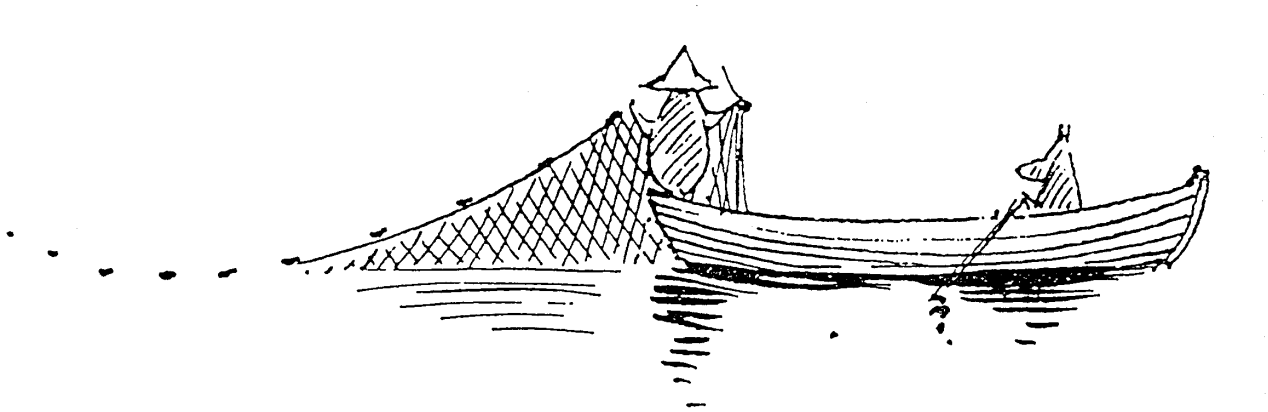
MOLECULAR DETECTION, TYPING AND EPIDEMIOLOGY OF *CLOSTRIDIUM BOTULINUM*

SEBASTIAN HIELM



Department of Food and Environmental Hygienie
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HELSINKI 1999

MOLECULAR DETECTION, TYPING AND EPIDEMIOLOGY OF *CLOSTRIDIUM BOTULINUM*

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ACADEMIC DISSERTATION

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Yliopistopaino

This work is dedicated to the memory of my good friend Timo Nieminen, DVM (1964-1999).

Bless what there is for being.

W. H. Auden

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We are conjured voiceless out of nothing and must return to an unknowing state. What happens in between is an uncontrolled dance, and what we ask for in love is no more than a momentary chance to get the steps right, to move in harmony until the music stops.

Louise Erdrich, in *Tales of burning love*

ABBREVIATIONS

AFLP, amplification fragment length polymorphism
AP-PCR, arbitrarily primed PCR
ATCC, American type culture collection
BoNT, botulinum neurotoxin
bp, base pair
CDC, Centers for Disease Control
cDNA, complementary DNA
CHEF, clamped homogenous electric field
CMM, cooked meat medium
CNS, central nervous system
DIG, digoxigenin
DNA, deoxyribonucleic acid
ELCA, enzyme-linked coagulation assay
ELISA, enzyme-linked immunosorbent assay
EMG, electromyography
ES, EDTA-sodium lauroyl sarcosine buffer
ESP, ES-proteinase K solution
EYA, egg yolk agar
HA, hemagglutinating protein
kb, kilobase[pair]
MLD, mouse lethal dose
MPN, most probable number
MRP, macrorestriction profile
NTNH, non-toxic non-hemagglutinating protein
PCR, polymerase chain reaction
PFGE, pulsed-field gel electrophoresis
PIV, Tris-NaCl buffer
PMSF, phenyl methyl sulfonyl fluoride
RAPD, random amplified polymorphic DNA
RE, restriction enzyme
REA, restriction endonuclease analysis
RFLP, restriction fragment length polymorphism
RNA, ribonucleic acid
rRNA, ribosomal RNA
S_D, Dice coefficient correlation
SIDS, sudden infant death syndrome, crib death
SNAP-25, synaptosomal associated protein-25
SSD, Statens seruminstitut, Denmark
TAE, Tris-Acetate with EDTA
TBE, Tris-Borate with EDTA
TE, Tris-EDTA buffer
TeTx, tetanus toxin
TPGY, trypticase-peptone-glucose-yeast extract
UPGMA, unweighted pair group method with arithmetic averages
VAMP, vesicle associated membrane protein

ABSTRACT

Molecular tools for the detection and typing of *Clostridium botulinum* and other neurotoxigenic clostridial species responsible for human botulism were developed and evaluated. Subsequently, the prevalence, contamination levels and biodiversity of *C. botulinum* type E in Finnish aquasystems and fish was studied using these tools.

Detection and toxin typing of viable neurotoxigenic clostridia was accomplished through polymerase chain reaction (PCR) –based amplification of serotype-specific botulinum neurotoxin (BoNT) gene sequences. For detection, environmental and fish samples were enriched in anaerobic trypticase-peptone-glucose-yeast extract (TPGY) broth for 3 days. Washed and boiled cells from an overnight culture of the enriched sample broth were used as template for the PCR. Depending on the choice of primers, a general BoNT, or a BoNT/A, B, E, or F fragment could be amplified by PCR, providing the corresponding sequence was present on the template. When enriched, the samples were subdivided into a 3-by-3 tube most probable number (MPN) series for quantification of contamination levels. All clostridial strains studied with the PCR assay gave identical results with the mouse bioassay.

The use of pulsed-field gel electrophoresis (PFGE) for typing nonproteolytic *C. botulinum* was pioneered. Using a formalin pretreatment of cells, the degenerative effect of DNases on clostridial DNA could be minimized. The average genome size of *C. botulinum* group II was estimated at 3.89 Mb. The best subtyping results were achieved using either *Sma*I or *Xho*I macrodigestion, preferably both.

Ribotyping was used for the first time as an identification tool for species responsible for human botulism. Inferior to PFGE in its discriminatory abilities, the strength of ribotyping using *Eco*RI and *Hind*III digestion lies in its taxonomical relevance, as it is able to clearly differentiate between strains of *C. botulinum* groups I and II.

The distribution of *C. botulinum* types A, B, E, and F in 110 natural aquatic sediments of the Baltic Sea and Finnish mainland was surveyed. Only *C. botulinum* type E was found, in 81% of the sea and 61% of the freshwater samples. Spore contamination levels were significantly higher in the sea (940 kg^{-1}) than in the freshwater (370 kg^{-1}) samples. The overall prevalence and spore counts correlated significantly with the offshore bottom oxygen content, depth and bioturbation activity, whereas there was no correlation with the bottom water temperature.

The distribution of *C. botulinum* types A, B, E, and F in 333 samples taken from 21 Finnish trout farms was examined. Again, only *C. botulinum* type E was found; in 68% of the farm sediments, in 15% of fish intestines, and in 5% of fish skins. Spore contamination levels were significantly higher in the sediments (2,020 kg^{-1}) than in the fish intestinal (170 kg^{-1}) samples. PFGE typing of 54 isolates generated 28 pulsotypes, suggesting extensive genetic diversity and that the discriminatory power of this method in *C. botulinum* type E is high.

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following original articles referred to in the text by the Roman numerals I to V.

I. Hielm, S., Hyytiä, E., Ridell, J. and Korkeala, H. (1996) Detection of *Clostridium botulinum* in fish and environmental samples using polymerase chain reaction. International Journal of Food Microbiology 31, 357-365.

II. Hielm, S., Björkroth, J., Hyytiä, E. and Korkeala, H. (1998) Genomic analysis of *Clostridium botulinum* group II by pulsed-field gel electrophoresis. Applied and Environmental Microbiology 64, 703-708.

III. Hielm, S., Björkroth, J., Hyytiä, E. and Korkeala, H. (1999) Ribotyping as an identification tool for *Clostridium botulinum* strains causing human botulism. International Journal of Food Microbiology 47, 121-131.

IV. Hielm, S., Hyytiä, E., Andersin, A.-B. and Korkeala, H. (1998) A high prevalence of *Clostridium botulinum* type E in Finnish freshwater and Baltic Sea sediment samples. Journal of Applied Microbiology 84, 133-137.

V. Hielm, S., Björkroth, J., Hyytiä, E. and Korkeala, H. (1998) Prevalence of *Clostridium botulinum* in Finnish trout farms: pulsed-field gel electrophoresis typing reveals extensive genetic diversity among type E isolates. Applied and Environmental Microbiology 64, 4161-4167.

INTRODUCTION

"Botulism is a relatively rare, always tragic, and in some respects still mysterious, neuro-paralytic disease affecting man and animals in various parts of the world, and generally resulting from the ingestion of a foodstuff containing toxic metabolites from one of several known types of anaerobic bacillus, *Clostridium botulinum*, whose natural habitat is the soil of certain regions… I propose at the outset to emphasize the tragic nature of the disease, along with its relative rarity, for these features have always had conflicting effects, positive and negative, in focusing the attention of research workers, health officials, and the general public upon prevention of the disease. Few diseases reveal more dramatically the ancient truth that in the midst of life we are in death. Like influenza, typhoid fever, and poliomyelitis, botulism is apt to select the fit, the strong, and the venturesome, striking quickly and inexorably like a bolt from the blue."

Claude E. Dolman, 1964

These are the opening words of a presentation called "Botulism as a world health problem", given at the first major international botulism symposium in Cincinnati, Ohio, in January 1964. Although held some years before my own birth, virtually every aspect of Dr. Dolman's talk is just as valid today, except perhaps for the reference to the other killers. Deadly influenza, typhoid fever and polio have today been replaced by more imminent health concerns in the more developed societies, but the intriguing menace of botulism persists. This is not to say that cases of botulism have increased dramatically in the last 35 years, but they have not decreased either.

The face of foodborne botulism has also changed, branching out from what was quintessentially a problem associated with inadequate canning or badly preserved meats to a very diverse health issue. Although 'traditional' cases are still frequently seen in connection with the home-canning of vegetables in the continental USA (Hatheway, 1995), the canning or dry-salting of meats in Europe (Lücke and Roberts, 1992), or Inuit food preparation (Wainwright et al., 1988), it is today apparent that a much wider selection of temperature-abused foods can be linked to foodborne botulism. Recent outbreaks have been caused by toxin elaboration in such uncharacteristic vehicles as

aluminum foil-wrapped baked potatoes (Angulo et al., 1998), cheese sauce (Townes et al., 1996), sevu (an Indian flour crisp; Chaudhry et al., 1998), mascarpone cheese (Aureli et al., 1996), peyote (a hallucinogenic cactus tea; Hashimoto et al., 1998), and hazelnut yogurt (O'Mahony et al., 1990). Another 'novel' risk food, especially in northern temperate regions with high natural *C. botulinum* type E contamination, is vacuum-packaged hot-smoked fish. Two dramatic outbreaks in the Great Lakes area in the USA (Dack, 1964) renewed the by then dwindling scientific interest in botulism in the 1960s, whereas two similar outbreaks in Sweden (Anonymous, 1991; Öberg, 1994) caused recent concern in Finland and instigated the present study.

In what is recognized as the worst botulism scenario of all, botulinum neurotoxin would be intentionally used to poison innocent groups of people. Whether used by warring armies in biological warfare or terrorists, the impact of botulinum toxin on densely populated modern cities would be devastating. Highly lethal and easy to produce in large quantities, the toxin is an ideal weapon for armies or other special interest groups which lack the technology to develop more sophisticated weapons. During the Persian Gulf War the Iraqi forces loaded 11,200 liters of botulinum toxin preparation into specifically designed missiles intended for aerosol dispersion over target populations, which fortunately were never fired (UNSCOM, 1995). In another scare, the Aum Shinrikyo terrorist cult 'bungled' and used the nerve gas sarin in their 1995 attack on the Tokyo subway system, instead of using the 15,000 to 100,000 times more toxic botulinum toxin that they had prepared as well (Shapiro et al., 1998).

For the epidemiologist every botulism outbreak is a rare and unforgiving challenge, a public health emergency that requires rapid recognition and smooth cooperation between authorities to prevent additional cases. Just as clinicians have to be prepared to quickly make the correct diagnosis in outbreaks of botulism, the epidemiologist must prepare for such outbreaks by surveying potential botulism hazards in the community. This thesis came about as a response to such a need. On a national level, we had no idea of the prevalence of different types of *C. botulinum* in the Finnish environment and in foods produced from Finnish raw materials. On an international level, we recognized a universal need for better detection and typing methods for *C. botulinum*. The mouse

bioassay, although sensitive enough, felt rather ill-fitting in the age of molecular biology and growing concern over animal experimentation. Also, for the epidemiologist interested in the dissemination and biodiversity of pathogenic agents in the food chain, the lack of a meaningful subtyping scheme has effectively restricted most work with *C. botulinum*. In the past decade, however, molecular typing techniques have brought microbiologists a new array of reliable and accurate inter- and intraspecies identification tools, which today are used to study the epidemiology of many food pathogens (Farber, 1996). These tools will hopefully in the near future also be employed for studies on *C. botulinum* biodiversity and epidemiology.

1. REVIEW OF THE LITERATURE

1.1. Botulism

Botulism is a severe neuroparalytic disease which is caused by the action of botulinum neurotoxins (BoNTs), produced by the anaerobic spore-forming bacterium *Clostridium botulinum* and some of its close relatives (Hatheway, 1992). The BoNTs, sometimes also called botulins in the vernacular, are regarded as the most potent toxins known to mankind (Lamanna, 1959). If left untreated, a severe case of botulism will within days lead to suffocation and death of the patient through paralysis of the respiratory muscles. Although the disease has probably been known to man for millennia, it was not described in the scientific literature until 1820 when the physician and poet Justinius Kerner reported on the data he had collected on 230 cases of sausage poisoning in Germany. Referring to the Latin word for sausage, *botulus*, Müller in 1870 coined the name 'botulism' for the newly described disease (Dolman, 1964). Following the advent of microbiology in the late 19th century, the causative organism was isolated and recognized as an anaerobic 'bacillus' in the now classical report by Emile van Ermengem (1897).

1.1.1. The causative organisms

As new reports of botulism outbreaks were published at the beginning of this century, it soon became evident that the new isolates from these outbreaks were not all similar to the van Ermengem strain (Leuchs, 1910). The clinical manifestations of the intoxications were all alike, but the cultural characteristics and growth requirements of different isolates differed. Through cross-neutralization tests of their respective toxins the different *C. botulinum* isolates were divided into two serotypes, A and B (Burke, 1919). Strains capable of producing botulism in livestock and wild animals were isolated in the 1920s, and these were designated serotypes C and D (Bengtson, 1922; Seddon, 1922; Thieler et al., 1927). Soon thereafter, a serotype E related to botulism outbreaks caused by fish foods was discovered (Gunnison et al., 1935). *C. botulinum* serotype E is most probably the same organism as *Bacillus ichtyismi* (Konstansov, 1914), responsible for the Russian variant of fish-derived botulism, ichtyism, which appeared for the first time in

the Russian literature as early as 1818 (Dolman, 1964). Møller and Scheibel (1960) isolated serotype F from a Danish botulism patient and Giménez and Ciccarelli (1970) isolated serotype G from Argentinean soil, which brought the number of known *C. botulinum* serotypes up to seven. Some strains of *C. botulinum* are capable of producing mixtures of two types of toxin, such as A+F, A+B, or B+F (Hatheway, 1992).

