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Molecular Epidemiology and Risk Assessment of Human Botulism in the Canadian Arctic

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MOLECULAR EPIDEMIOLOGY AND RISK ASSESSMENT OF HUMAN BOTULISM IN THE CANADIAN ARCTIC

A Thesis Presented to

The Faculty of Graduate Studies and Research

of

The University of Ottawa

By

Daniel Leclair

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ABSTRACT

On a *per capita* basis, the incidence rate of type E botulism attributable to aged marine mammal meat in Nunavik is very high, with an average rate greater than 3 reported cases per 10,000 population for the period from 1996 to 2004. All cases reported for this period were clustered in Northern villages along the Ungava Bay. A survey for the presence of *Clostridium botulinum* type E along the coastline of Nunavik indicated a prevalence of spores along the southern coasts of Ungava Bay and Hudson Bay, indicating a possibility of contamination of seal meat from environmental sources during butchering. The highest concentration of spores was found in the Koksoak River (>5400 spores/kg), which flows into southern Ungava Bay. Spores were also found on seal skin and in seal intestinal contents, although at levels lower than found in environmental sources.

Contamination of seal meat with *C. botulinum* type E spores may occur following contact with coastal rocks, tidal water, or shoreline soil, during the butchering process. Genomic analysis of coastal isolates of *C. botulinum* type E by PFGE indicated a heterogeneous population at the butchering sites, making it difficult to trace contamination sources.

Epidemiologically related strains of *C. botulinum* type E could be identified by PFGE. Several distinct subtypes were involved in botulism incidents originating from Inuit villages of southern Ungava Bay, corroborating the existence of a high genetic diversity of *C. botulinum* type E in the environment. Some of these subtypes were detected inland and in large rivers flowing into southern Ungava Bay.

The temperature used for *igunaq* preparation was the primary factor influencing the growth and toxigenesis of *C. botulinum* type E. All *igunaq* preparations incubated at 4°C were negative for BoNT/E. The use of traditional seal skin pouches or plastic

containers did not influence the time to toxicity of seal *igunaq* preparations aged at abuse temperatures (10 and 20°C), regardless of inoculation dose.

Changes in handling of seal meat during butchering to reduce contamination, combined with temperature control of the aging process to 4°C or less, should significantly reduce the risk of botulism from consumption of traditional Inuit foods.

Dedicated to my Family

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The PhD project on human botulism in the Canadian Arctic was very ambitious and logistically challenging. Nonetheless, Dr. Jeff Farber accepted with great enthusiasm and openness to meet with the challenge. It is with a lot of gratitude that I thank my supervisor, Dr. Farber, for his guidance, advice, and his precious time. Although Dr. Farber has a very busy schedule as the Director of the Bureau of Microbial Hazards, he always found the time to meet with me when I needed.

The extensive laboratory work carried out on this project was developed and conducted in the research laboratory of the Botulism Reference Service for Canada under the supervision of Dr. John Austin. I am very grateful to all the scientific advice and guidance that Dr. Austin provided me on various studies. This incredible amount of laboratory work, including the development of one of the largest bank of strains of *C. botulinum* type E in the world, could not have been done without the technical expertise of Burke Blanchfield, assisted by Brigitte Cadieux and Greg Sanders. They were wonderful friends and teachers with unlimited experience which allowed me, a wildlife vet, to gain knowledge and experience in the world of food microbiology and molecular bacteriology.

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Cheers,

Daniel

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LIST OF ABBREVIATIONS

BRS: Botulism Reference Service for Canada

CBI: *Clostridium botulinum* Isolation agar

CMM: Cooked meat medium

GPB: Gelatin phosphate buffer

h: hours

HFTA: Hunting, Fishing, and Trapping Association

mL: millilitre

min: minutes

MPN: Most probable number

NRC: Nunavik Research Centre

PFGE: Pulsed-field gel electrophoresis

QCS: Quality control samples

s: seconds

SPGY: Special peptone peptone glucose yeast extract broth

µL: microlitre

CHAPTER 1

INTRODUCTION, HYPOTHESIS, AND OBJECTIVES

INTRODUCTION

Foodborne type E botulism is predominantly reported in regions of the northern hemisphere and is often associated with the consumption of fish and fish products worldwide (Hauschild, 1993; Austin and Dodds, 2000). A series of control measures based on food safety inoculation studies and predictive modeling have been implemented by the industry to reduce the risk of botulism hazards from various packaged fish products (Hobbs, 1976; Austin and Smith, 2006; Skinner and Reddy, 2006). However, in Arctic regions, the disease is endemic in some areas and is frequently caused by botulinum toxin type E (BoNT/E) produced in Inuit traditional foods (Wainwright, 1993). Epidemiological studies of type E botulism in the North have identified fermented fish and marine mammals as common food sources of outbreaks (Dolman, 1974; Hauschild and Gauvreau, 1985; Wainwright *et al.*, 1988), but the factors influencing the lag phase of *Clostridium botulinum* type E toxigenesis in these products have not yet been characterized.

C. botulinum type E is ubiquitous in the environment and is naturally found in soil and aquatic habitats, particularly in coastal waters (Dodds, 1993a) . Type E is commonly isolated with other mesophilic clostridia from marine sediments (Smith, 1968; Matches and Liston, 1974), but strong evidence suggests that the organism is of telluric origin (Johannsen, 1963; Dolman and Ilda, 1963; Yamakawa and Nakamura, 1992). The ecology of *C. botulinum* type E in Arctic regions is largely unknown, but its distribution in marine sediments along the Alaskan shoreline has been characterized and generally follows the geographic pattern of human botulism (Miller *et al.*, 1972; Miller, 1975). Fish and marine mammals living in these waters may carry spores or become contaminated during handling of post-harvested fish or marine mammal meat processed directly on the shoreline by native populations.

The current handling practices of fish and marine mammals in the Arctic suggest that the contamination of raw meat from marine mammals with spores is probably high. Various preparation and fermentation methods are used by the Inuit to age marine mammal meat into *igunaq* (Weetaluktuk, 1997), a traditional dish frequently associated with botulism (Austin, 1996; Austin *et al.* 1997, 1999). Some of these methods were found to be highly hazardous with respect to botulism, but the safety of traditional or non-traditional fermentation methods has not been demonstrated. The germination and outgrowth of *C. botulinum* type E is known to be dependent upon water activity (a_w), pH, temperature, preservatives and background flora (Ando and Ilda, 1970; Hobbs, 1976), but none of these barriers are strictly applied for the safe production of traditional Inuit foods. Despite continual education efforts by public health authorities, the number of outbreaks and cases in endemic areas of the Canadian Arctic has not decreased (Austin, 1996; Austin *et al.* 1997, 1999). This introduction essentially describes the current state of knowledge on the ecology and epidemiology of *C. botulinum* type E, the factors influencing its growth and toxin production in marine foods, and the overall measures to control the organism.

1.1. *Clostridium botulinum* type E: the organism and toxin

1.1.1. Taxonomic classification.

Members of the genus *Clostridium* are motile, Gram-positive, rod-shaped, anaerobic, spore-forming bacteria ubiquitous in nature (Figure 1.1). The genus comprises 146 species, of which 34 are considered pathogenic (Sackebrandt *et al.*, 1999). *C. botulinum* is characterized by its ability to produce botulinum toxin (BoNT), the causative agent of botulism. The species was traditionally classified into seven distinct serotypes (A to G) based on the antigenicity of the neurotoxin they produced. This phenotypic trait is not exclusive to *C. botulinum*, as some strains of *C. butyricum* and *C. barati* have genes encoding type E (Poulet *et al.*, 1992) and F (Thompson *et al.*, 1993) neurotoxins, respectively, both of which have also caused botulism (McCroskey *et al.*, 1986; McCroskey *et al.*, 1991).

1.1.2. Phenotypic characteristics

Comparative analysis of the 16S rRNA gene sequences show that *C. botulinum* is phylogenetically divided into four distinct lineages (Collins and East, 1998), which agrees with the previous phenotypic classification that separates the species of *C. botulinum* into four groups (I to IV) based on their physiological characteristics. Type E and all strains of non-proteolytic *C. botulinum* types B and F belong to the physiologic group II and form a single phylogenetic group with 99.6-99.7% sequence similarity, corresponding to 4-6 base pair differences in the 16S rRNA genes (Hutson *et al.*, 1993). A number of reviews have summarized the physiological and biochemical traits that characterize each group of *C. botulinum* and strains of other clostridial species producing botulinum toxins and these traits are presented in Table 1.1 (Hobbs, 1981; Collins and East, 1998; Lund and Peck, 2000; Austin, 2001).

