrent CDI. The surgical removal of a section of infected intestine is also used to treat severe and recurrent CDI patients.

To prevent CDI, prescribing practices of antimicrobial drugs such as the type of antibiotics, frequency, and duration of therapy should be revised. The patients should be secluded in a separate room, and healthcare professionals should take precautions from the unintentional spread of the pathogens throughout the hospital. Disinfection of hospital facilities using sporicidal agents such as the chlorine-based disinfectants (sodium hypochlorite) and high concentration of hydrogen peroxides should be practiced. Ammonium- and alcohol-based sanitizers are effective against the vegetative cells, but this treatment may facilitate sporulation thus is discouraged.

Summary

Members of the genus *Clostridium* cause a variety of diseases in humans and animals, sometimes with fatal consequences. These organisms are anaerobic spore-forming rod-shaped bacteria and mostly associated with soil and sediments.

Three species, *Clostridium botulinum*, *C. perfringens* and *C. difficile*, have a significant importance because these pathogens are responsible for neuroparalytic botulism (intoxication), food poisoning (toxicoinfection), and antibiotic-associated diarrhea and pseudomembranous colitis (infection), diseases, respectively. *Clostridium botulinum* strains are grouped into proteolytic and non-proteolytic due to their ability to produce proteases. *C. botulinum* produces eight antigenically distinct botulinum toxins (A, B, C, D, E, F, G, and H). In foodborne botulism, the botulinum toxin is produced in the food during anaerobic growth. Botulinum toxin is an A–B-type toxin with a zinc-dependent endopeptidase activity. It cleaves SNARE protein complex, which is responsible for the release of neurotransmitter, acetylcholine, from the synaptic vesicles into the neuromuscular junction for transmission of nerve impulse. Lack of acetylcholine release impedes nerve impulse propagation resulting in the onset of flaccid paralysis. The symptoms appear as early as 2 h after ingestion of toxin, and the severity and progression of the disease depend on the amount of toxins being ingested. Early medical intervention involves administration of antibotulinal antisera. *C. perfringens* causes food poisoning, necrotic enteritis, gas gangrene, myonecrosis, and toxemia. It produces at least 20 different toxins and causes toxicoinfection. There are five types of *C. perfringens* (A, B, C, D, and E), classified based on the production of four types of extracellular toxins: alpha (α), beta (β), epsilon (ε), and iota (ι). *C. perfringens* type A strain is generally associated with the foodborne disease. After consumption of vegetative cells, the bacterium begins to sporulate as it encounters acidic pH of the stomach. The enterotoxin (CPE) is produced during sporulation. The enterotoxin binds to the claudin receptor in the tight junction (TJ) and forms a large protein complex with other membrane proteins to form a pore in the membrane that alters the membrane permeability to cause Ca^{2+} influx and fluid and ion (Na^{+}, Cl^{-}) losses. CPE alters the paracellular membrane permeability and promotes diarrhea. Food poisoning is generally self-limiting requiring bed rest and fluid therapy, but in rare cases, myonecrosis and necrotic enteritis diseases could be life-threatening thus require hospitalization and antibiotic therapy. *Clostridium difficile* is a nosocomial (hospital-acquired) human pathogen and causes *Clostridium difficile* antibiotic-associated diarrhea (CDAD) and pseudomembranous colitis. It produces toxin A (TcdA), toxin B (TcdB), and CDT which cause diarrhea and mucus membrane damage, inflammation leading to diarrhea, and sometimes life-threatening pseudomembranous colitis and megacolon and intestinal perforation. *C. difficile* association with meat animals and routine isolation from meats support its possible involvement as a foodborne pathogen. Prevention of *C. difficile* infection is possible by revising the antibiotic prescription practices such as the type of antibiotics, frequency, and duration of use. Probiotics supplement and fecal bacteriotherapy to repopulate the patient's gut with healthy microbiota, and the surgical removal of infected section of the intestine are used to control recurrent infection.

Further Readings

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