In a view still partly upheld by the latest edition of *Bergey's Manual* (Cato et al., 1986), Prévot (1953) proposed that all species responsible for botulism should be called *C. botulinum*. This decision was taken out of consideration for clinicians working with the disease, in spite of the already by then recognized large differences in the metabolical characteristics of different *C. botulinum* strains. Through early chromosomal DNA-DNA pairing studies (Lee and Riemann, 1970) it soon became apparent that the single-species decision did not hold up to modern nucleic acid-based taxonomical scrutiny, and *C. botulinum* was divided into three [sub]species called groups I to III (Holdeman and Brooks, 1970). Later this division was validated through 16S ribosomal RNA sequence analysis (Hutson et al., 1993; Collins et al., 1994). The non-disease forming serotype G (Giménez and Ciccarelli, 1970), found at the time of the subdivision, was at first termed *C. botulinum* group IV by Smith and Hobbs (1974), but has subsequently been given a species name of its own, *Clostridium argentinense* (Suen et al., 1988).

Clostridium botulinum group I

The type strain for *C. botulinum*, VPI 1550^T A (ATCC 25763), belongs to this group, which is also called proteolytic *C. botulinum*. All strains of serotype A and the proteolytic strains of serotypes B and F belong to this group. They have an optimal growth temperature of 37ºC, they do not grow below 10ºC, and they have spores which exhibit high heat resistance with typical D-values of 1.23 min at 112ºC (Hatheway, 1992). Proteolytic strains are not very sensitive to salt, and will grow at NaCl levels beyond 10% (Genigeorgis and Riemann, 1979). Strains of type A or B are commonly good toxin producers with 10^6 mouse lethal doses (MLD) of botulinum neurotoxin per ml in cultures, whereas type F strains generally produce toxin in the range of 10^3 to 10^4 MLD/ml (Hatheway, 1992). *Clostridium sporogenes* is the non-toxigenic counterpart in this

phylogenetic unit, showing very high (99.7%) rRNA sequence homology with group I *C. botulinum* strains (Hutson et al., 1993).

Clostridium botulinum group II

This group, also known as nonproteolytic or saccharolytic *C. botulinum,* consists of all serotype E strains as well as the non-proteolytic strains of serotypes B and F. They are psychrotrophs with an optimal growth temperature below 30ºC and they can grow at temperatures down to 3.3ºC (Schmidt et al., 1961), but their spores show much less heat resistance than other *C. botulinum* groups, with typical D-values of 0.6-1.25 min at 80ºC (Hatheway, 1992). Nonproteolytic strains are also more sensitive to salt, and will not grow at NaCl levels higher than 5-6% (Genigeorgis and Riemann, 1979). Type E cultures normally contain 10^5 MLD/ml, but the toxin has to be trypsinised to attain full strength, as the organisms in this group lack the endogenous proteolytic enzymes needed for this activation (Hatheway, 1992). Non-toxigenic organisms resembling group II isolates are encountered in the laboratory, possibly as a result of spontaneous BoNT gene loss, but these have not been given any specific species name (Hatheway, 1992; Collins and East, 1998).

Clostridium botulinum group III

All strains of serotypes C and D belong to this mainly non-proteolytic group, which causes botulism in susceptible animals. The C and D toxins are not pathogenic to humans *per os*, as the human gut does not seem to contain receptors for these toxins, pre-empting uptake into the blood stream (Simpson et al., 1996). As animal pathogens the strains in this group have a higher optimal growth temperature, 40ºC, and they do not grow below 15ºC. The spore heat resistance is intermediate, with typical D-values of 0.1-0.9 min at 104ºC (Hatheway, 1992). It has been shown that the toxicity of group III organisms is mediated by bacteriophages (Eklund et al., 1987). With a 98.5% rRNA sequence homology, the atoxigenic but phenotypically similar *Clostridium novyi* is the closest relative of *C. botulinum* group III (Hutson et al., 1993).

Other species capable of producing botulinum neurotoxins

Clostridium argentinense, formerly known as *C. botulinum* group IV (Smith and Hobbs, 1974) produces botulinum neurotoxin type G. Despite being a true BoNT which causes typical botulism symptoms in mice, type G has never been implicated in any botulism outbreaks in humans or animals (Hatheway, 1992). Toxin-producing organisms of this species have been isolated from Swiss autopsy specimens (Sonnabend et al., 1981, Sonnabend et al., 1985), but as the patients had shown no signs indicative of botulism prior to their deaths these findings must be considered coincidental.

Strains of *Clostridium butyricum* and *Clostridium barati* producing BoNT type E and F, respectively, have sporadically been implicated in botulism outbreaks since the 1980s. Being clearly defined species in their own right, the emergence of BoNT-genes in these related clostridia has rightly put into question the current taxonomy of *C. botulinum*, as it is awkwardly based on the single phenotypic trait of BoNT production (Prévot, 1953). DNA sequence studies have indicated that the ability to produce neurotoxins in both *C. butyricum* and *C. barati* is the result of lateral gene transfer between these species and *C. botulinum* (Collins and East, 1998). Interestingly, a majority of the *C. butyricum* and *C. barati* outbreaks have been associated with gut colonization: five cases of infant botulism (Hall et al., 1985; Aureli et al., 1986; McCroskey et al., 1986; Paisley et al., 1995; Franciosa et al., 1998) and one adult case (Hatheway and McCroskey, 1989). During the past year, however, two foodborne outbreaks caused by neurotoxigenic *C. butyricum* have been reported, one in China (Meng et al., 1997) and the other in India (Chaudhry et al., 1998).

1.1.2. Botulinum neurotoxins; structure and mode of action

BoNTs, with a lethal dose of less than 0.1 ng per kg in mice or humans (Gill, 1982), are along with tetanus toxin (TeTx, produced by *Clostridium tetani*) by far the most poisonous substances known (Montecucco and Schiavo, 1993). Assuming a global population of 5.6 billion people weighing on average 70 kg each, then only 39.2 g of BoNT would be sufficient to eradicate humankind (Arnon, 1997). The extreme toxicity of BoNTs is

primarily due to their absolute neurospecificity – they do not react with any substance except their target substrates in the presynaptic motoneurons (Schiavo et al., 1995).

The production of different BoNTs is encoded by their corresponding BoNT genes, which can be located either on the chromosome (apparently serotypes A, B, E and F), on bacteriophages (C and D; Eklund et al., 1987), or on a plasmid (G; Zhou et al., 1995). An integral part of the neurotoxin cluster are the genes for hemagglutinating proteins (HA) and non-toxic non-hemagglutinating protein (NTNH), which together with BoNT make up the virulent complex to be secreted. These non-toxic proteins are covalently bound to the neurotoxin and are thought to enhance toxicity and increase the stability of BoNT in the gastro-intestinal tract (Schantz and Johnson, 1992; East et al., 1994). In pure culture, and presumably in natural conditions as well, BoNT type A is shed during the late exponential (15 h) and stationary phases of *C. botulinum* growth (Call et al., 1995). Following these findings and transmission electron microscopy studies, it was hypothesized that BoNTs are partly excreted together with peptidoglycan layers during cell wall growth – thus it would seem that cell lysis is not a prerequisite for toxin release, as has long been presumed (Bonventre and Kempe, 1960; Montecucco and Schiavo, 1994).

The neurotoxin itself is released as a single polypeptide chain of 150 kDa, which is later nicked to generate two disulfide linked fragments, the heavy chain (H, 100 kDa) and the light chain (L, 50 kDa). The H-chain is responsible for cell binding and penetration at the nerve ending, whereas the L-chain is responsible for the enzymatic intracellular activity. The cell intoxication of the clostridial neurotoxins is a four-stage process involving (1) binding, (2) internalization, (3) membrane translocation, and (4) target modification in the cytosol (Montecucco and Schiavo, 1994). It has recently been established that the light chains are zinc-dependent endopeptidases (Schiavo et al., 1992), whose substrates are one of the three proteins of the docking complex responsible for releasing acetylcholine-containing synaptic vesicles into the synaptic cleft. Of these three substrates, SNAP-25 is cleaved by BoNT types A and E (Binz et al., 1994); VAMP/synaptobrevin is cleaved by BoNT B, D, F, G and tetanus toxin (Schiavo et al., 1992; Schiavo et al., 1994; Yamasaki et al., 1994) and syntaxin is cleaved by BoNT type

C_1 (Blasi et al., 1993). The structure of tetanus toxin is strikingly similar to BoNT, but whereas the BoNTs remain in the presynaptic motoneuron and cause a flaccid paralysis, the TeTx migrates retroaxonally along the motoneurons to the spinal cord's inhibitory interneurons and causes the characteristic spastic paralysis of tetanus (Montecucco and Schiavo, 1993). The result of blocking neuroexocytosis and neurotransmitter release is irreversible and the same for all toxin types, i.e., the nerve cell's end plate ceases to function. In botulism, a slow healing process occurs through non-collateral sprouting of new unmyelinated motoneuron terminals towards uncontracting muscles (Brin, 1997), rendering the denervation atrophy reversible on a macroscopic scale, after all. This re-innervation typically takes around four weeks, under which time the botulism patient must be kept under adequate supportive care, such as mechanical ventilation (Arnon, 1997).

Perhaps somewhat surprisingly, in the past two decades botulinum toxins have found a therapeutic application in the treatment of spastic muscular disorders (Scott, 1981). Minute amounts of purified BoNT type A (lately also types B and F; Lew et al., 1997; Mezaki et al., 1995) have been locally injected to successfully prevent muscle hyperactivity in such diverse conditions as strabismus, blepharospasm, torticollis, oromandibular dystonia, spasmodic dysphonia, and achalasia (Brin, 1995). New uses for this 'wonder drug' are under constant evaluation, including gastrointestinal smooth muscle disorders, skeletal muscle spasms following CNS injury, and even the cosmetic management of wrinkles and debarking of dogs (Blitzer et al., 1997; Jankovic and Brin, 1997). Problems related to antibody formation (limiting the duration of paralysis to 3-4 months) and systemic side-effects following toxin administration are anticipated to be overcome through future research efforts (Arnon, 1997). On a futuristic note, it has also been proposed that the extreme neurospecificity of BoNT heavy chains could be applied to deliver specific molecularly engineered molecules into nerve cells. Through replacement of the toxic light chain with an enzyme of a specifically desired therapeutic activity, target structures in the nerve endings could be reached without iatrogenic complications which might otherwise occur (Hambleton, 1997). The opposite has also been proposed, and Foster (1996) has envisioned taking the substrate specificity of the light chain to cells other than neurons, knocking out pathological vesicle-dependent functions that utilize the same target translocation proteins as the neural cells.

1.1.3. The disease

All four forms of [human] botulism (foodborne, infant, wound, and unclassified) cause illness through a common pathway regardless of the manner in which the toxin gains systemic access (Midura, 1996). The distribution of botulinum neurotoxin in the body is hematogenous, and since the relative bloodflow and density of innervation is largest in the head, this is where the typical signs of botulism are first seen (Arnon, 1997). All forms of botulism initially present with the same clinical symptoms, i.e., an acute onset of weakness in muscles innervated by cranial nerves, resulting in difficulty with speaking and swallowing, and double and blurred vision. This is followed by a progressive symmetrical flaccid paralysis descending from the muscles of the head and throat, which in severe cases causes death through paralysis of the airway and respiratory musculature (Hughes et al., 1981). In a comparison between botulism cases caused by different toxin types, it was found that intubation was required for 67% of type A patients, 52% of type B and 39% of type E (Woodruff et al., 1992). At present botulism patients are commonly intubated as part of the prophylactical management of the disease to maintain airway patency and avoid aspiration pneumonia (Arnon, 1997). Mental functioning is not impaired by BoNTs, so the patient remains alert and conscious throughout the disease (Hatheway, 1995). Recovery from botulism is a slow process which can take from weeks to months, but with proper supportive care patients normally recover without permanent sequelae (Arnon, 1997).

All forms of botulism are rare diseases and they are readily confused with other neural disorders before the right diagnosis is made. Often reported misdiagnoses include Guillain-Barré syndrome, myasthenia gravis, tick paralysis, drug reactions, stroke, or nervous system infections (Hatheway, 1995). Most of these can be ruled out with specific test equipment for each disorder, whereas the diagnosis of botulism can be strengthened by the use of electromyography (EMG). The typical finding in botulism patients is facilitation (potentiation) of the elicited muscle action potential at high frequency (50 Hz) stimulation (Arnon, 1997). Botulism is confirmed by detection of

BoNT in a patient's serum or stool, or in a sample of a food consumed before the onset of illness (Hatheway, 1988).

Foodborne botulism

This form is also known as 'classical' botulism, as it was the first form of the disease described in the literature. In foodborne botulism, the BoNT is secreted into foods by botulinogenic clostridia multiplying in it under suitable conditions for growth. As the toxin is preformed before ingestion, foodborne botulism represents an intoxication rather than an infection, which is the case with the other forms of human botulism.

A case of human foodborne botulism frequently starts with gastrointestinal disorders, i.e., nausea, vomiting, diarrhea (early) and constipation (later), which precede the onset of paralysis. Fever is virtually never seen in uncomplicated cases of foodborne botulism (Hughes et al., 1981). The incubation time is normally 18-36 h after ingestion of the contaminated food, but can be as short as 2 h or as long as 8 days (Arnon, 1997). If the intoxication is severe enough, the paralysis will descend to the respiratory musculature within days and the patient needs to be intubated to prevent suffocation and death. Trivalent (A, B and E) botulism antitoxin, if infused directly upon diagnosis, will enhance survival and shorten the course of illness by neutralizing any unbound BoNT still present in the patient's bloodstream (Tacket et al., 1984). Antibiotic therapy is not part of the treatment of uncomplicated cases of botulism, as subsequent bacterial lysis may cause additional release of BoNT into the intestinal lumen (Arnon, 1997). Since the introduction of intensive supportive care and mechanical ventilation in the 1950s the case/fatality ratio of foodborne botulism has decreased from 60% to its present state of less than 10% in developed countries (Shapiro et al., 1998). The estimated case/fatality ratio world-wide is 20% (Hatheway, 1995).

Infant botulism

Recognized as a distinct clinical entity only two decades ago (Pickett et al., 1976; Midura and Arnon, 1976), infant botulism has since 1979 been the most diagnosed form of

botulinal disease in the United States (Shapiro et al., 1998). From 1976 through 1996, 1444 cases were diagnosed in the United States (95% of all world-wide cases), with almost half of them occurring in the state of California (Paisley et al., 1995; Arnon, 1997; Shapiro et al., 1998). Infant botulism has been reported in all inhabited continents except Africa, and for reasons partly unknown, it is more common in developed than developing countries (Dodds, 1992b). Infant botulism has not been diagnosed in Finland, but of the other Nordic countries Sweden (Jansson et al., 1985) and Denmark (Balslev et al., 1997) have both reported one case, and Norway four cases (Tølløfsrud et al., 1998; Dr. Liv-Marit Rørvik, personal communication, June 1999).