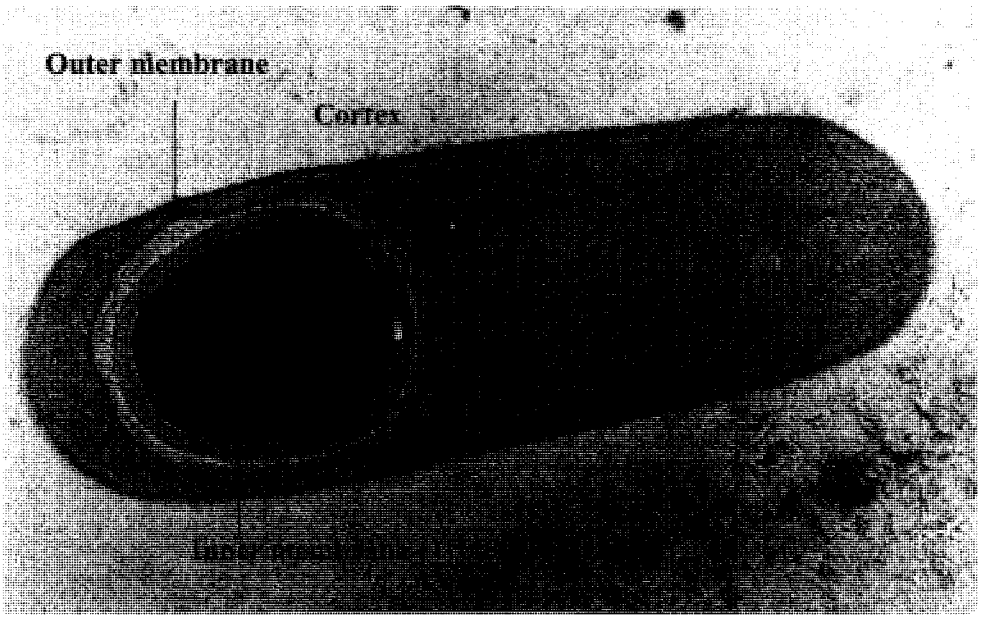


Figure 1.1. Transmission electron micrograph of a thin section of *C. botulinum* showing the endospore. Source: Botulism Reference Service for Canada.

Table 1.1. Physiological and biochemical characteristics of *C. botulinum* groups I to IV and other clostridial species producing botulinum toxins

Characteristics[e]	Groups					
	I	II	III	IV	*C. butyricum*	*C. baratii*
Toxin types	A, B, F	B, E, F	C, D	G	E	F
Proteolysis	+[a]	–	–	+	–	–
Liquefaction of gelatin	+	+	+	+	–	–
Fermentation of:						
Glucose	+	+	+	+	+	+
Fructose	±	+	±	–	+	+
Mannose	–	+	+	–	+	+
Maltose	±	+	±	–	+	+
Sucrose	–	+	–	–	+	+
Trehalose	–	+	–	–	+	–
Lipase	+	+	+	–	–	–
Lecithinase	–	–	–	–	–	+
Metabolic acids[b]	A, iB, B, iV	A, B	A, P, B	A, iB, B, iV, PA	A, B	A, B

Table 1.1. (Continued). Physiological and biochemical characteristics of *C. botulinum* groups I to IV and other clostridial species producing botulinum toxins

Characteristics[c]	Groups					
	I	II	III	IV	*C. butyricum*	*C. baratii*
Optimal growth temperature	35-40°C	18-25°C	40°C	37°C	30-37°C	30-45°C
Minimum growth temperature	10°C	3.3°C	15°C	ND[d]	10-15°C	ND
Lower pH limit for growth	4.6	5.0	ND	ND	4.0-5.2	ND
Inhibitory [NaCl]	10%	5%	ND	ND	ND	ND
Lower a_w limit for growth	0.94	0.97	ND	ND	ND	ND
$D_{100°C}$ of spores (min)[c]	25	<0.1	0.1-0.9	0.8-1.1	<1-5	ND

[a]For biochemical reactions: +, all strains are positive; -, all strains are negative; ±, some strains are positive and some are negative.
[b]Metabolic acids: A, acetic; B, butyric; iB, isobutyric; iV, isovaleric; P, propionic; PA, phenylacetic; PP, phenylpropionic.
[c]D-value: decimal reduction time required under specified conditions to cause one log or 90% reduction of the initial population of viable organisms.
[d]ND: not determined.
[e]Table was adapted from Collins and East (1998), Lund and Peck (2000), Austin (2001), Peck (2002).

1.1.3. Synthesis, structure and function of BoNT/E

The gene encoding botulinum neurotoxin (BoNT) type E was first localized in chromosomal DNA and partially sequenced by Binz *et al.* (1990). The complete sequence of the BoNT/E gene was later determined by Poulet *et al.* (1992) who showed that the sequence contained 4017 bp within a single open reading frame. The deduced amino acid sequence of type E toxin was composed of 1251 residues. BoNT/E is produced in culture supernatant, or foods as a progenitor toxin designated as the M toxin (12S or 300 kDa), consisting of a non-toxic component with no haemagglutinin (NTNH) activity and the neurotoxin (Sakaguchi *et al.*, 1966; Kitamura *et al.*, 1968). The neurotoxin is a single-chain polypeptide with a molecular weight of 150 kDa and possessing zinc-dependent endoproteinase activity. The protein is nicked at one-third of its length from the N-terminal into light (L) and heavy chains (H) of approximately 50 kDa and 100 kDa, respectively, joined by a single disulfide bond.

The biological activity of BoNTs specifically inhibits the release of acetylcholine at the neuromuscular junction by cleaving the proteins involved in the docking and fusion of vesicles containing acetylcholine (Austin, 2001). Figure 1.2 shows the mechanism of action of BoNTs and illustrates the three steps required to reach the target protein, i.e., binding, internalization and cellular action. The L-chain contains the zinc-binding motif common to metalloendoproteinases, allowing specific cleavage of SNAP-25, a presynaptic plasma membrane protein.

1.1.4. Lethal toxicity of botulinum toxins

All BoNTs induce toxicity in animals and humans by binding irreversibly to the pre-synaptic nerve endings and inhibiting the release of acetylcholine in the autonomic

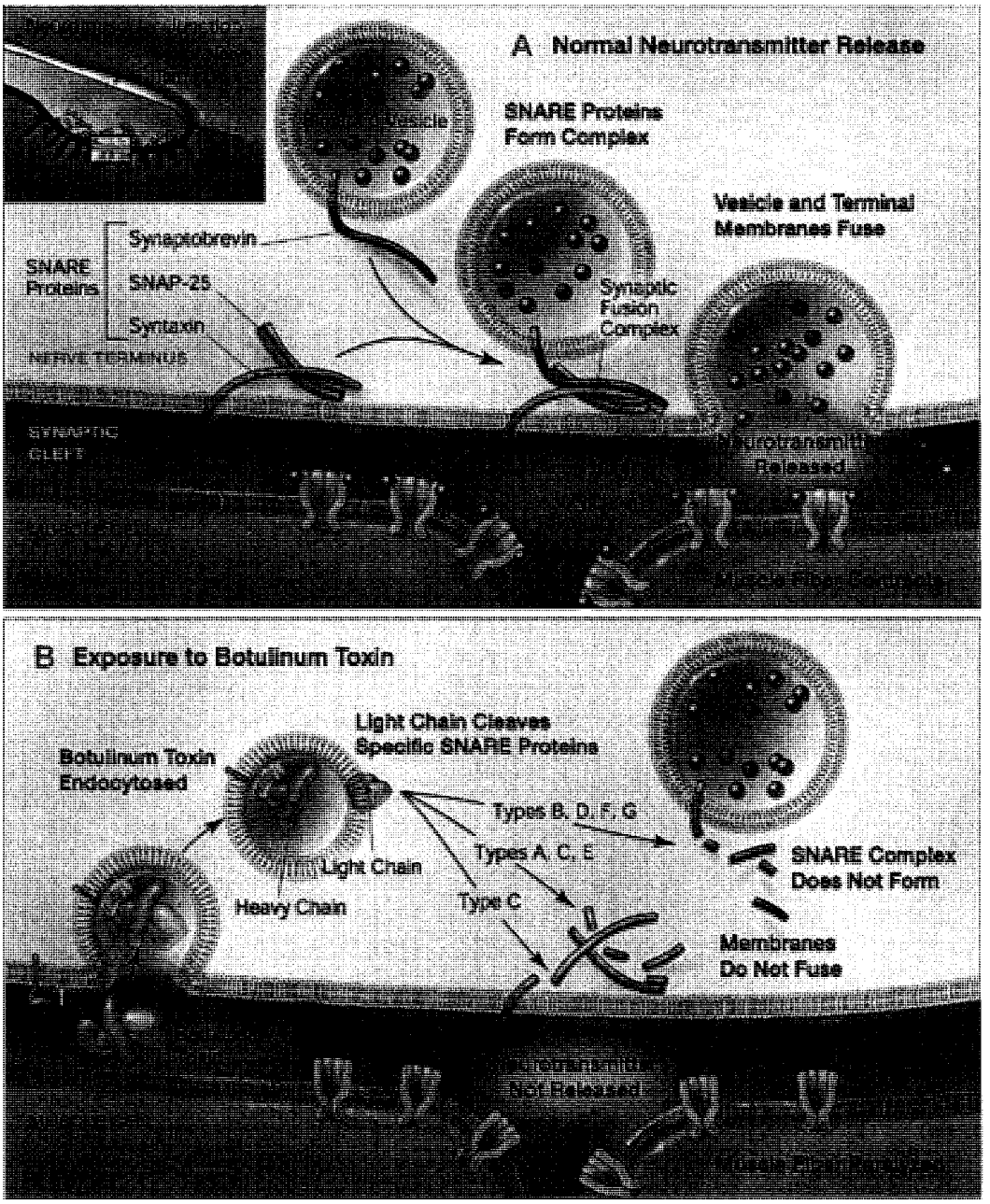


Figure 1.2. Mechanism of action of botulinum toxins. Panel A shows the release of acetylcholine at the neuromuscular junction mediated by the SNARE protein complex, i.e., syntaxin, synaptobrevin, and SNAP-25. Panel B illustrates binding, internalization, and enzymatic action of botulinum toxins. Botulinun toxin types B, D, F, and G cleave synaptobrevin; types A, C, and E cleave SNAP-25; and type C cleaves syntaxin. This figure was published by Arnon *et al.* (2001) and can be downloaded from http://jama.ama-assn.org/cgi/content/full/285/8/1059.