Infant botulism results from the ingestion, germination, outgrowth, and production of BoNT in the infantile gut by ingested spores of botulinogenic clostridia (Arnon, 1986). Originally the condition was only associated with *C. botulinum* types A and B, but the range of causative organisms has subsequently been broadened to include types E and F as well as toxigenic *C. butyricum* and *C. barati*. Honey is a food item epidemiologically linked with the disease, and it is known that honey can contain substantial numbers of *C. botulinum* spores; thus, feeding honey to infants less than one year old has been universally discouraged by health authorities. However, in recent years, less than 5% of the California cases have been fed honey before the onset of illness, which leaves the ingestion of dust as the only plausible source in most cases of infant botulism (Midura, 1996). The infection occurs in infants between 1 week and 12 months of age, with 50% of cases occurring between 1 and 3 months of age (Arnon, 1989). This extreme age distribution is only matched by two other diseases, sudden infant death syndrome (SIDS, crib death) and infant salmonellosis (Arnon et al., 1981). Although the SIDS connection has been substantiated in some 5 to 15% of autopsy specimens (Arnon et al., 1978; Sonnabend et al., 1985), it appears that infant botulism may explain only a small portion of crib deaths (Dodds, 1992b).

The initial neurological symptoms of infant botulism are largely the same as in other forms of botulism, although these are usually missed by parents and doctors because the infant cannot verbalize them. The first indication of illness is constipation (three or more days without defecating), but this sign is also often overlooked by parents (Arnon, 1997).

Infants are usually brought in for clinical inspection only after the paralysis has become more generalized – indicated by such symptoms as poor feeding, lethargy, a weak cry, decreased sucking, and lack of muscle tone (Shapiro et al., 1998). The overall appearance of these patients has given rise to the term 'floppy babies' (Onderdonk and Allen, 1995). The disease presents a very broad clinical spectrum, from asymptomatic carriers through various degrees of paralysis to even peracute death (Midura, 1996). Infant botulism is generally not a fatal illness and the treatment of patients has been restricted to supportive care in hospitals with close attention to nutrition and respiratory function. The case/fatality ratio among hospitalized patients is less than 1% (Arnon, 1997). Antibiotic therapy is contraindicated, as it does not seem to diminish the fecal excretion of *C. botulinum* and might cause the additional release of BoNTs into the gut. The use of equine antitoxin has also been avoided for fear of anaphylactic reactions and the development of hypersensitivity towards horse serum products in the infants (Hatheway, 1995). Instead, a human-derived antitoxin product, botulism immune globulin (BIG), has been developed for use in the treatment of infant botulism patients. In clinical trials the efficacy of this drug has been validated; hospital stays were reduced from an average of 5.5 weeks to only 2.5 weeks, with less time in intensive care units. This results in a reduction of average hospital costs from USD 128,000 to 59,000 per patient (Arnon et al., 1997).

Under special circumstances, an infectious disease similar to infant botulism can occur in adults. This has been documented only a few times, and most cases have a history of abdominal surgery, gastrointestinal abnormalities, and/or recent antibiotic treatment which has presumably upset the normal gut microflora (Shapiro et al., 1998). The United States' Centers for Disease Control (CDC) lists these in a fourth 'undetermined classification' category of botulism, together with all other botulism cases older than 12 months for whom no food or wound source of toxin has been determined (Centers for Disease Control, 1979).

Wound botulism

This rare form of botulism is analogous to tetanus, in that BoNT is disseminated from *C. botulinum* (usually group I organisms) growing *in vivo* in abscessed wounds. Most cases occur in physically active young males, who are presumably at higher risk for traumatic injuries (Arnon, 1997). Recently, wound botulism has emerged as a small-scale epidemic among [San Francisco] Bay Area drug abusers following subcutaneous injection of heroin (Passaro et al., 1998). The clinical signs are the same as in foodborne botulism, except for the absence of gastrointestinal symptoms and a longer median incubation period, 7 days (Shapiro et al., 1998). Specific treatment of wound botulism includes surgical debridement, antibiotics (penicillin) and trivalent botulism antitoxin (Arnon, 1997). The case/fatality ratio is rather high, 15% (Hatheway, 1995).

Animal botulism

Most vertebrate animal species are susceptible to botulinum neurotoxins, and develop botulism with similar clinical features as in humans (Critchley, 1991). A majority of cases are caused by *C. botulinum* group III, although cases with group I and II organisms also occur (Smith and Sugiyama, 1988). In general, animals present with a flaccid paralysis starting in the hind quarters with weakness, muscle tremors, stumbling, and recumbency (Hatheway, 1995).

Botulism in free-range beef cattle, notably in the southern hemisphere, has classically been associated with a phosphorus-deficiency leading to the chewing of carcasses (Smith and Sugiyama, 1988). Since 1987, more than 4.5 million cattle have died from botulism type C in Brazil, and it has been speculated that spore contamination of infrequent water-holes may play an even more important role than osteophagia (Dutra and Seifert, 1998). Cattle bred in more developed farming systems can also contract botulism if fed toxic poultry litter (Appleyard and Mollison, 1985) or silage feeds (forage poisoning; Smith and Sugiyama, 1988) contaminated with BoNTs. Horses are very sensitive to BoNTs, especially type B, and equine botulism occurs sporadically world-wide, both as

feed poisoning and as toxicoinfectious forms (Smith and Sugiyama, 1988). The feeding of low-quality, high pH and moisture content, plastic-wrapped big bale silage has been repeatedly associated with equine and bovine cases (Ricketts et al., 1984). In stressed foals, a disease known as 'shaker foal syndrome' refers to wound botulism in intestinal ulcerative lesions – this condition can be prevented by immunizing mares with botulinum toxoid before foaling (Bernard, 1997). Botulism cases have been reported in most other mammalian livestock species as well, but it seems that the disease is of greater consequence only for minks (which should be immunized against type C) and dogs (Smith and Sugiyama, 1988).

Avian botulism is usually caused by BoNT type C_1, to which most bird species seem susceptible. Duck species migrating through botulinogenic wetland environments at high densities are at greatest risk, and the losses in such epizootics annually reaches thousands to millions of birds throughout the world (Rocke et al., 1998). The neurotoxin is spread through consumption of unaffected fly larvae infesting the carcasses of earlier victims of the outbreak (Haagsma et al., 1971). Chickens are also susceptible to botulism; growth of *C. botulinum* occurs in the ceaca of unaffected birds who shed BoNT in their litter, thereby causing widespread botulism in their coprophagic mates (Roberts et al., 1973; Haagsma, 1974; Smart and Roberts, 1977). On some instances, botulism outbreaks in chickens have resulted from confocal spread of toxin from cannibalisation of rotting carcases left amidst intensively reared broiler flocks (Blandford and Roberts, 1970; Haagsma and Ter Laak, 1977).

Artificially raised salmonids are at risk of contracting botulism during warm summer weather, when environmental conditions favor growth of *C. botulinum* type E in either sediments or feeds. As with wildfowl, the possibility of a large epizootic is favored by high densities and elaboration of neurotoxin in rotting carcasses exposed to the susceptible fish (Eklund et al., 1984). Botulism in wild fish has never been described but may occur sporadically (Smith and Sugiyama, 1988).

1.2. Epidemiology of foodborne botulism

With 3,353 outbreaks and 9,767 cases documented world-wide between 1951 and 1989 (Hauschild, 1992), foodborne botulism is still the most frequently occurring form of the disease. In recent years an average of 449 outbreaks with 930 cases have been recorded annually, marking a clear rise in either actual numbers or the reporting of foodborne botulism. Of the 2,622 outbreaks in which the BoNT type was determined, 34% were type A, 52% type B and 12% type E. Only two foodborne outbreaks were due to BoNT type F during this period (Hauschild, 1992). More than 90% of foodborne botulism is caused by home-prepared or home-preserved foods (Hatheway, 1995).

In order for foodborne botulism to occur, four conditions must be met: (i) the raw materials or the food must be contaminated with spores or vegetative cells of *C. botulinum* from the environment or during processing; (ii) the processing of the food must be mild enough to enable survival of the spores, or unhygienic enough to allow recontamination; (iii) the food environment must support growth of *C. botulinum* during storage; and (iv) the food must be consumed without further heating (Eklund, 1992). Environmental surveys for *C. botulinum* spores have been undertaken since the beginning of the century, and these have provided researchers in the field with substantial knowledge about the world-wide prevalence of various types of *C. botulinum* (for an excellent review, see Dodds, 1992a). Most of these studies have shown that the incidence of botulism is largely dependent on the prevalence and contamination levels of *C. botulinum* spores in the local environment. The probability of germination, outgrowth, and toxin production in food increases with rising spore loads in the raw material (Genigeorgis, 1981). In addition, food cooking and preservation habits also play an important role in the epidemiology of foodborne botulism. As a rule, and certainly a paradoxical one, proteolytic strains are associated with botulism due to vegetable products while nonproteolytic strains are associated with botulism due to proteinaceous foods (Hatheway, 1995).

1.2.1. Foodborne botulism caused by group I or proteolytic strains

Foodborne botulism caused by the growth of proteolytic strains is traditionally associated with home-canned (USA) or fermented (China) vegetables or fruits (Hauschild, 1992). The *C. botulinum* contamination of vegetable foods is dependent on the prevalent spore type of the environment, as well as on the degree of soil contact in the growth and harvesting of the plants (Dodds and Austin, 1998). Vegetable home-canning, which is widespread in the United States, does not always employ sufficient heat to kill the thermoresistant *C. botulinum* group I spores, although group II spores are destroyed at lower temperatures (Hatheway, 1995). In China, with 986 outbreaks documented between 1958 to 1983, botulism is mainly caused by BoNT type A production in fermented beans (86% of all outbreaks; Hauschild, 1992). In addition to the USA and China, Italy and Spain also report a substantial portion of their respective botulism cases as caused by proteolytic *C. botulinum* – these mainly concern type B toxin elaboration in home-preserved vegetables in oil (Hauschild, 1992; D'Argenio et al., 1995). South American botulism outbreaks, although not very frequent, are generally associated with home-canned vegetables and *C. botulinum* type A, which is also the predominant serotype found in soil surveys of the continent (Dodds, 1992a; Hauschild, 1992).

1.2.2. Foodborne botulism caused by group II or nonproteolytic strains

Since they do not possess very heat resistant spores, nonproteolytic strains of *C. botulinum* usually cause foodborne botulism through foods that are produced with either no or only mild heat treatments (Hatheway, 1995). Type B intoxication following ingestion of inadequately salted hams or sausages is the predominant form of foodborne botulism throughout Central and Eastern Europe. Poland leads the botulism incidence statistics with a staggering 72% of outbreaks and 48% of cases reported world-wide between 1984-87, but numerous outbreaks occur in other Eastern European countries as well (Hauschild, 1992). The high botulism frequency in these countries is thought to result from two reasons: meats are not sufficiently heated during home production and they are generally stored without refrigeration (Lücke and Roberts, 1992). In Western

Europe, type B outbreaks result from delayed salt penetration during production of surface-cured hams, which enables outgrowth and toxigenesis of nonproteolytic *C. botulinum* spores occasionally present deep within the meats (Lücke et al., 1982). Being very rare and therefore of little practical consequence, the epidemiology of the nonproteolytic type F presumably follows that of type B. *C. botulinum* type E, on the other hand, is ubiquitous in temperate and arctic aquasystems and is a persistent risk associated with northern ethnic cooking. Traditionally, poorly fermented fish and marine meats have been the most commonly implicated foods, but in recent years a growing number of type E botulism cases linked to industrially produced vacuum-packaged hot-smoked fish have also been recorded.

1.2.3. The aquatic affinity of *Clostridium botulinum* type E

Although also a nonproteolytic group II organism, type E possesses a distinctly different epidemiology than all other types of *C. botulinum.* While spores of other BoNT-producing organisms are intermittently but universally dispersed in soils, type E spores have a strong affinity to the aquatic environment of the sub-arctic and temperate climatic zones (Eklund and Poysky, 1967; Kravchenko and Shishulina, 1967; Bott et al., 1968; Kazdobina et al., 1976; Yamakawa and Nakamura, 1992; Huss, 1980). Thus the foods of primary concern with type E botulism are fish and other aquatic animals caught in contaminated northern waters, such as the Baltic Sea, the Great Lakes area in North America, the Caspian Sea, the northern islands of Japan, Alaska, and arctic Canada (Dodds and Austin, 1998). The spores of type E are only resistant to mild heating, which means that implicated foods in type E outbreaks have seldom been subjected to any heat treatments harsher than pasteurization, if any. In practice, this means that fresh fish, which is served heated, is not a risk food, as heating for 10 min at 80ºC inactivates the toxin (Pierson and Reddy, 1988). The indigenous populations of Greenland, the Canadian north and Alaska have the highest botulism incidence of all people in the world, which is almost entirely (>90%) due to type E foodborne botulism (Wainwright et al., 1988). Foods frequently implicated in northern ethnic cooking are 'fermented' fish and marine meats, in which the pH drop has not been sufficiently swift due to a lack of fermentable carbohydrates, which has lead to putrefaction and *C. botulinum* outgrowth

instead (Dodds and Austin, 1998). Also in other parts of the world, fermented fish (usually uneviscerated) have caused type E botulism outbreaks. These fish dishes include isushi and kirikomi in Japan (Dolman, 1964); kapchunka among Russian immigrants in the United States and Israel (Telzak et al., 1990); moloha and faseikh in Egypt (French et al., 1992; Weber et al., 1993); rakefisk in Norway (Hauge, 1970; Rossebø and Maartmann-Moe, 1971; Ballangrud and Sellgren, 1983), kryddsill in Sweden (Johannsen, 1962; Christensson, 1970), to name a few.

In addition to the traditional ethnic foods listed above, vacuum-packaged hot-smoked salmonid fish have been implicated in several type E botulism outbreaks since the infamous Great Lakes outbreaks at the beginning of the 1960s (Dack, 1964). Especially in the countries around the Baltic Sea, the increased use of vacuum-packaging of hot-smoked whole fish has led to recent outbreaks in Sweden (Anonymous, 1991; Öberg, 1994) and Germany (Baumgart, 1970; Korkeala et al., 1998; Anonymous, 1998). This type of product fills all the criteria of a botulinogenic food mentioned earlier: (1) the raw material, although eviscerated, is heavily contaminated with *C. botulinum* type E spores (Bott et al., 1966; Huss et al., 1974a; Cann et al., 1975; Burns and Williams, 1975; Hyytiä et al., 1998); (2) the heat treatment currently applied in hot-smoking is not sufficient to kill even nonproteolytic spores (Eklund, 1982; Eklund et al., 1988; Peterson et al., 1997); (3) the amount of preservatives in the fish, most notably NaCl, has been lowered to ineffective levels by consumer demand, while the addition of sodium nitrite has been outlawed (Roberts, 1975; European Parliament and the Commission of the European Communities, 1995; Hyytiä et al., 1997); (4) the storage temperatures at the retail and consumer level are as a rule significantly higher than the prescribed 3ºC (Fieandt, 1995); (5) the spoilage flora of fresh fish, which might be antibotulinal (Okereke and Montville, 1991; Montville and Winkowski, 1998), are virtually obliterated during the hot-smoking process; leaving a product with a shelf-life of 4 to 6 weeks with little internal bacterial competition (Eklund, 1992; Lyhs, 1998); and (6) this type of food is traditionally consumed unheated.