systems at the neuromuscular junctions, while not affecting the cholinergic structures of the central nervous system (Wright, 1955). Lethal dose (LD) information of botulinum toxin types in various animal species is relatively scarce and has been the subject of several reviews (Gill, 1982; Lamanna, 1959; Wright, 1955). Gill (1982) has reviewed sources of data and recalculated lethal doses while taking into accounts the degree of purity of various toxins and the inactivation factors. Based on toxicity experiments conducted with several laboratory animals (Gill, 1982), the minimum lethal dose or the 100% lethal dose (LD_{100}) of type A botulinum toxin for humans has been estimated to be approximately 1 ng per kg of body weight.

More recently, Arnon *et al.* (2001) provided lethal doses of botulinum toxins for humans by extrapolation from primate studies using crystalline type A toxin. Thus, the lethal amounts for a 70-kg human have been estimated to be 0.09-0.15 μg intravenously or intramuscularly, 0.7-0.9 μg by inhalation, and 70 μg orally. However, pure botulinum toxin is many orders of magnitude less toxic when administered orally in laboratory animals than by other routes. The progenitor toxins or crude toxin complexes are more toxic than the derivative toxic form when administered orally, regardless of toxin types (Ohishi *et al.*, 1977; Sakaguchi and Sakaguchi, 1974). Therefore, it has been proposed that the non-toxic proteins of the toxin complex provide protection for the active component, against inactivation by proteolytic degradation in the gastrointestinal tract.

1.2. *Clostridium botulinum* type E in the environment

1.2.1 Ecology of type E spores

C. botulinum type E was originally considered an organism of marine origin as its prevalence and levels on land were much lower than in marine environments (Pedersen, 1961) and most previous cases of type E botulism were associated with fish and fish products (Johannsen, 1965). Worldwide, only a limited number of type E outbreaks have been associated with food types other than fish or sea mammals (Austin and Dodds, 2000). However, the occurrence of *C. botulinum* type E in freshwater and terrestrial environments suggested that the organism has a wider distribution and could be of terrestrial origin (Johannsen, 1963). The presence of *C. botulinum* type E in high concentrations in aquatic environments could be the result of terrigenous sedimentation by drainage to the sea via large river systems, where the spores remain in a viable state for many years. The Baltic sea is recognized for its high concentrations of *C. botulinum* type E spores, acting as a catchment basin that accumulates spores of telluric origin from watercourses that drain large areas of land masses. Dolman and Ilda (1963) suggested that certain littorals heavily seeded with spores may harbour suitable ecological niches for intermittent germination and multiplication. These niches would constitute a source of spores that could be ingested by bottom-feeders and ultimately by fish predators, which would contribute to the dispersion of spores in the coastal environment through their excreta and farther offshore by migratory fish, sea mammals, and oceanic current.

The terrestrial origin theory of *C. botulinum* type E was not supported by others who proposed that type E was rather an organism of aquatic origin capable of active multiplication *in situ* (Bott *et al.*, 1968; Huss, 1980). Type E has been found in high concentrations, i.e., up to 36,000 spores per gram in sediments of Green Bay, Lake

Michigan (Sugiyama *et al.*, 1970, 1972). It was suggested that such high concentrations resulted from both the passive accumulation of spores flowing through the river systems into the bay, and by an active multiplication of the organisms in the bay. Huss (1980) also showed that the highest levels of *C. botulinum* type E spores in Denmark were found in landlocked fiords and areas with very little hinterland. All sediment cultures from these shallow fiords were toxic and the estimated level of spores from a random sample was 240 spores per gram. Overall, *C. botulinum* type E was found in 90% of the samples from the aquatic environment of Denmark, although none was detected in cultivated soil and woodlands. Based on these findings, it was suggested that *C. botulinum* type E is a true aquatic organism which can multiply in aquatic environments.

According to Huss (1980), the concentration of type E spores in aquatic environments is not related to the amounts of rotting vegetation or dissolved carbohydrates. Rather than growing in bottom sediments, it is postulated that the organism could grow and multiply on dead fish and benthic fauna. This may occur as a result of the mass mortalities observed during warm weather conditions which cause the depletion of oxygen in the bottom environment. Hielm *et al.* (1998c) showed a positive correlation between spore counts and low oxygen content, depth, and absence of bioturbation in offshore sediments of the Baltic Proper. Whether *C. botulinum* type E grows in bottom sediments of the Baltic Proper or sporadically occurs in dead benthic fauna in Danish landlocked fiords, remains to be determined.

1.2.2. Occurrence of *C. botulinum* in Canadian soil and waters

The first reported case of botulism in Canada was recorded in 1919 (Dolman, 1974). The ubiquitous distribution of *C. botulinum* in Canada was demonstrated three

years later by Dubovsky and Meyer (1922), who recovered *C. botulinum* types A and B from virgin and manured soil samples from four and two provinces, respectively. Of 28 toxic cultures, one culture of soil from Alaska and seven from British Columbia could not be typed with available antitoxins; serotypes E and type F were discovered in 1936-37 and 1958-60, respectively (Lund and Peck, 2000). *C. botulinum* type E was first isolated in Canadian soil in Nanaimo, British Columbia, where the first type E outbreak was documented in Canada (Dolman and Kerr, 1947). The organism was later demonstrated in deep marine sediments of the coastal inlets and shoreline areas of British Columbia, but not in samples from Vancouver beaches (Dolman and Ilda, 1963). To demontrate that *C. botulinum* type E was of telluric and not of marine origin, the authors investigated the occurrence of type E in ocean bottom specimens collected at some distance from the coast of Alaska and the high Canadian Arctic. The authors failed to isolate the organism from any sea bottom samples and concluded that type E was of telluric origin with an affinity for marine environments.

Laycock and Loring, (1972) studied the distribution of *C. botulinum* in the Gulf of St. Lawrence in relation to the depositional processes and patterns of sedimentation. Only strains of *C. botulinum* type E could be isolated from sediments collected in the River and Gulf of St. Lawrence. All positive samples (15%) collected between Lake Saint-Pierre and the river estuary originated from the mouth of the river where deposition can effectively take place. As the shoreline sediments of the river estuary were mainly composed of old glacial deposits, the authors suggested that the source of spores was upstream, possibly from the Great Lakes. The distribution of *C. botulinum* type E in the River and Gulf of St. Lawrence was correlated with the terrigenous sedimentation patterns. The prevalence was generally higher in areas influenced by drainage basins. Accordingly, 69% of sediments collected from Baie de Chaleur

draining the highlands of New Brunswick and the landmass of the Restigouche and Matapedia river systems contained spores of *C. botulinum* type E. However, the authors postulated that multiplication of *C. botulinum* type E can possibly occur in shallow and brackish waters of Baie de Chaleur during the warmer months and seasonally contribute to the accumulation of spores in the bay.

The presence of multiple serotypes in marine environments was demonstrated in the eastern Canadian continental shelf by Laycock and Longard (1972). *C. botulinum* types B, C, and E were detected in 10% of the sediments collected from inshore fishing grounds off the coast of Nova Scotia. Type C was predominantly isolated from 12.5% of positive sediments collected from offshore fishing grounds. Overall, the prevalence of *C. botulinum* types B, C, and E was relatively low at 0.9, 8.4, and 1.7%, respectively. The highest prevalence (40%) of *C. botulinum* type E was found near the coastline in the sediments of St. Margaret's Bay. *C. botulinum* type E was also isolated from offshore fishing grounds north of Newfoundland, where 31% of the sediments located between 40 and 150 miles from Notre Dame Bay were positive. In contrast, all bottom samples collected from the Grand Bank were negative for *C. botulinum*.

1.2.3. Distribution in the Arctic

The distribution of *C. botulinum* type E in Arctic regions has not been extensively studied, except in Alaska. The presence of *C. botulinum* (untyped) in the Alaskan environment was first suspected in a single soil sample collected from a bank of Copper River in 1921 (Dubovsky and Meyer, 1922). Dolman and Ilda (1963) were later unable, during an investigation of the North Pacific coastline, to show the presence of *C. botulinum* type E in 80 silt samples collected in Hooper Bay on the northwest coast of Alaska. No spores were isolated from offshore sea-bottom samples of Bristol Bay north

of Alaska peninsula, Bering Sea, Chukchi Sea, as well as from soil samples collected on St. Paul Island, a flat and isolated island situated about 480 km west of the Alaska peninsula. Similarly, no offshore sediment samples collected from the Canadian far North near the Sverdrup Islands contained the organism. Based on these environmental surveys, the authors concluded that strains of *C. botulinum* type E present in the coastal regions of the North are not derived from the ocean bottom distant from the coast, but rather from land sources (Dolman and Ilda, 1963).

Miller *et al.* (1972) targeted their investigations in coastal areas of northwest Alaska near communities affected with endemic botulism. The environmental survey revealed the presence of *C. botulinum* type E in 74% (17/23) of shoreline samples from Point Hope, Kotzebue, and Elephant Point, where marine mammals were frequently butchered. The predominance of spores was later confirmed in coastal areas of southern and western Alaska, from which the organism was detected in 36 of 80 beach soil samples (41%) from five of 12 investigated beaches (Miller, 1975). In comparison, only seven of 115 non-coastal soil samples (6%) collected inland along rivers, streams and ponds contained spores of *C. botulinum* type E (Miller, 1975). The author identified areas near Kotzebue where 90% of sediments, 73% of beach soils and all water samples collected along these beaches were found positive for *C. botulinum* type E. It was suggested that the continual discharge of organic materials originating from butchered marine mammals along the shoreline and from effluents of crab, fish, or shrimp canneries contribute to the accumulation of spores in these areas.

Huss (1980) has studied the distribution of *C. botulinum* in the natural environment of Iceland, Greenland, and the Faroe Islands. In general, a low occurrence of *C. botulinum* types A, B or E was observed in Iceland and the Faroe Islands with only 3.7% (2/54), 4.3% (3/69) and 1.3% (1/75) of marine sediments, freshwater

sediments, and soil samples, respectively containing spores. In contrast, 86% of 21 marine sediments from the Greenland waters harboured *C. botulinum* type E spores. The distribution of *C. botulinum* type E in northern Siberia has not been reported yet, but spores of type E have been found in soils of five widespread regions of the former U.S.S.R. (Kravchenko and Shishulina, 1967).

1.2.4. Genetic biodiversity

While evaluating the performance and usefulness of new genotyping methods, several studies have examined the genetic diversity among strains of *C. botulinum* group I and group II of different origins and continental regions (Hielm *et al.*, 1998a, 1999; Hyytiä *et al.*, 1999a; Skinner *et al.*, 2000; Keto-Timonen *et al.*, 2005; Nevas *et al.*, 2005a). Hielm and co-workers (1998a) initially showed that several strains of *C. botulinum* group II, predominantly composed of type E strains, were genetically heterogeneous based on pulsed-field gel electrophoresis (PFGE) analysis. The isolation of studied strains spanned over 60 years throughout northern Europe and North America and probably explained the observed genetic diversity. However, several type E clones were isolated over a 45-year period and from different marine species of North America. Isolate *C. botulinum* Beluga E recovered from a fermented white whale flipper from Alaska, RS-1 from a Pacific red snapper of the Pacific coast of the United States, and isolate R-9087 E from a smoked rainbow trout of Canada, yielded identical PFGE patterns despite their origins and widespread locations. Similarly, isolate C-60 E recovered from dry mutton produced in Faeroe Islands, and isolates C-51 E and C-94 E originating from seal meat butchered in Greenland were all found to be indistinguishable by PFGE.

A limited number of studies have reported on the genetic biodiversity existing among strains of *C. botulinum* type E originating from the same ecosystem. Hielm *et al.* (1998b) have studied the genetic diversity of *C. botulinum* type E isolated from Finnish trout farms, including traditional earth pond farms and marine net cage farms. By comparing PFGE patterns of 54 isolates recovered from sediments, fish skins and fish intestines, 24 different subtypes and a variety of clonal pairs originating from different geographic locations, species, and environments were identified. Up to five different *C. botulinum* type E subtypes were isolated from a single rainbow trout farm, indicating the presence of local genetic biodiversity in areas of frequent contamination with spores. Using a combination of PFGE and randomly amplified polymorphic DNA (RAPD), Hyytiä *et al.* (1999b) identified 62 different subtypes among 92 type E isolates (or 67.4%) recovered from fresh fish and fishery products of Finland and Germany. The high genetic biodiversity was observed among type E isolates from fish and fish products of Finland, but not from Germany. Various subtypes were isolated from different fish species and also from individual fish, regardless of geographical locations of Finland. Overall, the genetic diversity appeared to be higher among isolates of fish originating from lakes and farms, than those captured from the sea or the coastal environment.

1.3. *Clostridium botulinum* type E in foods

1.3.1 Occurrence of *C. botulinum* type E in fish and marine species

Fish-borne botulism has been documented in the Russian literature since 1818 (Hobbs, 1976). Since then, the incidence of fish-borne type E botulism outbreaks has been reported worldwide and prompted research in prevalence and levels of *C. botulinum* type E in fish and fishery products and its associated risk in many countries. This review essentially describes prevalence data in fish and marine species from North America and Scandinavia, but additional data from other parts of the world can be found in the excellent review of Dodds (1993a). In Canada, the existence of type E in fish intestinal samples was shown for the first time in mullets caught in Lake St. Martin in southern Manitoba during a survey of the Federal Department of Fisheries (Dolman and Ilda, 1963). Apart from this report, there is no data on the occurrence of *C. botulinum* type E in freshwater fish and marine fish in the Canadian waters.

The prevalence of type E in fish was extensively studied in the United States following the occurrence of three botulism outbreaks associated with smoked fish caught in the Great Lakes between 1960 and 1963. Several research studies were conducted to determine the distribution and sources of spores in the Great Lakes, and these have been reviewed by Sugiyama *et al.* (1972). *C. botulinum* type E was found widely distributed in fish of the Great Lakes with a prevalence ranging from 1 to 9% among the lakes (Green Bay not included) (Bott *et al.*, 1966b). Although the intestines of fish were seen as the natural reservoir of spores, it was demonstrated that the organism does not multiply in fish intestines. The prevalence was found to be higher in fish captured in some of the large bays of the Great Lakes (Bott *et al.*, 1966a), particularly in Green Bay of Lake Michigan, where 57% of fish and 97% of bottom and shoreline deposits contained spores (Bott *et al.*, 1968).

Field surveys were also carried out on fish and shellfish harvested in waters of a number of Pacific and Atlantic coastal states. Overall, spores of *C. botulinum* were detected in 14% of anadromous fish and 15% of bottom fish along the Pacific coast of the United States, of which 91% were type E and the rest, either type A, B, or F (Craig *et al.*, 1968). Interestingly, the prevalence of spores in Chinook salmon and Coho salmon captured in the Columbia River, Alaska was relatively high with 20 and 31%, respectively, compared to those caught in ocean waters which had 7 and 18% prevalence, respectively. In contrast, Houghtby and Kaysner (1969) found a lower prevalence of *C. botulinum* type E in salmon caught in Alaskan, Oregon and Washington coastal waters. Only 6 of 1083 (0.5%) samples collected from 589 salmon entering the Alaskan canneries and 11 of 288 (3.8%) samples from 144 salmon harvested in Oregon and Washington coastal waters harboured type E spores. The majority of these positive samples were gills taken from salmon caught in rivers, supporting the previous observation that fish caught in rivers are more likely to harbour spores of *C. botulinum* type E than fish caught in offshore waters. The low prevalence of spores of *C. botulinum* in viscera was attributed to the lack of feeding during upstream migration.

Similarly, Miller (1975) later found a low prevalence of *C. botulinum* type E spores in 41 salmon collected directly from processing tables in villages of Alaska. Type E was detected in gills of two salmon and all enrichment cultures of heads, viscera, flesh, and roe of fish were negative. The prevalence and levels of type E spores in intestinal contents of various fish species harvested in the Gulf of Maine was also relatively low at 4.5%, and less than 10 spores per 100 grams, respectively (Nickerson *et al.*, 1966).

The highest prevalence of spores, predominantly of type E, was observed in shellfish, including clams, oysters, and crabs from Oregon coastal waters, ranging from 30 to 68%. The presence of multiple serotypes of *C. botulinum* was demonstrated in oyster beds of Mobile Bay along the Alabama coast, but only type E was present in oysters (Presnell *et al.*, 1967). Up to 87.5% of marine sediment samples collected from crab fishing areas of Cheseapeake Bay contained spores of *C. botulinum* type E. These results indicates that the natural environment of shellfish constitutes a source of contamination during harvesting prior to their arrival at processing plants (Cockey and Tatro, 1974).