1.3. Detection of *Clostridium botulinum* and its serotypes

The basic principles of the detection and isolation of *C. botulinum* from clinical, food and environmental samples have remained essentially unchanged since van Ermengem's (1897) first report more than a century ago. Through incorporation of serotype testing by Leuchs (1910) and Burke (1919), the method has evolved to its present-day form, the mouse bioassay (Kautter et al., 1992). Although universally acknowledged as the most sensitive method available for detecting the biological activity of BoNTs in samples, the mouse bioassay has been criticized as being slow, laborious, expensive, and lacking in specificity. Furthermore, the increasing public resistance to animal testing makes it clear that there is a need to replace the bioassay with a reliable *in vitro* test (Wictome and Shone, 1998).

1.3.1. Isolation and cultural characteristics

Isolation of *C. botulinum* almost invariably starts with anaerobic enrichment of the sample in a non-selective liquid media, e.g. Robertson's cooked meat broth (CMM) or trypticase-peptone-glucose-yeast extract (TPGY; Lilly et al., 1971), for 3-10 days at 26° to 35°C. After a preliminary mouse challenge with the sample/broth, it is streaked onto non-selective anaerobic plating media, usually horse blood agar and egg yolk agar (EYA), where typical signs like slight haemolysis and the lipase reaction are evident after 3 days of incubation at 30°C (Varnam and Evans, 1991). Usually cultures are heat- or ethanol-treated prior to plating to kill off vegetative contaminant cells, which greatly improves subsequent isolation (Kautter et al., 1992). Although selective media for *C. botulinum* have been developed (Mossel and DeWaart, 1968; Dezfulian et al., 1981), their use has remained limited. The efficiency of selection in the media has been questioned (Hobbs et al., 1982), since the antibiotics used seem to inhibit some strains of type E and to alter the appearance of type G (*C. argentinense)* colonies (Varnam and Evans, 1991).

The optimal and minimum growth temperatures for group I strains is 35-40°C and 10°C, for group II strains 18-25°C and 3.3°C, and for group III strains 40°C and 15°C,

respectively (Hatheway, 1992). *C. botulinum* cultures in TPGY broth are turbid with a smooth or flocculent sediment.

The cells of all strains of *C. botulinum* are straight to slightly curved rods with round ends, from 2 to 10 μm in length and 0.5 to 2 μm in width (Smith and Sugiyama, 1988). The spores are oval and subterminal, and in group I strains they usually swell the sporangium (Cato et al., 1986). The cells are motile by peritrichous flagella and usually occur singly, or sometimes as pairs or short chains. Most cultures retain the Gram stain well, becoming Gram-negative only after sporulation sets in (Smith and Sugiyama, 1988).

On blood agar, colonies of *C. botulinum* group I are 3-4 mm in diameter, raised, opaque, often yellowish, with an irregular mucoid margin surrounded by a narrow but complete haemolysis. Group II colonies are smaller, 1-3 mm in diameter, translucent to semiopaque, with a mottled 'mosaic' internal structure, slightly irregular with lobate margins surrounded by small zones of β-haemolysis. Group III colonies resemble the former, except that they are slightly bigger, 2-5 mm in diameter. Group IV (*C. argentinense)* colonies are smaller (0.5-2.5 mm), smooth on the translucent gray surface, with lobate margins that are usually non-hemolytic (Smith and Sugiyama, 1988). Egg yolk agar shows the lipase reaction on fats in the medium, otherwise colonies generally present with similar characteristics as on blood (save haemolysis). All strains of *C. botulinum* are positive for lipase, which shows as a thin mother-of-pearl layer covering and surrounding colonies, most easily seen in oblique light. *C. argentinense (C. botulinum* group IV), *C. barati*, and *C. butyricum* are all lipase-negative, which makes differentiation of these species from contaminating clostridia difficult and isolation improbable when using only standard techniques (Smith and Sugiyama, 1988). On the other hand, non-toxigenic organisms of *C. botulinum* groups I-III are also lipase-positive, rendering toxin detection the only available method for differentiation between non-toxic and toxic organisms within the groups (Varnam and Evans, 1991).

1.3.2. The mouse bioassay

Confirming the identity of *C. botulinum* and other BoNT-producing clostridia is done by toxin detection. In outbreaks of botulism, it is customary to assay suspect foods, patient serum and feces samples, and enrichment cultures for the presence of toxin (Varnam and Evans, 1991). In prevalence surveys, the toxin and serotype is determined from enrichment cultures alone, preferably in connection with a quantification of the *C. botulinum* spore contamination level by the most-probable- number (MPN) technique.

Botulinum neurotoxin detection

Similar methods are applied to food, serum, feces and enrichment cultures. According to Kautter et al. (1992), solid samples are first homogenized in an equal volume of gelatin-phosphate buffer at pH 6.5, then cold-centrifuged to separate the toxic supernatant from the solids. Liquid samples, such as serum or enrichment broth supernatants, can be assayed directly. To fully activate toxins of nonproteolytic organisms, it is advisable to trypsinize part of the sample prior to toxicity testing. Similarly, another part should be left untrypsinized so as not to degrade the toxin of proteolytic types. Separate pairs of 15 to 20-g mice are injected intraperitoneally (i.p.) with 0.5 ml of undiluted fluid, as well as with the same amount of 1:2, 1:10, and 1:100 dilutions in gel phosphate buffer. This is repeated for the trypsinised test samples. As a control for the heat-labile BoNTs, two mice are injected with 0.5 ml of boiled and cooled undiluted test fluid. All the mice are periodically observed for 4 days (at least 48 hrs) for signs of botulinal intoxication, as their death by itself is not sufficient evidence for toxicity. The classical symptoms of botulism in mice are: ruffling of the fur, labored but not rapid breathing ('wasp waist'), weakness of the limbs, gasping for breath (opening of the lower jaw), and death due to respiratory failure. The minimum lethal dose (MLD) of the toxin can be calculated from the highest dilution killing both mice.

Serotype determination

The second stage of the mouse lethality test is to identify the serological toxin type by protecting some mice with specific monovalent (types A through F) antisera, which may be ordered from the Centers for Disease Control (Atlanta, Georgia, USA). Mice can either be protected with the different antisera 1 h prior to toxin injection (Kautter et al.,1992) or otherwise the toxin can be neutralized with the antisera for 1 h prior to its injection into mice (Centers for Disease Control, 1987). Following either approach, the result is the same: mice treated with the antiserum corresponding to the injected toxin type will be unaffected and survive, whereas mice receiving any other type of antiserum will die with typical signs of BoNT intoxication.

1.3.3. Immunological neurotoxin detection methods

Numerous attempts to replace the mouse bioassay with immunology-based methods for the *in vitro* studies of BoNTs have been described in the literature. To aid in isolation, a fluorescent-antibody (FA)-based test for the identification of vegetative cells of different physiological groups of *C. botulinum* was developed (Glasby and Hatheway, 1983), as well as an agar-plate immunodiffusion assay for serotypes A, B, E, and F (Lilly et al., 1984). An assay for rapid (5 min) type A and B antibody detection using fiber-optic biosensors and evanescent wave spectroscopy was developed by Ogert et al. (1992) and Kumar et al. (1994), respectively. Lacking both sensitivity and reliability, none of these methods has come into wider use or commercial application. The only method which has gained any wider acceptance has been the enzyme-linked immunosorbent assay (ELISA); its use in BoNT detection was pioneered in the 1970s by Notermans et al. (1978; 1982) and Kozaki et al. (1979). A single antibody ELISA with a monoclonal capture antibody and a polyclonal antibody-enzyme conjugate to detect BoNT type A was described by Shone et al. (1985). The test was able to detect 5-10 MLD/ml, had no cross-reactivity, and appeared to be unaffected by food constituents. Although still less sensitive than the mouse bioassay, the test paved the way for continued research into the use of ELISA's for BoNT detection.

ELISA

The work with ELISA's continued with equally sensitive assays for BoNT type B (Gibson et al., 1987, 1988) and a streptavidin-biotin complex amplified ELISA for type A (Ekong et al., 1995), but due to the possibility of false negative results and the fact that the assays do not measure biological activity, the tests have not been commercialized. Another improved version for the detection of types A, B and E was developed by Doellgast et al. (1994), using ELISA-ELCA (enzyme-linked coagulation assay) amplification technology. In addition to being as sensitive, it was reported to have several advantages over the mouse bioassay, including earlier detection and no pre-treatment of samples. An ELISA-ELCA application for measuring type E toxin in fish fillets has also been described (Roman et al., 1994). Although expensive, these assays are used in some laboratories for screening samples, but have so far not been authorized for official or clinical use, perhaps due to the system's inability to differentiate between active and inactive neurotoxin (Wictome and Shone, 1998).

Endopeptidase assays

The extreme biological specificity of the clostridial neurotoxins, with each type having its own precise substrate sequence on its peptide target (VAMP, SNAP-25, or syntaxin) in the presynaptic motoneuron, has been utilized in a novel 'second generation' ELISA, the endopeptidase activity assay (Hallis et al., 1996). It involves toxin cleavage of covalently immobilized genetically engineered peptide substrates with its C-terminal attached to a solid-phase, which exposes a toxin type-dependent N terminus that is recognized by specific antibody-enzyme reagents. The enzymatic amplification and visualization of the immunological reaction with horseradish peroxidase results in a quantifiable substrate color change dependent on the biological activity of the measured toxin. Although the system is still in development, it is envisaged that it may in the near future come to replace the mouse bioassay as the BoNT detection method of choice, especially in the food industry. However, as the endopeptidase assays are incapable of measuring such

facets of toxicity as gastrointestinal uptake, cell binding and internalization, there will still be a limited need for *in vivo* tests as well (Wictome and Shone, 1998).

1.3.4. Detection of BoNT genes by PCR amplification

The polymerase chain reaction (PCR) renders possible billion-fold amplification of specifically determined nucleic acid fragments to amounts visible to the naked eye (Saiki et al., 1985; Mullis and Faloona, 1987). The template region to be amplified is specified with an oligonucleotide primer pair, which can be designed by computer so as to be universally specific for any known genome. In microbial detection, PCR has been applied to the partial amplification of group- or species-determining genes for virtually all relevant bacterial, viral, fungal, and protozoan species (for a review, see Persing, 1993). Due to the aforementioned practical and ethical disadvantages of the mouse bioassay, the detection of BoNT genes by PCR was an early application of the new technique. Ferreira et al. (1992) pioneered the use of PCR with *C. botulinum* by detecting BoNT serotypes A, and soon Szabo et al. (1992; 1993) presented a method for detection of types A through E in pure cultures; later the method was expanded to work in food, soil and feces samples as well (Szabo et al., 1994a). This group also successfully applied the PCR method to an array of pathology and soil samples in the investigation of an equine botulism outbreak in Australia (Szabo et al., 1994b). Franciosa et al. (1994) designed a different set of PCR primers for the amplification of type A, B, and E BoNT genes, which gave the correct toxin type on 209 previously bioassayed clostridial strains at the CDC. That study also presented evidence of unexpressed 'silent' type B toxin genes in type A toxigenic strains. Ferreira et al. (1994) added primers for BoNT type F to the American PCR detection scheme, making it suitable for detecting all neurotoxin types experienced in human botulism. Meanwhile, a third group in France presented a PCR method intended for food sample studies that covered types A, B, E, F, and G as well, making it possible to use PCR to type all BoNTs (Fach et al., 1995). Fach et al. (1996) later added a nested PCR procedure for the detection of types C and D in animal outbreaks to the French scheme.

Most of the PCR detection techniques for BoNT genes share the sensitivity and specificity of the mouse bioassay, which make them ideal for the determination of toxin type in pure cultures and in most clinical and environmental enrichment cultures as well. In complex food samples, however, the rate of DNA amplification can be severely reduced by substances (fat, proteins, chemical compounds, etc.) that act as inhibitors to the sensitive PCR reaction (Rossen et al., 1992). This problem can usually, but not always, be overcome by enrichment and other more specifically designed PCR-pretreatments of samples (Lantz et al., 1998), although it clearly limits the use of PCR gene detection directly on foods. Another key issue to bear in mind during PCR investigation of BoNTs is the fact that it is only the gene, not the active toxin, which is amplified. A toxic food with no *C. botulinum* cells present will be PCR-negative, whereas a non-toxic sample with *C. botulinum* spores present will be PCR-positive for the BoNT structural gene. Thus there is still a need for the mouse bioassay or other methods measuring the biological activity of toxins in botulism outbreak studies, whereas the need for such methods has clearly diminished in surveys for *C. botulinum* in clinical, food and environmental samples.

Direct ethidium bromide BoNT gene visualization

In most instances, direct visualization of amplification products followed by documentation through standard photography or digital imaging is the fastest way to obtain results in PCR detection of microbes (Persing, 1993). Intercalating the amplified DNA strand with the fluorescent dye ethidium bromide following electrophoresis makes it visible under UV-light (366 nm) through re-emission of energy in the red-orange region (590 nm) of the spectrum (Sambrook et al., 1989). By comparing the product fragments to molecular size markers in the electrophoretic gel the size of the product can be determined, so as to see whether it matches the size of the expected PCR product or not. If there is no PCR product at all it signifies that there was no template for the specific primers in the sample, hence it is negative for the BoNT gene specified by the selected primer pair. This approach means that every sample has to be assayed with as many type-specific primer pairs as can be expected to be found in it, which makes this type of approach more suitable for laboratory studies where researchers merely want to

ascertain whether a known type of *C. botulinum* is present or not. In less controlled surveys it would be of greater interest to study at first whether any type of *C. botulinum* is present and subsequently proceed to toxin typing – in analogy with the classical mouse bioassay. Starting with a 'general' BoNT primer pair which is able to identify a sequence common to all BoNT genes this can be achieved in two ways, either by double-PCR (Fach et al., 1996) or by differentiating within the amplified PCR product with a type-specific DNA probe (Campbell et al., 1993; Fach et al., 1995). Nested PCR is a method in which a second round of amplification is done with type-specific primers on the products generated in positive samples with the first PCR, thus obviating all unnecessary toxin type determination in *C. botulinum*-negative samples. DNA probing has been used in many PCR-based BoNT detection protocols for signal amplification if there has been a low yield of PCR-product in samples with low template concentrations (Szabo et al., 1992; 1993; Ferreira et al., 1993; 1994; Franciosa et al., 1994), but its main application in this context is to specify the BoNT toxin type in 'general'-BoNT sequences produced in *C. botulinum*-positive samples by PCR.

Toxin type-specific DNA probe hybridization

In another approach, the PCR-generated 'general'-BoNT DNA is transferred from electrophoretic gels onto a nylon membrane. Using Southern hybridization (Sambrook et al., 1989), toxin type-specific oligonucleotide probes are attached to their corresponding DNA sequences within the amplified fragments. The attachment is confirmed by washing the membrane; the remaining probe is then visualized either by using enzymatic digoxigenin-labeling (Fach et al., 1993) or chemilumenescence (Campbell et al., 1993). A bound probe can be stripped by boiling and washing with detergents, and the membrane can be re-probed with a second probe, until the toxin type of all BoNT products on the gel has been determined.