The prevalence of *C. botulinum* type E has been extensively studied in wild fish and farmed fish from Scandinavian countries (Johannsen, 1963; Cann *et al.*, 1965a; Huss *et al.*, 1974a; Hielm *et al.*, 1998c), particularly in Denmark and Sweden, where previous outbreaks of human botulism were linked to the consumption of fish and fish products (Dolman and Ilda, 1963). The initial survey of Johannsen (1963) revealed a high prevalence of type E spores in the Baltic Sea, with a 100% prevalence in sediments and fresh herring caught in the Sound. The author suggested that the Baltic acts as a natural reservoir for spores originating from inland areas which settle in sediments of the Baltic or flow into Skagerrak and the North Sea by current. However, none of the enrichment cultures of sediments and fish intestines of surface and bottom feeders from the North Sea were found positive for type E toxin, despite a continuous flow of spores originating from the Baltic Sea (Cann *et al.*, 1965a). It has been postulated that the high salinity level in the North Sea, possibly combined with other environmental factors, might affect the viability of spores in ocean waters (Hielm *et al.*, 1998c). Fish from traditional offshore fishing grounds of the North Sea were found less frequently

contaminated with *C. botulinum* type E spores than fish harvested in the Baltic Sea, and were thus considered safer with regards to botulism hazards (Cann *et al.*, 1965a).

High contamination rates with spores of *C. botulinum* type E have been observed in Danish trout raised in farms and varied from 5 to 100% in winter and 85 to 100% in late summer (Huss *et al.*, 1974a). These high rates of contamination spurred the development of measures to reduce various sources of contamination from the farm environment (Huss *et al*, 1974b). The use of lime treatment at higher concentrations was successful in reducing contamination of ponds with *C. botulinum*, but its safety to fish was not assessed (Dodds, 1993a). There has been little quantitative data (Nickerson *et al.*, 1966) on the levels of contamination in fish until Hielm *et al.* (1998c) determined the levels of *C. botulinum* spores in intestinal contents and skin samples of Finnish farmed trout using an MPN procedure by which different serotypes were detected by PCR directed against the toxin genes (Hielm *et al.*, 1996). Overall, 15% of trout intestines and 5% of skin samples (includes gills, head, peritoneum, and skin) were contaminated with a mean count of 166 and 310 of *C. botulinum* type E spores per kg, respectively. The levels of spores in sediment and fish were lower in self-cleaning freshwater ponds than in marine net cage farms or in the regular freshwater ponds not equipped with self-cleaning systems.

The occurrence of spores of *C. botulinum* in migrating and non-migrating species of marine mammals remains largely unknown worldwide. Some data can be found from Dolman and Ilda (1963) who reported that none of the 15 fish and seal intestines collected during an environmental survey at St. Paul Island west of the Alaska Peninsula yielded a positive culture for *C. botulinum*. Miller (1975) later studied the occurrence of spores in colonic contents of 44 marine mammals of different species

hunted by the Inuit of Alaska and found only a single beluga whale sample (2.3%) to be positive for *C. botulinum* type E.

1.3.2 Occurrence *C. botulinum* type E in fishery and meat products

In Canada from 1961-1984, up to 24 botulism outbreaks were caused by fish and fishery products in northern British Columbia, affecting 53 people, mostly First Nations people, and causing nine mortalities (Dolman, 1974; Hauschild and Gauvreau, 1985). Most of these incidents were due to the consumption of traditionally prepared salmon eggs. A survey involving 26 lots of freshly fermented eggs has reported a mean pH of 5.9, but the level of spore contamination has not been studied (Hauschild and Gauvreau, 1985). Similarly, during the same time period but in the Canadian Arctic, 53 botulism outbreaks with 123 cases and 35 fatalities were related to the consumption of raw, parboiled and aged marine mammal meat. The frequency and levels of contamination with spores in raw and aged meat products derived from marine mammals are currently unknown in the Canadian Arctic and other circumpolar regions.

Surveys have been conducted in the United States on the prevalence of *C. botulinum* type E in fishery products following the occurrence of botulism outbreaks in the 1960s involving smoked whitefish chubs caught from the Great Lakes. Pace *et al.* (1967) isolated *C. botulinum* at all stages of the processing lines of smoked fish producers. The prevalence of spores ranged from 7.6 to 41.7% in fresh eviscerated fish, but the rates of contamination drastically decreased following the heating process, ranging from 1.3 to 1.5% in freshly smoked fish. However, Fantasia and Duran (1969) obtained a higher prevalence rate in finished fish products with 14.1% of the smoked chubs being contaminated with *C. botulinum* type E. In comparison, 4.6% of smoked

fish from the Pacific Northwest of the United States were contaminated with type E (Hayes *et al.*, 1970).

The occurrence and levels of *C. botulinum* in retail fishery products marketed worldwide varied greatly depending on the fish species, preparation method or the country of origin (Hobbs, 1976; Austin and Dodds, 2000). Salted carp from the Caspian Sea and dressed rockfish from California were the two fishery products with the highest reported prevalence with 63% and 100%, respectively and also with the highest contamination levels with calculated most probable number (MPN) counts of 490 and 2400 spores/kg, respectively. The prevalence of type E in smoked fish produced from Scandinavian countries was as low as 2% and as high as 20%, with a maximum calculated MPN count of 11 spores per kg. To determine the extent of contamination of Finnish fish with type E, Hyytiä *et al.* (1998) examined the intestinal contents and skins of several fish species purchased from local market places using the PCR-MPN procedure developed by Hielm *et al.* (1996). Overall, between 10 and 40% of marketed fish were contaminated with type E. The range of spore counts varied from 30 to 1900 per kg in intestinal contents and from 30 to 2730 spores per kg in skin samples. These surveys clearly showed that *C. botulinum* type E is prevalent in marketed fish and that recommended food safety measures should be implemented and maintained to prevent botulinum toxin formation in fishery products.

In comparison to fish, the prevalence of *C. botulinum* type E in meat and poultry products has not been extensively studied. The low background levels of spores of *C. botulinum* measured in meat and the rare occurrence of type E outbreaks associated with meat products worldwide (Johannsen, 1963; Austin and Dodds, 2000), probably explain this knowledge gap. The levels of spores found in various meat products were generally

low, regardless of serotype, ranging from less than 0.1 to 7 spores per kg of meat (Hauschild, 1989).

1.3.5. Factors affecting BoNT/E development in fish and meat products

Johannsen (1965) initially identified temperature, pH, and buffering capacity, oxygen tension, redox potential, a_w, essential amino acids, fatty acids, and the background microflora as factors influencing the growth and toxin production of *C. botulinum* type E in foods. According to Johannsen (1965), the risk of toxin formation in vacuum packed foodstuffs is primarily dependent on the nature of the product and not on packing, which has no influence on the redox potential of the food. Few studies have evaluated the effect of redox potential (E_h) on the growth and toxin production of *C. botulinum* type E in fish or meat products, probably because of inherent difficulties in accurately measuring the E_h of foods. The rate of BoNT/E production in fish appeared not to be influenced by the initial redox environment. Huss *et al.* (1979) showed that the times to toxicity were similar whether the inoculated fish were fresh (E_h values of 102 to 202 mV) or spoiled (E_h values of -191 to -261 mV). In fact, germination of *C. botulinum* type E spores occurred rapidly whether the initial redox potential was low or high in broth, and intrinsically spores can reduce the redox environment through their germination activities (Ando and Ilda, 1970). In addition, the E_h decreases rapidly during the storage of fish products. Hyytiä *et al.* (1999c) showed that the E_h decreased to -224 to -369 mV in vacuum packaged unprocessed fish inoculated with type E spores during the first week of storage at 8°C and remained in the lower negative range for at least five weeks.

Unexpectedly, the levels of oxygen in packaged fish appear to have little influence on the time to toxicity of *C. botulinum* type E. The ability of *C. botulinum*

type E to grow under aerobic conditions was demonstrated by Lyver *et al.* (1998a), who showed that the organism can grow and produce BoNT/E in sterilized nuggets packaged in air. More recently, Dufresne *et al.* (2000a) showed that trout fillets packaged with various concentration of oxygen up to 100% all became toxic after 5 days of storage at 12°C. The authors also demonstrated that the use of a low gas barrier film with an oxygen transmission rate (OTR) of 10,043 cc/m^2/day at 24°C did not prevent BoNT/E production in trout packaged in air, vacuum, or modified atmosphere and stored at 12°C. Furthermore, toxin production was detected even earlier in vacuum cold smoked trout fillets packaged in barrier films of high OTR stored at 8°C, than those packaged in films of low OTR (Dufresne *et al.*, 2000b). As a safety margin, the authors proposed to use a film with an OTR between 5,000-10,000 cc/m^2/day at 24°C to ensure the occurrence of spoilage before toxin development in packaged smoked trout fillets accidently stored under temperature abuse conditions.

Storage temperature is by far the most important factor that influences the growth and toxin production of *C. botulinum* type E in foodstuffs. Cockey and Tatro (1974) showed that temperature influenced the toxigenesis of *C. botulinum* type E in autoclaved crabmeat homogenate. Toxin production was detected in homogenized crabmeat depleted of its natural flora as early as 1, 2, and 8 days when incubated at 29, 24, and 10°C, respectively, regardless of the strain of type E used. However, Solomon *et al.* (1977) showed that autoclaved crabmeat inoculated with various strains of *C. botulinum* type E did not support growth and toxin production if storage temperatures were kept at 4 or 8°C for at least 180 days. The authors concluded that, under these conditions the use of strictly controlled refrigeration would not pose a public health risk with regards to botulism. Similarly, the presence of BoNT/E was not detected in untreated crabmeat, containing a normal microflora, which was inoculated with 10^3 to

10^4 type E spores per gram, packaged in four commercial-type containers, and held at 4°C for 21 days (Harrison *et al.*, 1995).

In fish, fresh tilapia fillets surface inoculated with 10^2 spores of *C. botulinum* type E per gram packaged in a high barrier film under vacuum or selected atmospheres such as 100% air, 75% CO_2 and 25% N_2 did not become toxic after 47 or 90 days of storage at 4°C (Reddy *et al.*, 1996). The authors attributed these results to the presence of a competitive psychrotrophic microflora preventing growth and toxin development of *C. botulinum* type E. These results were consistent with others who found the use of 4°C to be effective in preventing toxin production in fish packaged under vacuum or a modified atmosphere (Reddy *et al.*, 1997a,b; Dufresne *et al.*, 2000b). However, toxin was detected in vacuum packed crawfish tails and catfish fillets stored at 4°C after 30 and 46 days of storage, respectively, or two to three weeks after the onset of spoilage (Reddy *et al.*, 1997a; Lyon and Reddmann, 2000). In contrast, none of the inoculated catfish packaged in air, or crawfish packaged in air permeable bags, were toxic at 4°C after 54 and 30 days of storage, respectively. Vacuum packaging of salmon fillets and salmon homogenate tissue was more conducive to toxin production than salmon packaged under modified atmospheres with CO_2 (Garcia and Genigeorgis, 1987; Garcia *et al.*, 1987). A low temperature of 5°C failed to prevent BoNT/E production in vacuum packed herring deep inoculated with 10^2 spores per pack after only 15 days of storage (Cann *et al.*, 1965b). The combined effect of deep inoculation, which does not reflect the natural surface contamination of fish products by spores (Hyytiä *et al.*, 1999c), and the use of vacuum packaging, probably contributed to the short lag period.

Cockey and Tatro (1974) also demonstrated that the time to toxicity of *C. botulinum* type E in crabmeat also varies as a function of the levels of spores in the inoculum. Toxin production in autoclaved crabmeat homogenate stored at 4°C occurred

at 55, 82, and >120 days using inocula of 10^6, 10^3, 10^2 spores per sample, respectively. The time to toxicity for *C. botulinum* type E in deep inoculated vacuum packed herring was shorter with the highest dose of 10^6 spores per pack at any temperatures tested (Cann *et al.*, 1965b). Garcia and Genigeorgis (1987) observed the development of toxigenesis in vacuum packed salmon tissue homogenate inoculated with 10^4 spores after 15 days of storage at 4°C, as compared to 36 days for an inoculum of 10 spores. However, in another study, Garcia *et al.* (1987) showed that the levels of spores had no influence on the time to toxicity of *C. botulinum* nonproteolytic types B, E, and F in fresh salmon fillets packaged under any atmosphere at 4°C for 60 days, all of which remained nontoxic. The effect of inoculum dose on time to toxicity in vacuum packed fish stored at 10 and 20°C was not log linear or significantly different among spore levels ranging from 10^2 to 10^5 spores per pack (Cann *et al.*, 1965b). Furthermore, toxin production was detected in packs stored at 5°C as early as 15 days with an inoculum dose of 10^2 spores, whereas packs inoculated with doses ranging from 10^3 to 10^5 spores showed toxicity much later after 32 days of storage.

Lindroth and Genigeorgis (1986) showed that the lowest level of spores inducing toxin production in red snapper stored under modified atmospheres at 4°C for 21 days was 10^3 spores per fish homogenate. The authors concluded that the low natural background level of *C. botulinum* spores in red snapper will not initiate toxigenesis under these conditions. The presence of background flora may inhibit growth and toxin production of *C. botulinum* type E. Lyver al. (1998b) showed that natural lactic acid bacteria present in raw surimi nuggets packaged in air contributed to the inhibition of growth of *C. botulinum* type E incubated at 4, 12, and 25°C by decreasing the pH value of nuggets to 4.0-4.9. The authors also demonstrated that the presence of *Bacillus* spp. in cooked surimi nuggets packaged in air, were responsible for the growth inhibition of

C. botulinum type E by producing bacteriocin-like inhibitory substances. Garcia and Genigeorgis (1987) observed a decreased risk of toxigenesis in salmon tissue homogenates with the increased levels of microbial flora in fish, inhibiting growth and/or competing against the non-proteolytic *C. botulinum* types B, E, and F.

Christiansen *et al.* (1968) clearly showed that the development of type E toxin in smoked fish heated at 82°C for 30 min depends on the brine concentration in fish. It was shown that a brine concentration of >3% was necessary to prevent the outgrowth of *C. botulinum* type E spores that survived the smoking process. Hyytiä *et al.* (1997) demonstrated that vacuum packed cold-smoked rainbow trout containing 3.4% NaCl and 166 mg/kg of nitrite, became toxic after 6 weeks of storage at 4°C using a low inoculum (810 cfu/kg) of non-proteolytic types B, E, and F. However, when an inoculum composed of only type E strains was used, no toxicity was observed in fish samples containing the same concentration of NaCl and 109 mg/kg of nitrite, indicating a difference among strains in growing capacity and toxin production under these conditions. A curing method using 3.4% NaCl and the addition of nitrate at 686 mg/kg appeared more effective in reducing the risk of toxigenesis, particularly with a low level of spore inoculum containing non-proteolytic types B, E and F strains.

Most of the previous inoculation studies described were qualitative and generally involved a low number of growth parameters at specific levels. However, other researchers have used factorially designed experiments and developed mathematical models to predict toxigenesis of *C. botulinum* type E in foods (Lindroth and Genigeorgis, 1986; Meng and Genigeorgis, 1993). The use of modelling allows the quantitative measurement of effects of different inhibitory parameters and their interactions on microbial growth or toxin production in foods, enabling the industry to predict the outcome when product formulations change (Dodds, 1993b). Using five

incubation temperatures and seven levels of spore inoculum, Lindroth and Genigeorgis (1986) determined that the probability of toxigenesis of non-proteolytic *C. botulinum* types, B, E, and F in red snapper stored under three different atmospheres was significantly influenced by temperature, storage time, interactions of atmosphere and temperature, as well as temperature and time. The probability (*P*) of one spore initiating growth and producing toxin in fish stored until spoiled was found to be 0.001% at 4°C. However, toxin production occurred in fish samples inoculated using a minimum of 10^3 spores per sample at 4°C after 21 days of storage, regardless of atmosphere conditions. By combining data from the linear regression used for lag phase and from the logistic regression for the exponential phase of the *P* curves, the authors generated best fit equations to describe the relationship of *P* of toxigenesis and the predictor variables. In general, the predicted *P* values were close to those observed in the experiments.

Using a similar approach, Genigeorgis's group also developed models to predict toxigenesis of the same non-proteolytic strains in salmon fillets and tissue homogenates stored under modified atmospheres at low and abusive temperatures (Garcia and Genigeorgis, 1987; Garcia *et al.*, 1987). Meng and Genigeorgis (1993) then developed a model to predict the lag phase of *C. botulinum* type E toxigenesis in vacuum packed turkey breasts as a function of temperature, sodium lactate, sodium chloride, and spore inoculum using regression analysis. The model explained 94.5% of the variation in results, of which 65% was influenced by temperature. All meat samples containing sodium lactate remained negative at 4°C for up to 120 days, but became toxic after 60 days without added sodium lactate and 1% sodium chloride (Genigeorgis *et al.*, 1991). These models allow one to determine the lag phase using the described predictor variables at any level within the range of conditions tested. Hyytiä *et al.* (1999c) compared the predicted and observed growth and toxigenesis of *C. botulinum* type E in

vacuum packaged fishery products and found several discrepancies. The two predictive microbiological modelling programs had several limitations. The models did not permit the inclusion of all relevant predictor variables (i.e., limited range of spore loads, initial levels microbial population, temperatures, NaCl concentrations) at levels that would reflect more accurately the conditions of the food environment influencing the probability of toxigenesis of *C. botulinum* type E in fish products.