1.4. Genotyping methods

In any type of microbial epidemiology study it is of essential importance to establish the degree of relatedness between isolates. In outbreak studies the concept of clonality

between etiologic agents is of greatest importance, as clonal isolates from different sites is taken as proof of a common source. By 'clonal' one refers to a population in which all members are the non-sexual progeny of a single cell which thus are genetically identical or at least nearly so (Dijkshoorn and Ursing, 1997). Within a taxonomically defined bacterial species there is a large degree of genetic diversity ranging from clonality to virtual unrelatedness. The process of examining a set of characteristics to determine the extent of relatedness of isolates from a single species is called subtyping, often merely referred to as typing. Historically, subtyping has been accomplished by testing for one or more phenotypic markers: biotyping, serotyping, phage and bacteriocin typing, and antibiotic susceptibility testing are examples of such methods. Although many of these methods are still employed in typing today, the inherent disadvantages of testing only for expressed characteristics are such that phenotyping is increasingly being replaced by methods that differentiate between certain macromolecular structures of bacteria instead. Molecular typing can be broadly defined as any method used to discriminate between isolates based on the composition of proteins, lipopolysaccharids and fatty acids, or nucleic acids (Swaminathan and Matar, 1993). Of these, the most prominent type of molecular typing focuses on nucleic acids, and is also known as DNA-fingerprinting, DNA-based typing, genomic fingerprinting, genotyping or DNA-typing, and is the methodology with the most applications in bacterial epidemiology today. As most methods produce one-dimensional graphs out of DNA, one author has suggested that 'DNA-fingerprinting' is the most appropriate and descriptive term (Vaneechoutte, 1997).

DNA-fingerprinting involves direct or indirect analysis of chromosomal or extrachromosomal bacterial DNA, allowing us to reveal whether and to what extent DNA-sequence polymorphism is present in the population. According to Farber (1996) its advantages over other typing methods are numerous: (i) essentially all bacterial isolates are typeable, since they all contain extractable DNA; (ii) the same typing strategy can be applied to most bacterial species, since the structure of DNA is universally identical; (iii) genomic DNA in an organism is a stable characteristic, independent of growth conditions or sample preparation methods; (iv) genotyping results can easily be interfaced with statistical computer programs; (v) most genotypic methods are amenable to automation; and (vi) the discriminatory power, i.e., the ability to distinguish between

two closely related strains, of genotyping is generally much higher than that of phenotyping. Although few DNA-fingerprinting analyses conform with each of the aforementioned advantages, they have replaced classical phenotyping in many laboratories and will most likely continue to do so (Vandamme et al., 1996).

The actual result of most genotypic analyses resembles a ladder, which comes out of electrophoretic separation of differently-sized DNA fragments through an agarose gel. The relative movement of DNA molecules in an electric field is inversely proportional to their size, which results in the large fragments ('bands') migrating only a short distance from the loading well while the small fragments end up further down. Fragmentation of DNA is usually accomplished by the use of restriction enzymes (RE), which are endonucleases of bacterial origin that cut DNA at specific 4-, 6-, or 8-base recognition sites. Variations in the array of fragments generated by a specific RE are called restriction fragment length polymorphisms (RFLPs), and are the result of mutations in the target DNA such as sequence rearrangements, insertions/deletions, and base substitutions within the RE cleavage sites. All REs can detect the first two types of changes, whereas base substitutions are only recognized if they are contained in a RE recognition site (Swaminathan and Matar, 1993). In many PCR-based typing methods the DNA fragments are generated through amplification rather than digestion; this notwithstanding, the gel patterns appear very similar to RFLPs. When evaluating polymorphism, the possible similarities of gel profiles ('fingerprints') of different bacterial strains are compared with each other. Adjoining lanes on the gels are relatively easy to compare, but to establish the degree of inter-relationship between fingerprints of a larger set of isolates a computer-assisted approach using digitized gels and cluster analysis is preferable (Gerner-Smidt et al., 1998) .

1.4.1. Chromosomal restriction endonuclease analysis and ribotyping

The first chromosomal DNA-based genotypic method to be described, restriction endonuclease analysis (REA), exists today mainly as a precursor to ribotyping. In REA, isolated chromosomal DNA is digested with a suitable frequently-cutting restriction enzyme (RE), and electrophoresed to separate the cut fragments by molecular weight.

The size of the fragments generally range from 1,000 to 20,000 bases (1-20 kb), and since bacterial chromosomes are several megabases (Mb) in size, the resulting REA pattern contains several hundred 'bands'. Such complex fingerprints are very difficult to interpret and impossible to analyze by computer, which has lead to the dismissal of REA in favor of other genotyping methods.

Ribotyping (Grimont and Grimont, 1986) increases the interpretability of the restriction endonuclease assay through selective probing and visualization of fragments containing all or parts of the genes coding for ribosomal RNA (rRNA). The ribosomal operons (*rrn* loci) contain three highly conserved genes coding for the 5S, 16S, and 23S rRNA – all 'housekeeping genes' essential for cell division and reproduction. The universal structure of the operon makes it a perfect target for hybridization, as a single probe can be used to subtype all bacteria. To safeguard against DNA damage in these vital genes the *rrn* operon is usually present in multiple (2 to 11) copies in most bacterial cells, presenting in ribotyping as a reasonable number of highlighted fragments enabling good discrimination (Farber, 1996). In practice, ribotyping is done by Southern transfer of the REA gel onto a nylon membrane, which is subsequently hybridized with a 16S+23S complementary DNA probe made directly from rRNA by reverse transcription (Blumberg et al., 1991). Other types of probes are also used for ribotyping, such as plasmid *rrn* DNA or synthetic oligonucleotides as well as commercially available *E. coli* 16S+23S rRNA cDNA (Farber, 1996).

As parts of the ribosomal RNA operon are among the most highly conserved gene sequences known, its sequencing has become the 'gold standard' of modern taxonomy (Pace, 1997; Collins and East, 1998) and phylogeny studies (Vandamme et al., 1996).
Likewise, as the variability of RFLPs are highly dependent on the phylogeny of the isolates tested, ribotyping was initially intended mainly for taxonomical studies. This has not deterred researchers from using ribotyping as a subtyping method as well, and the method would seem to be of greatest use in studies demanding a somewhat lower discriminatory power, e.g., in dividing strains into more or less accepted subspecies. The *rrn* operons represent only about 0.1% of the chromosomal DNA, so for actual strain typing the use of a more discriminatory method would usually be advisable (Farber,

1996). However, as the protocol is applicable to most bacterial isolates, ribotyping's greatest strength lies in its ability to identify isolates for which no background information is available. This feature has sparked the development of the RiboPrinter® (Qualicon Inc., Wilmington, Del., USA), which is the first automated genotyping machine available. By comparing a sample's profile with other ribotyping profiles stored in its computer database, this robot can identify – and to some extent subtype– any bacterial species it has encountered before, from a plated colony in 8 h.

Ribotyping has never been applied to *C. botulinum*, despite the frequent use of this technique in epidemiological studies of related species, such as *Clostridium difficile* (Chachaty et al., 1994; Kristjánsson et al., 1994; Samore et al., 1997) and *Clostridium perfringens* (Forsblom et al., 1995; Schalch et al., 1997).

1.4.2. Pulsed-field gel electrophoresis typing

Pulsed-field gel electrophoresis (PFGE; Schwartz and Cantor, 1984) renders possible the generation of a subtyping fingerprint with a suitable amount of DNA fragments without having to resort to probe hybridization methods (Swaminathan and Matar, 1993). PFGE typing (macrorestriction) essentially follows the same principles as other (microrestriction) RFLP techniques, the major difference being the larger molecular size of the cut DNA fragments. In PFGE typing the entire chromosomal DNA is digested with a restriction enzyme that recognizes rare 6- or 8-base sequences (termed a 'rare-cutter'), which ideally produces 5 to 20 fragments ranging from approximately 10 to 800 kb in length (Maslow et al., 1993). This approach must have been the logical consequence of frustration over the blurry images of REA, but it was not achievable until two major discoveries were made in the 1980s. Firstly, to be able to reproducibly work with visible amounts of DNA fragments that are in the kilobase-megabase range, one needs a DNA isolation procedure that is so delicate that the genome of the cells remains unsheared throughout the process. To accomplish this, an *in situ* DNA isolation method was devised: the cells are embedded in agarose prior to lysis with lysozyme and/or some other lytic agent, after which contaminating cellular proteins are digested with proteinase K, which is subsequently inhibited with phenylmethylsulfonyl fluoride

(PMSF). The preparation of good quality DNA with the regular procedure takes six days (Maslow et al., 1993), but lately three-day (Matushek et al., 1996) and even one-day (Leonard and Carroll, 1997) protocols have been described. The agarose-imbedded and entrapped DNA is finally digested with a rare-cutting RE to produce the desired array of fragments. Secondly, as DNA fragments larger than 25-40 kb are not sieved through agarose in standard electrophoresis an improvement was developed, initially intended for the separation of yeast chromosomes (Schwartz and Cantor, 1984). Pulsed-field gel electrophoresis signifies a periodical changing of orientation in at least two electrical fields that do not coincide, which theoretically enables the separation of molecules up to 12 Mb in size, which is more than enough for bacterial macrodigestion (Farber, 1996). Many factors are known to affect the mobility of fragments during PFGE: temperature, agarose and buffer concentrations, pulse and run times, voltage gradients, and the electrical field angle and shape (Maslow et al., 1993). In regard to the configuration of the electrical field, it is at present generally accepted that angles between 110° and 130° seem to be the appropriate compromise for sufficient band sharpness and economic electrophoresis time (Römling et al., 1994). A 120° angle is featured in the most popular PFGE configuration at present, the clamped homogenous electric field electrophoresis system (CHEF; Chu et al., 1986; Vollrath and Davis, 1987).

Depending on the RE used, the fingerprints generated through PFGE typing consist of a limited set of easily discernible fragments, which makes interpretation and possible digitization of the profiles easy. The variability between strains reflects relatively stable but random changes in sequence throughout the whole genome, giving PFGE typing excellent discriminatory power and reproducibility. A number of studies have shown that isolates which seem clonal by PFGE typing are unlikely to show substantial differences when tested by other typing methods (Tenover et al., 1995), except perhaps AFLP typing (Vos et al., 1995). PFGE has been criticized for the time-consuming *in situ* DNA isolation protocols required, but such is the power of this method that it has been adopted as the official typing strategy in epidemiological investigations of the major bacterial foodborne pathogens in the USA (PulseNet; Swaminathan, 1998).

PFGE analysis has been extensively used as a subtyping tool for the nosocomial pathogen *C. difficile* (Chachaty et al., 1994; Kato et al., 1994; Kristjánsson et al., 1994; Talon et al., 1995; Samore et al., 1997) and also for food-poisoning strains of *C. perfringens* (Ridell et al., 1997; Pyhälä et al., 1998). Only one report on PFGE genome analysis and size determination of *C. botulinum* (group I) strain 62A has been published (Lin and Johnson, 1995). The technique seems to be an ideal method for typing clostridial strains, but widespread use of PFGE has been hindered by the relatively high proportion of isolates that are untypeable because of persistent DNases, which result in shearing of the DNA (Lin and Johnson, 1995; Samore et al., 1996).

Another application of PFGE besides subtyping is the calculation of bacterial genome size by adding the estimated sizes of resolved restriction fragments, cut preferably by a variety of suitable rare-cutting restriction enzymes. It is said to be the most direct and accurate method available (Lin and Johnson, 1995). The genome sizes of many species have been estimated by this procedure, including certain clostridia: *C. botulinum* 62A, 4.04 Mb (Lin and Johnson, 1995) and *C. perfringens*, 3.6 Mb (Cole and Canard, 1997).

1.4.3. PCR-based subtyping

To distinguish them from all other genotyping methods, the PCR-based subtyping methods always contain a phase of DNA amplification. As a rule this means that subtyping can be achieved quickly with a minimal number of target cells, which might be of importance if for example one is studying non-culturable bacteria or historical specimens (Swaminathan and Matar, 1993). Some of these methods might only utilize PCR amplification (RAPD, PCR ribotyping, Rep-PCR), whereas others might employ REs before (amplified fragment length polymorphism l. AFLP typing) or after (PCR-RFLP) the amplification step. Random amplification of polymorphic DNA (RAPD; Williams et al., 1990), also called arbitrarily primed PCR (AP-PCR), is the most commonly used of these methods and will be discussed further. Other PCR-based subtyping methods also exist in food microbiology (for overviews, see Farber, 1996; Vaneechoutte, 1997), although none except AFLP typing (Vos et al., 1995; Janssen et al., 1996) seem to hold any greater promise for genome coverage and reproducibility.

In RAPD, the fingerprint's DNA fragments are generated through non-selective amplification of template DNA from the isolate. In 'normal' PCR the stringency of the reaction inhibits the production of any other sequence than the one limited by the primer pair, whereas in RAPD several products of differing sizes and amounts are amplified. This is achieved by using single 9- or 10-mer primers with no known sequence homology, which at low-stringency conditions (low annealing temperature and high DNA polymerase and $MgCl_2$ content) anneal to several locations on the target DNA. Through a series of approximately 45 thermocycles, sufficient amounts of DNA are amplified to enable electrophoretic separation and visual inspection of the fingerprint. The choice of primer is often quite empirical; what seems to work for one bacterial species does not necessarily work for the other. RAPD is a very fast and inexpensive method, independent of prior sequence information of the target; and with no need for restriction, it is probably the simplest genotyping method available to date (Swaminathan and Matar, 1993; Vaneechoutte, 1997). The use of RAPD is quite widespread in hospital epidemiology and in other settings where there is a need for quick proof regarding presumed clonality of isolates, but in any long-term epidemiological surveys the inherent irreproducibility of the chaotic RAPD reaction is seen as a crucial drawback. RAPD has been used extensively in the typing of *C. difficile* (McMillin and Muldrow, 1992; Killgore and Kato, 1994; Samore et al., 1996; Rafferty et al., 1998), but its suitability for typing of *C. botulinum* is still under evaluation (Hyytiä et al., 1999b).

The AFLP typing concept was originally envisioned as a universal DNA fingerprinting method (Zabeau and Vos, 1993), and has indeed found applications in a wide range of taxonomical studies. It was quickly adapted for the study of plant genetics, but in recent years has also generated a high degree of interest as a method in bacterial taxonomy as well (Vos et al., 1995; Janssen et al., 1996). In AFLP typing the robustness of RFLP analysis is combined with the power of PCR amplification, while taking advantage of the semi-automated high resolution electrophoresis inherent in present-day DNA sequencer units. AFLP typing consists of three stages: (1) digestion of the whole genomic DNA with two REs and ligation of restriction halfsite-specific adaptors to all restricted fragments; (2) selective amplification of these fragments with a PCR primer pair that

have corresponding adaptor- and restriction-site-sequences as their target sites, and (3) electrophoretic separation of the approximately 30 to 50 PCR products (size range: 80-550 bp) on a polyacrylamide gel (Janssen et al., 1996). The relationships between generated AFLP profiles should preferably be analyzed by computerized cluster analysis, which enables an objective classification and documentation of the complicated profiles. To date, AFLP typing results have been consistent with modern bacterial taxonomy and have expressed excellent discriminatory power in both species and strain differentiation (Janssen et al., 1996; Kokotovic et al., 1997). AFLP typing of *C. botulinum* or other clostridia has not to the author's knowledge, been described in the literature.