1.3.6. Control of *C. botulinum* type E in fishery and meat products

Control measures are needed to ensure that any residual spores of *C. botulinum* are destroyed or their growth inhibited before any fishery products reach the consumers. Inhibition of *C. botulinum* type E growth and toxin production can be achieved by controlling growth parameters commonly used for control of foodborne pathogens and spoilage organisms, including the use of heating temperature, storage temperature, pH, salt content, a_w, chemical additives, or a combination of them. Hobbs (1976) reviewed the threshold values of these growth parameters for *C. botulinum* type E and their applications in fishery products. The minimum growth temperature for *C. botulinum* group II is 3°C and inhibition of growth can be achieved if the temperature is constantly maintained below this value. The lower limits of pH, a_w, and sodium chloride for the growth of *C. botulinum* group II are 5, 0.97 and 5%, respectively, under optimum conditions (Austin, 2001). However, these inhibitory factors can interact together and growth can be inhibited by a_w values as high as 0.99 when the temperature is maintained at 10°C for a period of time and similarly, less salt is required at less optimum temperatures to inhibit the growth of *C. botulinum* group II (Hobbs, 1976).

Destruction of spores can be achieved by irradiation or thermal processing. Irradiation doses required to inactivate spores of *C. botulinum* will adversely affect the

organoleptic quality of foods (Hobbs, 1976). The maximum radiation dose absorbed by fish without causing undesirable organoleptic effects is 3 kGy (Hobbs, 1976), which is six times less than the dose required for a 5-log reduction of *C. botulinum* spores (Tauxe, 2001). Thermal processing can be used to control spores of *C. botulinum* in fish, but the resultant product must be acceptable to consumers. Spores of *C. botulinum* group II are heat-sensitive and a total exposure time of 17 min at 82.2°C was effective in destroying an inoculum of 10^6 type E spores in whitefish chubs (Crisley *et al.*, 1968). A heat pasteurization process of 82.2°C for 30 min is currently in force in different parts of the United States for the control of *C. botulinum* type E in smoked fish. However, it is recognized that the regulated process does not completely eliminate spores and hence, additional control measures are needed to prevent the germination and outgrowth of spores (Hobbs, 1976). The contamination rate with *C. botulinum* decreased from 20% in whitefish chubs at a stage immediately prior to smoking, to 1% in the finished product using a heating regime of 82.2°C for a minimum of 30 minutes (Pace *et al.*, 1967). Nonetheless, for safety reasons, the authors recommended that all fish processed under these conditions be limited to a shelf life not exceeding 7 days at 4°C.

Strains of *C. botulinum* group II are more heat sensitive than group I strains. However, heating processes may not be sufficient to ensure the full destruction of group II spores and, in practice, storage at refrigeration temperatures is usually used in combination with a restricted storage time. The possibility that meat products may be temperature-abused during storage, transport, or in the home exists, and this situation may require additional inhibitory factors such as pH, salt (low a_w), or nitrite to inhibit the growth of *C. botulinum* group II.

1.3.7. Inactivation of BoNTs in foods

As opposed to the heat resistant clostridial spores, BoNTs are heat-labile and can be inactivated by boiling contaminated foods for 5 min. Freezing and irradiation have also been investigated, but heating is the only practical method to inactivate botulinum toxins in foods (Siegel, 1993). Toxins are more resistant to radiation than spores and irradiation cannot be used for detoxifying foods without affecting the organoleptic quality (Siegel, 1993). The *D* value for inactivation of type E botulinum toxin was determined to be as high as 21 kGy (Skulberg, 1965). However, the presence of proteins, nucleic acids and other related substances in high amounts in foods would provide additional protection to type E botulinum toxin from radiation inactivation and would necessitate the use of higher radiation doses (Miura *et al.*, 1968).

Freezing is not considered a suitable method for inactivating botulinum toxins in foods. Yao *et al.* (1973) showed that type E botulinum toxin can retain its original toxicity after 264 days of storage at –15°C in salmon fluid extract. Woodburn *et al.* (1979a,b) showed that the heat inactivation rate of BoNTs was biphasic in canned salmon paste. A temperature of 85°C for a maximum of 5 min allowed for the inactivation of 10^5 MLD_{50} of botulinum toxins A, B, and E in salmon paste (Woodburn *et al.*, 1979a). The authors recommended the use of 85°C as the end-point temperature for home-canned fish heated in a 177°C (350°F) oven to ensure inactivation of BoNTs, regardless of toxin types. Type E botulinum toxin can also be inactivated through normal cooking procedures used for fish (Licciardello *et al.*, 1967). Deep-fat frying for 3 min at 190.6°C or pan frying for 5 min per side at 204.4°C was found adequate to inactivate toxic fish fillets containing 10^3 to 10^4 MLD_{50}.

1.4. Type E botulism

1.4.1. Clinical forms and manifestations

Five forms of botulism have been described in the literature and include foodborne, infant, wound, adult intestinal, and inadvertent (Arnon *et al.* 2001; Marks, 2004). Foodborne botulism is caused by the ingestion of foods contaminated with pre-formed botulinum toxins, predominantly types A, B, or E. Infant botulism occurs in children younger than 1 year who ingest spores of group I *C. botulinum* (Austin and Dodds, 2000). The spores are able to germinate, outgrow, colonize and produce toxins *in vivo* due to the immature intestinal flora of the infant (Caya *et al.*, 2004). Adult intestinal botulism develops like infant botulism in patients affected with an abnormality of the gastrointestinal tract following previous surgery, Crohn's disease, or antibiotic therapy exposure. Wound botulism was originally caused by infection of traumatic wounds of the extremities, but more recently contamination of needle puncture sites of intravenous drug users has become commonly reported (Caya, 2004). Inadvertent botulism usually results from accidental exposure of laboratory workers through the inhalational route or from therapeutic use of botulinum toxins (Caya *et al.*, 2004; Marks, 2004). Since the September 11 event, inhalational botulism has been identified as a potential biological risk. BoNTs have been previously produced for military use and constitute a potential weapon of mass destruction (Arnon *et al.* 2001).

The clinical onset of foodborne type E botulism in northern regions usually occurs within 12-36 h following ingestion of native foods containing pre-formed botulinum toxin (Wainwright, 1993). Gastrointestinal symptoms, including nausea, vomiting, diarrhea, and abdominal pain usually precede the development of neurological symptoms. The neurological disorder is initially characterized by cranial nerve deficits and parasympathetic blockade of the exocrine glands manifested by dysphagia,

dysarthria, dry mouth, and several ocular signs and symptoms, including blurred vision, ptosis, dilated and fixed pupils, and diplopia. The paralysis of extraocular muscles is due to the paralysis of cranial nerves III, IV, and VI, while dysphagia is caused by the paralysis of cranial nerve IX (Sobel, 2005). Paralysis of cranial nerve VII may also occur and is responsible for the inexpressive faces presented by some patients. A symmetrical flaccid paralysis then progressively develops downward with weakness, dyspnea and dizziness, affecting all skeletal muscles including the diaphragm and leading to respiratory distress, anoxia and death. The paralysis of voluntary muscles affects, in order, the muscles of the neck, shoulder, and upper and lower limbs (Sobel, 2005). Other symptoms (Barrett, 1991; Castrodale, 2005; Sobel, 2005) include hypotension, intestinal ileus, constipation, and urinary retention as a result of autonomic dysfunctions involving both the parasympathetic and sympathetic nervous systems (Ball *et al.*, 1979).

The epidemiological reviews of type E botulism affecting the native populations of Alaska between 1947 and 1986 revealed that nausea, vomiting, dry mouth, weakness of the extremities, diplopia, dyspnea, blurred vision and dysphagia were the most common symptoms observed among patients, ranging from 42 to 90% in frequency (Wainwright *et al.*, 1988; Barrett, 1991). In comparison to other toxin types, type E patients exhibit the shortest incubation period (90% $\leq$1 day) and are more susceptible to developing nausea, vomiting, and dryness of the mucous membranes than any other toxin types (Barrett, 1991; Woodruff *et al.*, 1992). The disease is generally more severe in patients affected by BoNT/A, of which 67% of the cases require intubation, as compared to 39% for those caused by BoNT/E (Woodruff *et al.*, 1992). Patients who develop respiratory muscle failure also demonstrate symptoms of dizziness, dyspnea, and weakness more frequently than those who do not require assisted ventilation.

Mechanical ventilation is used to maintain tissue oxygenation and prevent the development of anoxia caused by the pharyngeal collapse obstructing the air flow and the lack of tidal volume resulting from respiratory impairment (Sobel, 2005). However, many cases manifesting nonspecific and minimal neurological symptoms have been recorded (Wainwright *et al.*, 1988). These cases were designated as mild botulism cases and are probably often underreported in sporadic illness.