2. AIMS OF THE STUDY

The present studies, dealing with the molecular detection, typing and epidemiology of *Clostridium botulinum*, were conducted in order to:

1. evaluate PCR detection of botulinum neurotoxin genes in pure culture, fish and environmental samples

2. evaluate the quantification of PCR detection results using the most-probable-number (MPN) method for determination of *Clostridium botulinum* spore contamination levels

3. develop pulsed-field gel electrophoresis as a genome analysis tool for *Clostridium botulinum* group II

4. develop ribotyping as a characterization tool for *Clostridium botulinum*

5. determine the prevalence of *Clostridium botulinum* types A, B, E, and F in Finnish freshwater and Baltic Sea sediment samples

6. study the effect of selected environmental parameters on the prevalence and spore contamination levels of *Clostridium botulinum* type E in natural sediments

7. determine the prevalence of *Clostridium botulinum* types A, B, E, and F in fish and sediments from Finnish trout farms

8. study the genetic biodiversity of *Clostridium botulinum* type E isolates as revealed by pulsed-field gel electrophoresis subtyping

3. MATERIALS AND METHODS

3.1. Bacterial strains (I-III, V)

The strains that were used in the methodology (I-III) and biodiversity (V) studies belong to culture collection of the Department of Food and Environmental Hygiene at Helsinki University or were received as donations or bought from the following institutions: American Type Culture Collection (ATCC), Manassas, Virginia, USA; Bundesinstitut für Fleischforschung, Kulmbach, Germany; Institut Pasteur, Paris, France; National Public Health Institute (KTL), Helsinki, Finland; Statens Seruminstitut (SSD), Copenhagen, Denmark; University of California, Davis, USA. The Davis strains had been collected from various sources by Dr. Seppo Lindroth and were generously supplied to us by the National Veterinary and Food Research Institute in Helsinki, Finland.

In all, 50 strains were used in the studies, belonging to the following bacterial species: 16 strains of *Clostridium botulinum* group I (10 producing toxin type A, 4 B, and 2 F); 21 strains of *C. botulinum* group II (4 B, 14 E, and 3 F); and one strain each of *C. argentinense* (producing toxin type G), *C. histolyticum, C, perfringens, C. putrefaciens, C. septicum,* and *C. sporogenes*. Seven strains were used solely in the evaluation of the PCR detection method (I): two strains each of *C. tetani* and *Bacillus cereus*, and one strain each of *B. coagulans, B. sphericum,* and *B. subtilis*. Additional information on the origin of these strains, if available, is given in tables in the original publications (I-III).

3.2. Sampling (I, IV-V)

Freshwater and Baltic seashore sediment samples of approximately 100 to 300 g each were collected in studies I and IV from random sites, representing various types of aquasystems. Offshore samples from deep sedimentation bottoms in the Baltic Sea were collected by the Finnish Institute of Marine Research in conjunction with recording hydrography and biology data for 42 regular sampling stations (IV). In study V, samples were collected from fish farm sediments, either from the inside of ponds or from the

immediate vicinity of marine net cages. All 235 sediment samples were gathered in late 1994 (I), or in 1995-1996 (IV, V). All samples were placed in individual plastic vessels and transported chilled to the laboratory.

Fifty-nine whole, eviscerated fish (1 to 2 kg) and 181 intestinal samples were obtained from fish farms and placed in plastic bags on ice immediately after the fish were stunned and eviscerated (I, V). The 21 sampling sites, of which one was located in Sweden and the rest in Finland, represented different local trout farming practices: 10 marine net cages, 7 traditional earth ponds, and 3 concrete ponds with a suction device for the removal of sedimented bottom matter. Seventeen farms, including all of the marine net cage type, farmed only rainbow trout (*Oncorhynchus mykiss*), two farmed lake trout (*Salmo trutta lacustris*), two farmed sea trout (*Salmo trutta trutta*), of which one also farmed whitefish (*Coregonus lavaretus*).

3.3. Quantitative MPN analysis of samples (I, IV-V)

The sediment and intestinal samples were homogenized as such prior to enrichment and quantitative analysis. The skin samples consisted of the head, skin, peritoneum, gills, and fins of a single fish, which were homogenized with 100 ml sterile 0.1% peptone water prior to enrichment. From each sample nine subsamples were taken: 3×10 g, 3×1 g and 3×0.1 g, and placed in 90 ml (10 g sample) or 10 ml (1 and 0.1 g sample) of trypticase-peptone-glucose-yeast extract broth (TPGY; Food and Agriculture Organization, 1991) and incubated anaerobically for 3 to 5 days at 26ºC. The results of this most probable number (MPN) series were subsequently calculated into an estimation of the amount of spores per sample.

3.4. PCR detection (I-V)

To avoid PCR inhibition by the sample constituents, 1 ml of sample enrichment broth was transferred to 10 ml of fresh TPGY broth for overnight enrichment. From this broth, 1 ml of cells were removed and washed to make a template for the PCR. For the detection of *C. botulinum* types A, B, E and F through BoNT amplification, we used the

protocols described by Ferreira et al. (1994) (type F) and Franciosa et al. (1994) (all other types). In addition to the toxin type primers, in study I we also used a general BoNT primer for the detection of all *C. botulinum* types (Campbell et al., 1993) and a 16S rRNA universal primer as a PCR positive control (Franciosa et al., 1994). Dynazyme DNA polymerase (Finnzymes, Espoo, Finland) and a 96-well thermal cycler (MJ Research, Watertown, Mass., USA) were used for the PCR. The size of the amplified PCR product was determined in agarose gels by comparison with standard DNA fragments.

3.5. Isolation of *Clostridium botulinum* strains (I, IV-V)

Isolation of strains was done by streaking alcohol-treated PCR-positive cultures on anaerobic egg yolk agar (American Public Health Association, 1992). Plates were grown for 3 days, from which lipase-positive colonies were inoculated into tryptone-peptone-glucose-yeast extract (Difco, Detroit, Mich., USA) broth. All cultures were incubated at 26°C in an anaerobic cabinet with an internal atmosphere of 85% N_2, 10% CO_2, and 5% H_2 (MK III; Don Whitley Scientific Ltd., Shipley, England). The species and serotype of *C. botulinum* isolates were ascertained by botulinum neurotoxin specific PCR. If many isolates were obtained from a single sample, only one was kept for the culture collection.

3.6. Mouse bioassay (I)

The neurotoxicity and toxin type of each strain of *C. botulinum*, as well as all naturally contaminated samples PCR-positive for BoNTs in study I, were assayed according to the Nordic Committee on Food Analysis (1991a; 1991b). The toxin type was determined by specific neutralization with monovalent antisera (Biological Reagents Program, CDC, Atlanta, Ga., USA). The bioassays were approved by the Faculty of Veterinary Medicine's Committee on Animal Experimentation.

3.7. DNA preparation (II-III, V)

In situ DNA extraction (II-III, V) was performed on overnight cultures in the late log-phase, which were chilled on ice and resuspended in PIV buffer containing 3.5-4.0% (v/v) formaldehyde solution (Merck & Co., Darmstadt, Germany) and left on ice for 1 h. Cell suspensions were mixed with an equal amount of 2% (w/v) low melting temperature agarose (InCert agarose; FMC Bioproducts, Rockland, Maine, USA) and cast in GelSyringe dispensers (New England Biolabs; Beverly, Mass., USA). The plugs were lysed for 2 h (II-III, V) or overnight (II) in lysis solution (containing 20 U/ml mutanolysin) with gentle shaking at 37°C and the isolation was completed with a single 1 h (II-III, V) or three overnight (II) ESP washes at 50°C. Phenylmethylsulfonyl fluoride inactivation of proteinase K and restriction endonuclease digestion of the agarose embedded DNA was performed as recommended by the manufacturer.

In study III, conventional DNA isolation was performed according to the method of Marmur (1961), with slight modifications. Cells were resuspended in TE solution containing 8.3 mg lysozyme/ml and 167 IU mutanolysin/ml. The mixture was incubated at 37°C for 15 min (proteolytic strains) or 2 h (non-proteolytic strains). RNA was removed from the DNA solution by adding 160 μg/ml DNase-free RNase (Sigma Biochemicals, St. Louis, Missouri, USA). The purity and yield of DNA were determined spectrophotometrically.

3.8. Restriction enzyme macrodigestions and PFGE (II, V)

In study II, 15 rare-cutting restriction enzymes (*Apa*I, *Asc*I, *Avr*II, *Bss*HII, *Cla*I, *Eag*I, *Mlu*I, *Nae*I, *Not*I, *Nru*I, *Rsr*II, *Sac*II, *Sma*I, *Xba*I, *Xho*I; New England Biolabs) were initially tested for cleavage of *C. botulinum* DNA. In study V, *Apa*I, *Mlu*I, *Sma*I, *Xho*I, and *Xma*I [New England Biolabs] were used. All DNA extractions and digestions were repeated at least twice. The presence of potentially confounding plasmids in the DNA isolates was determined by subjecting undigested samples to PFGE prior to macrodigestion in study V.

All samples in study II were electrophoresed with a Gene Navigator system (Pharmacia, Uppsala, Sweden), and in study V with a CHEF-DRIII system (Bio-Rad Laboratories, Hercules, Calif., USA), through a 1% (w/v) agarose gel (SeaKem Gold; FMC Bioproducts) in 0.5× TBE buffer (Amresco, Solon, Ohio, USA). Switch times were ramped from 1 to 18 s over 20 h at 10ºC and 200 V (Pharmacia) or from 1 to 26 s over 22 h at 14ºC and 6 V/cm (Bio-Rad), unless otherwise stated. Low Range, MidRangeI, Yeast chromosome and/or Lambda Ladder PFG markers (New England Biolabs) were used for fragment size determination. The gels were stained for 30 min in 1 liter of used running buffer containing 0.5 mg of ethidium bromide and were destained in running buffer until appropriate contrast was obtained for standard photography and/or digital imaging with an Alpha Imager 2000 documentation system in study V (Alpha Innotech, San Leandro, Calif., USA).

3.9. Genome size determination (II)

The genomic DNA of 19 *C. botulinum* group II strains was digested with three rare-cutting enzymes (*Mlu*I, *Sma*I, and *Xho*I) and separated under multiple PFGE ramps to move all fragments into the linear range of the gel. Fragment sizes were estimated by measuring their respective running lengths in the gel in relation to the closest molecular weight markers. The genome sizes for each strain were derived by separately adding the sizes of the cut fragments in each macrodigestion.

3.10. Ribotyping (III)

Initially, 13 restriction enzymes (*Bam*HI, *Bgl*I, *Cla*I, *Eco*RI, *Eco*RV, *Hind*III, *Nae*I, *Pac*I, *Sac*I, *Spe*I, *Sph*I, *Ssp*I, and *Xba*I [New England Biolabs]) were tested for their suitability to ribotype *C. botulinum*; at first we tested two strains, VPI 1550 A^T (group I) and 250 E (group II). A 1-mm thick agarose-imbedded slice of DNA, or alternatively, 10 μg of DNA in solution, was cleaved as recommended by the manufacturer. The digested DNA was separated in a 0.8% agarose gel at 25 V for 18 h in 1× TAE buffer (Amresco). Digoxigenin (DIG)-labeled lambda *Hind*III digests (Molecular weight marker II; Boehringer Mannheim) were used as size markers in the gels. Following electrophoresis,

Southern transfer and hybridization were performed as outlined previously (Björkroth and Korkeala, 1996). The cDNA probe was prepared from *Escherichia coli* 16S and 23S rRNA (Boehringer Mannheim) and labeled by incorporating DIG-modified dUTP (Boehringer Mannheim) by reverse transcription (AMV-RT; Promega, Madison, Wisc., USA) as presented by Blumberg et al. (1991). Ribotyping membranes were documented with a Hewlett Packard ScanJet 4c/T tabletop scanner (Boise, Idaho, USA).

3.11. RFLP pattern analysis (III, V)

In study III, the numerical analysis of ribotyping banding patterns (ribopatterns) in the molecular size range between 0.6-26 kb was performed independently using the GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium). The similarity between restriction profiles was expressed as a Dice coefficient correlation, according to the equation $S_D = [2n_{AB} / n_A + n_B] \times 100$, where n_{AB} is the number of matched fragments, and $n_A + n_B$ the total number of fragments in profiles A and B (Applied Maths, 1996). The position tolerance for band matching was set at 0.8% of the total length of the pattern. A numerical index for the discriminatory ability of both the *Eco*RI and *Hind*III ribotyping assay was calculated according to Hunter and Gaston (1988). Arrangement of banding profiles into a dendrogram was accomplished using the unweighted pair group method with arithmetic averages (UPGMA). As a means to assess the faithfulness of representation of the similarity matrix, the cophenetic correlation (Applied Maths, 1996) of each dendrogram was calculated with GelCompar software.

In study V, *Sma*I/*Xma*I macrorestriction profiles (MRPs) in the molecular size range between 50-350 kb were also analyzed using GelCompar software and the Dice coefficient correlation. The position tolerance for band matching was set at 1.4% of the total length of the pattern (300 kb). Arrangement of *Sma*I/*Xma*I MRPs into a dendrogram was accomplished using the unweighted pair group method with arithmetic averages (UPGMA). MRPs generated by cleavage of DNA with *Apa*I, *Mlu*I, and *Xho*I were visually inspected and compared. The genotypes resulting from MRP analyses were clustered at a similarity level of 76%, referring to a possible epidemiological relatedness according to the guidelines set out by Tenover et al. (1995).

3.12. Statistical analysis (IV-V)

In all analyses, logarithmic transformations of the most-probable-number *C. botulinum* spore counts kg^{-1} were used. Percentages were compared by using the χ^2 test, medians by using the Mann-Whitney test, and means by using the *t*-test. In study IV, the values for the linear correlation (*r*) between spore counts and independent variables were generated through simple regression analysis. Statistical analyses were done with Statgraphics Plus software (Manugistics, Rockville, Md, USA).

4. RESULTS

4.1. PCR-based BoNT gene detection (I)

The use of washed and heated bacterial cells as templates for PCR was successful for 26 strains of *C. botulinum* and six other strains of *Clostridium* spp. and five strains of *Bacillus* spp. with all tested primer sets. The 16S rRNA, general BoNT and toxin type-specific (A, B, E and F) primer pairs consistently generated a single, typical product of constant size: 763, 1184/1038, 2278, 1284, 762 and 1138 bp, respectively. Mouse bioassay results corresponded completely to those of the PCR assay. Natural and inoculated fish and sediment samples were only tested with the general BoNT and type E primers. The BoNT/E primer pair was superior to the general BoNT primers when testing natural samples, as many template suspensions that were positive for the type E toxin gene were negative for the general BoNT sequence.

Note: During a visit to the Institute of Food Research in Reading, England, in spring 1996, I was supplied with an alternative sequence for the general BoNT primer 1 by the research group who wrote the original paper on the general BoNT PCR amplification (Campbell et al., 1993). This primer, BoNT 1.1, performs better and produces a 1184 bp product with all serotypes, including A, with primer BoNT 2. The sequence for the new primer BoNT 1.1 is:

5'-CC(C/A) TAT (A/G)TA GG(A/T) C(C/T)T GCT TTA AAT ATA GG(A/T) A(A/T)T-3'

There is also a printer's error in the original paper (I) in the sequence of primer 48F (p. 360, Table 1). The actual sequence by Ferreira et al. (1994) reads as follows:

5'-GCT TCA TTA AAG AAC GGA AGC AGT GCT-3'

4.2. Genomic analysis of *C. botulinum* group II by PFGE (II, V)

4.2.1. DNase inactivation by different *in situ* DNA preparation methods (II)

Using a regular *in situ* DNA isolation procedure only one out of the 21 *C. botulinum* group II strains yielded a visible PFGE pattern. The best results for these strains were achieved using a large amount of late log-phase cells (8-ml cultures) and formaldehyde fixation on ice. For most strains, shortening of the isolation to a 2-h lysis step at 37°C, a 1-h ESP wash at 50°C, and a 1-h TE wash at 50°C, had no effect on the PFGE outcome. With this isolation procedure, lanes sometimes had less smearing due to degraded DNA but the restriction fragments lacked the intensity seen with DNA isolated with the 3-day procedure. The use of an ultrashort method (30 + 30 min.) gave lower DNA yields and resulted in reduced fragment visibility and increased smearing of PFGE lanes. The size and surface of the *in situ* DNA plug had no effect on the results; neither did the use of double strength lysis solution. The remaining DNase inactivation procedures that were tested (high temperatures *pro lysis*, omission of the 37°C lysis step, use of a sucrose-saturated lysis solution) did nothing to reduce DNase damage in affected strains and had a negative effect on the general outcome of the isolation; i.e., they resulted in increased smearing of PFGE lanes. One strain in study II was consistently untypeable with PFGE due to extensive DNase activity (92 E), and the genomic size of another strain (211 E, a.k.a. Vancouver herring l. Dolman VH E) could not be estimated because of marked DNA degradation. Six isolates (10 %) out of the 60 isolated from Finnish trout farms were also untypeable by PFGE due to particularly active DNases (V).

4.2.2. Suitable restriction enzymes for PFGE analyses (II, V)

Of the enzymes tested for DNA cleavage and PFGE typing of *C. botulinum* group II in study II, *Apa*I, *Eag*I, *Mlu*I, *Nae*I, *Nru*I, *Sac*II/*Ksp*I, *Sma*I, and *Xho*I produced convenient numbers of fragments (between 10 and 20). *Bss*HII and *Rsr*II generated only large

fragments (one below 500 kb, the rest above), and were unsuitable for basic PFGE interpretations. *Asc*I and *Not*I were altogether unsuitable, as they generated none or only one very large fragment under regular PFGE conditions. *Avr*II, *Cla*I, and *Xba*I generated too many small-sized (<100 kb) fragments. Reproducibility of banding patterns between different DNA lots was excellent with all enzymes, although *Nae*I gave some problems with incomplete digestions. For strain differentiation, *Apa*I, *Mlu*I, *Nru*I, *Sma*I, and *Xho*I, gave the most revealing patterns, and these can be recommended for subtyping *C. botulinum* group II. *Mlu*I, *Sma*I, and *Xho*I were found to be the most suitable for genome size determination.

In study V, 14 trout farm isolates were undigestable with *Sma*I, presumably due to CG methylation of the restriction site. This resulted in one intact DNA band in the MRP, which was easily distinguished from DNase smearing. The genomes of these 14 isolates were digested with *Xma*I, an isoschizomer of *Sma*I with the same restriction site. The cleavage point of *Xma*I (C▼CCGGG) is different from that of *Sma*I (CCC▼GGG): this makes *Xma*I indifferent to CG methylation while *Xma*I produces restriction fragments identical in appearance to the fragments produced by *Sma*I.

4.2.3. Genome size determination (II)

The number of fragments generated by digestion of 19 *C. botulinum* group II genomes with *Mlu*I, *Sma*I, and *Xho*I ranged from 9 to 22, 13 to 18, and 11 to 18, respectively. With *Mlu*I, the smallest detected fragment was 24 kb and the largest was 1,192 kb; with *Sma*I, they were 6.7 and 1,664 kb; and with *Xho*I, they were 29 and 874 kb. The mean genome sizes obtained with the different restriction enzymes were 3,872 (*Mlu*I), 3,881 (*Sma*I), and 3,918 kb (*Xho*I), resulting in an overall genome size estimate of 3,890 ± 170 kb for *C. botulinum* group II.

4.3. Ribotyping of *C. botulinum* (III)

Of the 13 restriction enzymes tested, *Cla*I, *Eco*RI, *Eco*RV, *Hind*III and *Spe*I digestion resulted in good cleavage of *C. botulinum* DNA with revealing ribotypes. Eventually two cost-efficient enzymes were selected for the ribotyping of all strains in this study; *Hind*III because of good overall banding patterns, and *Eco*RI because it is the default restriction enzyme in the automated ribotyping system (RiboPrinter®). Ribopatterns generated from conventionally isolated DNA and from agarose-imbedded DNA plugs intended for PFGE were equally readable; bands in the latter case were somewhat less defined, but this did not interfere with the reading of the results. All strains were typeable with both restriction enzymes, although for *C. botulinum* group II strains *Eco*RI-generated patterns from 3 kb upwards were muddled and difficult to interpret. The intraspecies variation (Dice similarity index) between strains was moderate with the *Hind*III and *Eco*RI ribotyping assays, whereas differences were striking between the two *C. botulinum* species.

*Eco*RI digestion of 27 *C. botulinum* and 5 other *Clostridium* spp. strains resulted in 25 ribotypes, which clustered according to the presently accepted clostridial taxonomy. The clostridial species diverged at a Dice similarity level of 35 ± 18% (mean ± standard deviation) in the *Eco*RI dendrogram. The discriminatory index for *Eco*RI ribotyping of the strains was 0.982. *Hind*III digestion of the 32 strains resulted in 19 ribotypes, which clustered according to the presently accepted clostridial taxonomy and were consistent with the *Eco*RI ribopatterns. The species diverged at a Dice similarity level of 35 ± 8% in the *Hind*III dendrogram. *C. botulinum* type E strain comparison was easier to perform on *Hind*III ribotypes than on the muddled *Eco*RI profiles. In order to better assess the subtyping abilities of ribotyping on nonproteolytic *C. botulinum* strains, 41 type E isolates from Finnish trout farms (study V) were included in the analysis. This expansion of the study material raised the total number of *Hind*III ribotypes from 19 to 33, at the same time raising the discriminatory index for *Hind*III ribotyping of the strains from 0.954 to 0.964.

4.4. Prevalence of *C. botulinum* in natural aquatic sediments (II)

Of the 110 sediment samples gathered from the Baltic Sea and Finnish mainland, 78 (71%) were PCR-positive for the BoNT/E neurotoxin gene, indicating a presence of *C. botulinum* type E. *C. botulinum* type A, B and F organisms were not found.

TABLE 1. Distribution of *Clostridium botulinum* type E in natural aquatic sediments.

Sample type	No. of samples examined	% of positive samples*	Mean MPN spores kg^{-1} in positive samples*
Freshwater	56	61_a	372_a
Seashore samples	12	58_a	500_{ab}
Offshore samples	42	88_b	1020_b
Total	110	71	690

* Percentages and means with different subscripts within a column differ significantly ($P < 0.05$), as determined by the χ^2 test and the *t*-test, respectively.

Of the three types of aquasystems studied, the offshore sediments showed significantly higher prevalence (88%) and spore counts. This was especially true for the Baltic proper samples (n=22), which were 100% positive for *C. botulinum* type E and had the highest level of spore contamination as well, on average 1652 kg^{-1}.

In regard to the bottom hydrography and biology data recorded at the offshore sampling stations and *C. botulinum* contamination levels, some interesting results were seen. There was a significant correlation between spore counts and oxygen content (r = -0.596) and depth (r = 0.363); if analyzed together by multiple regression analysis, the model could be shown to explain (R^2) 42% of the variance of the observed data. Bottom temperature had no effect on the spore counts in any sediment sample set. Actively bioturbated offshore bottoms had a significantly lower *C. botulinum* type E prevalence (78% *vs.* 100%) and a lower mean spore count (240 *vs.* 1,710 kg^{-1}) than non-bioturbated bottoms.

4.5. Prevalence of *C. botulinum* in Finnish trout farms (V)

C. botulinum type E, but no other toxin types, was found in 20 (95%) of the 21 trout farms in study V. The detected prevalences and spore counts for sediment, intestinal and skin samples were 68% and 2,020 kg^{-1}, 15% and 166 kg^{-1}, and 5% and 310 kg^{-1}, respectively. The levels of *C. botulinum* type E contamination of sediment and fish intestinal samples were considerably lower in the self-cleaning freshwater ponds than in the other two farm types (Table 2).

TABLE 2. Sediment and fish sample prevalence and spore counts of *Clostridium botulinum* type E in different types of Finnish trout farms.

Farm type	Sediment samples			Fish intestinal samples		
	No. of samples	% positive samples*	Log spores kg^{-1}†	No. of samples	% positive samples	Log spores kg^{-1}
Freshwater pond	42	60_b	2.11_b	54	19_a	1.32_b
Self-cleaning freshwater pond	14	14_a	1.22_a	17	6_a	1.20_a
Marine net cage	69	84_b	2.40_c	94	14_a	1.29_b
Total	125	68	2.17	165	15	1.29

* Values followed by different subscripts within a column are significantly different, as determined by the χ^2 test ($P < 0.05$).

† Mean logarithmic spore count for all samples in a group. Values below the most-probable-number detection limit (30 spores kg^{-1}) were assigned a value of 15 (log = 1.18) spores kg^{-1} for statistical calculations. Values followed by different subscripts are significantly different, as determined by the *t*-test ($P < 0.05$).

4.6. Diversity and cluster analysis of *C. botulinum* type E PFGE subtypes (V)

The *Sma*I/*Xma*I digests of the 54 isolates generated 24 different MRPs, forming 15 clusters at a similarity level of 76%. In these MRPs, this level of similarity corresponds roughly to a three-band difference. Analysis of MRPs generated with digestion by *Apa*I, *Mlu*I, and *Xho*I

was used for distinguishing between isolates that seemed clonal in *Sma*I/*Xma*I digestion. The discriminative value of *Apa*I, *Mlu*I, and *Xho*I was slightly higher than that of *Sma*I/*Xma*I (producing 25, 27, and 28 MRPs, respectively), but since the profiles were not as easy to interpret as the *Sma*I/*Xma*I MRPs, they were not used for the numerical analysis. Indistinguishable pulsotypes, indicating clonality, were obtained for a wide variety of sample pairs, such as sediments and fish, freshwater and marine, different fish and mammal species, and European and American samples. Isolates from a single farm, however, generally represented different pulsotypes, implying extensive local genetic diversity in *C. botulinum* type E. No commonly shared markers could be detected in *C. botulinum* type E MRPs of strains that had been isolated in association with cases of botulism.

5. DISCUSSION

5.1. Use of PCR-based gene detection for identification of *C. botulinum* (I-V)

The simplified PCR protocol for detection of *C. botulinum* BoNT genes that was developed for the analysis of fish and environmental samples (study I) was used in all subsequent studies. In all studies the PCR assay was used to ascertain the species and serotype of pure *C. botulinum* cultures, whereas in study I, IV and V it was also used to evaluate the *C. botulinum* prevalence and spore counts of fish and sediment samples. Although Franciosa et al. (1994) thought that the PCR approach was as labor-intensive and merely more ethical than the mouse bioassay, it is my experience that PCR is better suited than the bioassay for large-scale laboratory studies. PCR gene detection is certainly cheaper to perform and more ethical than animal testing, although the bioassay or some equivalent to it will always be needed for toxicity testing.

When determining the identity of pure cultures of *C. botulinum*, the serotype-specific primers and general BoNT primers worked equally well, giving results consistent with the mouse bioassay. However, in the study of mixed cultures, such as fish or sediment enrichment broths, the serotype-specific primers were more sensitive and/or not as easily inhibited as the general BoNT primers – hence the later prevalence studies (IV and V) were conducted using only the serotype-specific PCR assays. The 16S rRNA universal primers always gave a positive result in PCR of bacterial samples, even when applied to heavily contaminated and presumably PCR-inhibitive template suspensions; hence the routine use of this assay as a positive PCR sample control was discontinued after study I. Instead, one *C. botulinum* and one pure water template was included in every PCR sample plate as positive and negative controls for the PCR reagents. This notwithstanding, negative PCR-results could sometimes be seen when examining samples containing inhibitory substances, as has been reported by several other food microbiologists as well (Rossen et al., 1992; Lantz et al., 1998). However, the possibilities of a false negative result for an entire sample can be effectively minimized by using the MPN approach for

the quantification of results. Inhibition of PCR is seemingly arbitrary, and the possibility of all tubes in a three-dilution, nine-tube enrichment series being PCR-inhibited and falsely negative is negligible. Even so, a simple sample preparation method eliminating PCR-inhibitory components and suitable for a wide variety of foods remains high on the wish list of many laboratories. Such a procedure might also render possible quantitative PCR-detection of bacteria straight from food samples, still a utopian desire for workers with most food pathogens.

5.2. Applicability of PFGE analysis for nonproteolytic *C. botulinum* (II, V)

Using regular *in situ* DNA isolation procedures, *C. botulinum* group II strains cannot be characterized by PFGE. The reason for this can be the low yields associated with DNA isolation in this subspecies, further hampered by DNA degradation problems caused by extracellular nucleases. Our studies show that formaldehyde fixation of cells prior to lysis can to a large extent prevent DNase-related problems. With some strains, the results can be further improved by shortening of isolation steps at critical temperatures. On the other hand, some formaldehyde treated cultures gave clearer PFGE patterns with a 3-day DNA isolation procedure. We have found that optimal results can be obtained by performing both isolation procedures on the same agarose plug: after two hours of lysis we transfer half of the syringe plug into ESP for one hour and TE-buffer for one hour, while the other half goes through the 3-day protocol. This method has the benefit of rapid results, while in the end it gives the researcher the option to choose the plug with the better isolation result for further genotyping studies. Of the 69 *C. botulinum* group II strains studied 7 (10%) could not be characterized by PFGE due to extensive DNase damage. This unwelcome trait could not be anticipated by colony or broth appearance, and the strains had typical, positive PCR results for the *C. botulinum* type E toxin sequence. The successful use of ultra-short lysing steps (30 min) and ESP-washes (30 min) suggests that slow penetration of chemicals into the agarose plugs is not the reason for the DNase problem. The indistinguishable outcome with both agarose plug sizes, either using 1-mm slices or whole syringe plugs, also warrants this conclusion. However, a surprisingly large number of nonproteolytic *C. botulinum* cells, at least 8 ml TPGY broth at late-log growth, is needed to

obtain sufficient amounts of DNA for a 1-ml gel plug for PFGE analysis. This cell mass is about five to ten times greater than the amount of other gram-positive bacteria used for PFGE in our laboratory. As similarly low DNA yields are not as pronounced with other clostridia, this seems to be a special feature of *C. botulinum* group II strains, possibly a result of the resistance of cell wall structures against lysis.

With PFGE analysis, the average genome size of 19 *C. botulinum* group II strains was determined to be 3.89 Mb (range: 3.59 to 4.15 Mb). This result corresponds well to the earlier study of the genome size of *C. botulinum* group I strain 62 A, which was estimated at 4.04 Mb using a similar approach (Lin and Johnson, 1995).We estimated the fragment sizes manually, and we obtained more accurate results by comparing the fragments in relation to the two closest marker fragments, as opposed to a calculation of fragment sizes through measurement of the migration distance from the well, plotted on a standard curve (Tenover et al., 1995). Fragment size estimates generated through standard curves, even those based on a cubic spline formula, can easily be off by at least 10% at the top and bottom of the gel, where fragment migration is non-linear.

Cluster analysis of the MRPs of the *C. botulinum* type E isolates from Finnish trout farms confirmed that there is indeed great diversity between strains of this serotype, as we had hypothesized in study II. Hyytiä et al. (1999a) came to the same conclusion when studying *C. botulinum* type E isolates from fish and fishery products in Finland and Germany. As opposed to our reference strains, the isolates in the fish farm study (V) were of a fairly restricted origin, both spatially and temporally. Even so, 18 different *Sma*I/*Xma*I MRPs were generated, and by the use of the additional restriction enzymes *Apa*I, *Mlu*I, and *Xho*I, some seemingly clonal isolates could be further differentiated. No such differentiation could be seen with the clonal isolates in our first study, which led us to propose *Sma*I as the principal macrorestriction enzyme for *C. botulinum* group II. However, as a result of additional digestions, especially with *Xho*I, a total of 28 different pulsotypes could be detected in the 54 typeable *C. botulinum* type E isolates. Whether this means that *Xho*I is epidemiologically more relevant than *Sma*I in PFGE typing of *C. botulinum* type E isolates remains, however, to be validated by future typing studies.

With regard to the clonal pulsotypes, it is interesting to notice that these by no means were restricted to samples from the same farm, animal species, or even country. The MRPs of the 12 North American type E reference strains characterized earlier (study II) fell in right in the midst of the dendrogram of the isolates of the trout farm survey. Ten (83%) of these 12 strains have been isolated from actual botulism outbreaks, yet their MRPs expressed no shared markers which might indicate the existence of a genetic virulence mechanism in these strains as compared to the survey isolates. The proportion of clonal isolates between the sediment and fish sample groups was larger than the proportion of clonal isolates when multiple samples from one farm were examined. These findings would imply that: (i) *C. botulinum* type E is an organism adapted to northern aquatic environments, from which it occasionally contaminates animal or human food chains at random, and (ii) there is or has been extensive spread and exchange of *C. botulinum* type E strains in the northern temperate regions, the vehicle of which has not been determined.

The successful use of MRPs for typing *C. botulinum* group II isolates confirmed that this technique is a very powerful method for this species, with broad clinical, industrial, and perhaps even legal implications. The ability of PFGE to distinguish a majority of 42 type E isolates obtained from a very limited ecological niche, 21 Finnish trout farms, implies that in future botulism outbreaks investigators may be able to link patient isolates to incriminated foods with a certainty not possible a few years ago (Townes et al., 1996). For the fish and seafood industry, the typing of *C. botulinum* type E strains isolated from raw material, processing routes, and/or products is a new tool in the identification of control points and implementation of hazard analysis critical control point systems in manufacturing processes.

5.3. Ribotyping as a characterization tool for *C. botulinum* (III)

The aim of this study was to determine the usefulness of ribotyping in characterizing the two groups/species of *C. botulinum* responsible for human botulism. Ribotyping of the clostridial strains in this study validated the taxonomical changes proposed for *C. botulinum* when it was split into [sub]species in the 1970s (Holdeman and Brooks, 1970).

The relational clustering of species within 16S rRNA gene sequence clusters I and II *Clostridium* (Collins et al., 1994) was reproduced by *Hind*III ribotyping of the clostridial strains in this study. *C. sporogenes* clustered with the *C. botulinum* group I ribotypes, which can be seen as further proof that this species is indeed the non-toxic counterpart of a single phylogenetic unit, proteolytic *C. botulinum*.

As expected, typeability *per se* was not a problem in ribotyping – three strains untypeable by PFGE were perfectly digested with the frequently cutting *Eco*RI and *Hind*III, despite obvious degradation of the agarose-imbedded DNA. With *C. septicum* ATCC 12464, however, typeability problems arose with the selected restriction enzymes, as *Eco*RI generated only one fragment highlighted by the rRNA operon in this strain. The number of operon copies cannot be the cause of this particular problem, as this strain displayed good ribopatterns with *Hind*III. Similar typeability problems were encountered with the *Hind*III ribotyping of the *C. botulinum* group I strains, in which only 3 to 7 fragments were generated, leading to a low discrimination index of only 0.694.

The comparative numerical analysis of the ribopatterns mostly accentuated differences between the clostridial species that were equally detectable by visual inspection of the ribotyping membranes. However, the computational approach adds tremendous power to within-species cluster analyses and enables the creation of a database for the identification of newly typed clostridial isolates.

The discrimination indexes were surprisingly high for the ribotyping assays, and the 0.90 criteria for a confident typing method (Hunter and Gaston, 1988) was met for all assays except *Hind*III ribotyping of *C. botulinum* group I. However, the difficulties in interpreting the large bands of *Eco*RI ribopatterns of *C. botulinum* group II definitely adds some uncertainty to the correct analysis of bands in this assay. For species identification, and for the typing of other clostridial isolates except *C. botulinum* group II, however, *Eco*RI worked well, which would seem to justify the use of this enzyme as the default in the RiboPrinter® microbial characterization system.

The successful use of agarose-imbedded DNA for ribotyping may increase interest in applications of ribotyping for *C. botulinum*, as researchers working with PFGE need not go into the cumbersome mini-prep isolation of clostridial DNA. This way ribotyping could easily be used to add a taxonomic dimension to the mostly random discriminating powers of PFGE typing. On the other hand, for microbiologists not familiar with the perplexities of PFGE typing, manual or automated ribotyping provides an alternative epidemiological tool. When using either *Eco*RI or *Hind*III ribotyping, the results show excellent group identification in *C. botulinum*.

5.4. Prevalence of *C. botulinum* type E in the Baltic catchment area (IV-V)

The high prevalence of *C. botulinum* type E in the sediments and Finnish farmed fish studied corresponds well with earlier surveys conducted in the Southern Baltic (Johannsen 1962; 1963; Cann et al., 1965; Bach et al., 1971; Zaleski et al., 1973; 1978; Ala-Huikku et al., 1977; Huss, 1980). Study IV shows that *C. botulinum* is distributed over most of the Baltic catchment area, indicating that it indeed is one of the most heavily contaminated regions known with regard to *C. botulinum* type E. Study V showed that the prevalence and contamination rates in farmed trout, although lower than in the sediments, are high enough to pose a relevant botulism risk for the consumer. Somewhat alarmingly, studies by our group have shown that the 15% prevalence of *C. botulinum* type E in fresh farmed fish is matched by almost equally high numbers in fishery products at the retail level. Out of 214 vacuum-packaged hot-smoked salmonids, ten (5%) were found to contain *C. botulinum* type E spores, the prevalence being exceptionally high (10%) in whole-smoked whitefish (Hyytiä et al., 1998). In studies IV and V, both spore counts and prevalence numbers were higher in the marine samples than in the freshwater samples, an observation shared by most *C. botulinum* type E surveys of the Baltic catchment area so far (Johannsen 1962; Zaleski et al., 1973; Huss, 1980). *C. botulinum* prevalence studies on fish farms and farmed fish have also been conducted in several other countries, e.g. Denmark (Huss et al., 1974a), Norway (Tjaberg and Håstein, 1975), Great Britain (Cann et al., 1975), Scotland (Burns and Williams, 1975), and in the Aral Sea in Russia (Kazdobina et al., 1976). In the light of these and

our own studies it would seem that in areas with low surrounding *C. botulinum* contamination levels of *C. botulinum* increase as fish farming increases, whereas this effect is much less marked in environments which already have high levels of natural *C. botulinum* type E contamination.

The observation in the offshore samples in study IV that low oxygen content, depth and absence of bioturbation positively correlated with *C. botulinum* type E spore counts point to anoxic bottom multiplication or, at least, suitable conditions for spore survival in these conditions. Whatever the origin of *C. botulinum* type E in the Baltic Sea, terrestrial or aquatic, it is an indisputable fact that the spores thrive in the brackish and shallow waters of this inland sea. Little is known about the factors influencing anaerobic spore-formers in natural environments but it is possible that oxygen, possibly combined with high salinity in oceans, exerts a substantial stress on the viability of *C. botulinum* type E spores. This might explain the comparably lower prevalence (37%) we found in the well-oxygenated northern parts of the Gulf of Bothnia and why the North Sea, although subjected to a steady influx of spores from the neighboring Baltic Sea, is not contaminated with *C. botulinum* type E (Johannsen, 1963; Cann et al., 1965; 1967).

The finding in study V that contamination levels were remarkably lower in the freshwater ponds equipped with a self-cleaning apparatus than in the other types of farms is interesting. It seems that the removal of bottom sludge effectively eliminated *C. botulinum* type E multiplication in the self-cleaning ponds. The argument that "bottomless" marine net cages might be less botulinogenic than freshwater ponds has been used to promote marine trout farming in the Baltic Sea. However, although direct sediment feeding is indeed effectively hindered in net cages, it seems that other factors, such as the accumulation of biomass within the net cages and the overall higher *C. botulinum* type E contamination rates in the marine environment, outweigh most of this benefit. Furthermore, the widespread use of ground baltic herring as a cheap additional feed in the marine farms is likely to increase the level of contamination of net cage-farmed trout; Hyytiä et al. (1998) found that 40% of herrings caught in the Baltic Sea contain *C. botulinum* type E spores. On some marine farms, prototypes of funnel-shaped net cages equipped with a sediment suction device are on trial in an effort to reduce

nutrient escape; our findings for the self-cleaning freshwater ponds give us reason to believe that this sort of apparatus could also be used to lower *C. botulinum* type E contamination rates in the produce of such farms.

6. CONCLUSIONS

1. PCR detection of botulinum neurotoxin genes is a highly suitable method in screening for viable *C. botulinum* cells in pure culture, fish and environmental samples. Due to its high specificity, speed, cost-efficiency, safety and favorable ethics it can and should replace the mouse bioassay in studies aiming to demonstrate the existence of the *C. botulinum* organism. In most botulism outbreak studies PCR detection will not supplant the use of the bioassay, as PCR does not detect nor measure the biological activity of neurotoxins. Until problems concerning PCR inhibition in food and soil samples can be prevented the incorporation of an enrichment step in the detection protocol effectively concentrates the target cells while getting rid of many PCR-inhibitory sample constituents.

2. An indirect quantification approach, such as MPN tables, is a good method of determining the *C. botulinum* spore contamination levels of samples. Although PCR quantification techniques have been described for the direct enumeration of microbes in samples, their use in connection with un-enriched and presumably PCR-inhibitive food and soil samples cannot be recommended. Furthermore, the use of multiple subsamples and several dilutions of the samples substantially lowers the possibility of a positive sample being falsely labeled negative due to PCR-inhibition in a single reaction tube.

3. The suitability of PFGE typing as a characterization method for nonproteolytic *C. botulinum* was demonstrated for the first time in studies II and V. The mean genome size of *C. botulinum* group II was determined to be 3.89 Mb. PFGE analysis of macrorestriction digests using *Sma*I and *Xho*I seems to be a valuable subtyping method for *C. botulinum* isolates. The MRPs created by these two rare-cutting enzymes contain approximately 12 to 18 well-defined fragments of which most are brought into the linear portion of a regular PFGE gel ramped from 1 to 26 s over 22 h. In most practical aspects PFGE typing is superior to ribotyping with *C. botulinum* group II isolates; the method has excellent reproducibility and

the discriminatory index is higher. The MRPs are well-defined and thus easy to interpret either manually or by computer, which is essential in cluster analysis or in creating strain identification databases. However, PFGE of *Sma*I and *Xho*I digests is definitely not a taxonomical method for clostridia; intraspecies differences in the MRPs can be larger than interspecies differences.

4. The suitability of ribotyping as a characterization method for *C. botulinum* was demonstrated for the first time in study III. All clostridial isolates in our study were typeable by *Eco*RI and *Hind*III ribotyping, whereas 10% of the isolates were untypeable by PFGE due to DNase degradation of the isolated DNA (II, V). The ribotyping profiles of clostridia were taxonomically coherent, and it seems that there are few genotypic approaches except rRNA gene sequencing to match the species identification abilities of this method. In comparison with PFGE typing the obvious drawbacks of ribotyping are the difficulties with clostridial RFLP interpretation and reproducibility, as well as a lower discriminatory index. Results with manual ribotyping of clostridia are essentially similar whether using agarose-imbedded or conventionally isolated DNA, which should increase interest in ribotyping as an auxiliary characterization method to be used in connection with PFGE typing.

5. When determining the prevalence of *C. botulinum* types responsible for human botulism in Finnish freshwater and Baltic Sea sediments, only spores of type E were found, although in very high numbers. The prevalence and spore contamination level in 56 Finnish freshwater samples was 61% and 372 kg^{-1}; in 12 Finnish seashore samples 58% and 500 kg^{-1}; and in 42 Baltic Sea offshore samples 88% and 1,020 kg^{-1}, respectively. All Baltic Sea offshore sites were positive for *C. botulinum* type E except for five sites up in the well-oxygenated northern end of the Gulf of Bothnia.

6. The overall prevalence and spore counts of *C. botulinum* type E in aquatic sediments correlated significantly with offshore bottom oxygen content, depth, and bioturbation activity, whereas there was no correlation with bottom water

temperature. These findings indicate the possibility of *C. botulinum* type E multiplication or at least, suitable conditions for spore survival, in anoxic sediments in the Baltic Sea.

7. When determining the prevalence of *C. botulinum* types responsible for human botulism in Finnish fish and sediments from Finnish trout farms, only spores of type E were found. The prevalence and spore contamination level in 125 sediment samples was 68% and 2,020 kg^{-1}; in 165 salmonid intestinal samples 15% and 166 kg^{-1}; and in 43 salmonid skin samples 5% and 310 kg^{-1}, respectively. The prevalence and level of *C. botulinum* type E spore contamination was equally high in traditional freshwater ponds and marine net cages, whereas it was significantly lower in ponds equipped with a self-cleaning system for the removal of sedimented bottom matter.

8. The genetic biodiversity of *C. botulinum* type E as revealed by PFGE subtyping is great. MRPs of isolates obtained from the same sampling site may express less than 30% similarity, thereby establishing the value of PFGE typing as a subtyping tool for botulism outbreak or food industry contamination studies. Clonal profiles, on the other hand, can be found from a variety of different samples over a wide temporal and spatial range. Thus it seems that the observed variety in MRPs is a product of a true diversity between neurotoxigenic clostridial lineages rather than a result of a rapidly mutating genome. No shared genotypic markers could be detected in *C. botulinum* type E strains that had been associated with cases of botulism, which seems to indicate that at least type E intoxication is the result of passive spore contamination of susceptible and botulinogenic foods.

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