1.4.2. Laboratory confirmation and case definition

The diagnosis of foodborne botulism is primarily based on clinical examination and food history of patients. Toxicity testing and culture of specimens are the best methods used for confirming the diagnosis, but are not confirmatory in all cases (CDC, 1998). To conduct an extensive epidemiological review of Alaskan outbreaks of botulism during the period from 1947 to 1985 period, Wainwright *et al.* (1988) defined a confirmed case of foodborne botulism as a clinical illness with one or more of the following symptoms and laboratory findings: nausea and vomiting, dysphagia, diplopia, dilated and fixed pupils, blurred vision, ptosis, dry throat or mouth, dysarthria, respiratory impairment, or weakness; demonstration of botulinum toxin in food, serum, feces, or gastric specimens or isolation of *C. botulinum* from fecal culture. Individuals with clinical manifestations of botulism who ate the same food as the laboratory-confirmed cases were considered to be part of the same outbreak and counted as confirmed cases. In another study describing the clinical management of botulism cases in Alaska between 1973 and 1986, only patients with the positive detection of botulinum toxin in serum, feces, gastric aspirate, or food were considered cases. Detection of *C. botulinum* in clinical specimens alone was not considered sufficient for a laboratory confirmed case (Barrett, 1991). In situations where no laboratory

confirmation was obtained, clinical illness associated with the consumption of high-risk foods for botulism and which had three or more of the following symptoms were considered suspect cases: nausea and vomiting, dysphagia, diplopia, dilated and fixed pupils, and dry throat or mouth (Wainwright *et al.*, 1988).

1.4.3. Clinical management of botulism

Foodborne botulism is a reportable disease managed as a public health emergency due to the severity of the intoxication, its high mortality rate if not treated promptly, and the potential health and economic impacts of suspect foods produced in large quantity and widely distributed. Early recognition of botulism symptoms and signs is therefore critical for rapid diagnosis and administration of treatment, and identification of common food sources to prevent further cases from occurring. The antitoxin treatment has significantly contributed to the decrease in mortality-case ratio since its introduction and remains a key component of botulism therapy. The early administration of antitoxin can prevent the progression of moderate illness and reduce the duration of assisted ventilation in severe cases (Marks, 2004). The mode of action of the antitoxin is unknown, but it presumably acts by neutralizing the circulating toxins. The antitoxin is particularly effective during the first 24 h of illness when high amounts of toxins are in circulation (Caya *et al.*, 2004). Although type E botulism is predominant in Arctic regions, the use of a trivalent (ABE) antitoxin is recommended as type A and type B outbreaks have been observed in Alaska since 1975 and 1973, respectively (Barrett *et al.*, 1977). However, since 1999, only vials of bivalent types A and B and monovalent type E have been available (Castrodale, 2005). Laboratory confirmation of toxin type is not necessary to start antitoxin therapy, since it can take up to 72 h to obtain conclusive results from the mouse bioassay (Sobel, 2005). Administration of antitoxin usually

begins when typical signs and symptoms of botulism are present and progressive in patients having a 3 to 5 day food history involving a food frequently associated with botulism (Wainwright *et al.*, 1988).

To eliminate unabsorbed toxin, all clinical and asymptomatic cases exposed to the suspect foods should promptly receive cathartics, enemas or gastric lavage, except those with an atonic bowel (Eisenberg and Bender, 1976b). Patients with any respiratory impairment should be placed in an intensive care unit and have their vital signs closely monitored, as they may require mechanical ventilation through endotracheal or nasotracheal intubation if their condition deteriorates (Barrett, 1991; Marks, 2004). As the paralytic illness may last several weeks, the continuous and dedicated intensive care becomes critical for the recovery of patients while minimizing any complications they may develop during prolonged hospitalization.

1.4.4. Epidemiology of type E botulism

A number of epidemiological reviews (Dolman, 1960; Dolman and Iida, 1963) on type E botulism have been published since the first type E outbreak was documented by Hazen in 1938. The first laboratory-confirmed outbreak of botulism in Canada occurred in Nanaimo, British Columbia in 1944, and was caused by the consumption of home-canned salmon containing botulinum type E toxin (Dolman and Kerr, 1947). The early reports of type E botulism showed that the disease occurred mainly in the northern regions of Canada and in the United States and Japan. Fish, fish eggs, and traditional preparations of marine mammals produced locally were the vehicles most often implicated (Dolman, 1960). Until 1960, most type E outbreaks occurring in Canada were confined geographically to northern British Columbia and Labrador and were categorized by population groups and food types (Dolman and Iida, 1963). By the end

of 1960, a total of 33 outbreaks were recorded in Canada affecting 105 people and causing 60 deaths for a fatality rate of 57%. Of 18 laboratory-confirmed outbreaks, 11 were caused by type E botulinum toxin associated with uncooked fish or seal products. Most early type E outbreaks from the United States occurred in Alaska or the Great Lakes area during the period from 1899 to 1969, and were linked to fish and marine mammal products, particularly beluga flippers (Dolman, 1960; Gangarosa *et al.*, 1971). In Alaska, a total of 10 outbreaks related to marine products were recorded, which affected 26 persons, of whom 11 died. In Japan, the first type E outbreak was only documented in 1951, but by the end of 1960, a total of 32 outbreaks affecting 187 persons causing 60 deaths were recorded. The vast majority of these cases were associated with the consumption of *izushi*, a traditional fermented relish made of raw fish, rice, and diced vegetables. Based on retrospective studies, up to 10 *izushi*-borne outbreaks were traced between 1930 and 1952 (Dolman, 1960). Prior to 1960, while type E antitoxin was still unavailable, the fatality rates ranged from 32 to 72% in different parts of the world. The introduction of type E antitoxin for therapeutic use in endemic areas contributed to the decrease in the fatality rate worldwide. The therapeutic value of type E antitoxins was demonstrated during the first clinical trials in Canada and Japan in the early 1960s; the fatality rate was only 3.5% in 85 patients who received the type E antitoxin in Japan (Dolman and Iida, 1963).

In contrast, a relatively low number of type E outbreaks were reported in Scandinavian countries and other circumpolar regions. In Denmark, the first recorded type E outbreak was in 1951 when six persons developed symptoms of botulism following consumption of uncooked vinegar herring. Another fish-borne incident followed 10 years later when a person became sick after ingesting salted herring which had been left in a barrel for several months (Dolman and Ilda, 1963). Three fish-related

type E outbreaks were later documented in southern Sweden in the early 1960s, affecting six persons and causing one death. The first type E outbreak in Greenland recorded in 1967, and involved 20-25 persons who ate meat from a putrefied sperm whale. Three persons and several dogs died from eating meat that contained 1,000 MLD per mL of meat extract (Müller and Thomsen, 1968).

Although the disease gained recognition among the public health community, and the risks associated with specific traditional dishes were better known, the occurrence of type E botulism persisted and became endemic in native populations of Alaska, British Columbia, and regions of the Canadian Arctic. In Canada, type E botulism was by far the most frequent reported type. Of 38 outbreaks reported between 1961 and 1973, 18 were caused by raw or parboiled marine mammals in Arctic regions and 15 were due to putrid salmon eggs in the Indian communities of the Pacific coast, affecting a total of 86 persons of whom 30 died (Dolman, 1974). The number of reported type E outbreaks associated with raw, parboiled or aged marine mammals continued to increase in the Canadian Arctic during the 1971-1984 period, particularly from northern Quebec (Nunavik) and the Northwest Territories, which accounted for 59% (36/61) of the total outbreaks in Canada (Hauschild and Gauvreau, 1985). In contrast, the number of outbreaks associated with salmon eggs in British Columbia decreased with 7 outbreaks or 11% of Canadian outbreaks affecting 15 persons and causing 2 deaths. This decrease was attributed to the educational efforts aimed at discouraging the fermentation of salmon eggs in the Indian communities. Overall, the Inuit populations were involved in 77% of the outbreaks, with 98 cases and 18 mortalities. This population was identified to be at high risk for botulism, with an overall incidence rate of 30 cases per 100,000 population annually (Hauschild and Gauvreau, 1985).

In Alaska, a total of 21 outbreaks of type E botulism were recorded between 1947 and 1974 involving 46 people and causing 13 deaths for an overall mortality rate of 28% (Eisenberg and Bender, 1976b). All 21 outbreaks occurred in villages of Western and Southeastern Alaska near the coast and along the rivers, following the distribution pattern of spores in the environment (Miller, 1975). Marine mammals, particularly beluga, followed by fish and fish eggs, and more rarely beaver tails, were the incriminated vehicles. The mortality rates significantly decreased over the years and can be reduced by prompt recognition of the disease, adequate therapy and supportive care, as well as thorough epidemiological investigations (Eisenberg and Bender, 1976b). Wainwright *et al.* (1988) reviewed botulism cases in Alaska from 1947 through 1985, and observed an increase in the number of outbreaks and cases for the 1976-85 period, where there was a total of 38 outbreaks and 92 cases, of whom four died. In comparison, the incidence of botulism in 1966 was 1.2 cases per 100,000, which increased to 15.2 cases in 1985. The increase was associated with changes in aging processes such as the use of plastic bags, indoor aging (near the stove), and improved surveillance (Eisenberg and Bender, 1976a; Wainwright, *et al.*, 1988). Sobel *et al.* (2004) showed that the incidence rate in Alaska decreased to 1.9 cases per 100,000 between 1990 and 2000, approximately 190 times the rate for the United States as a whole. Up to 58 incidents affecting 103 persons (case-fatality rate was 3%) were documented during this period and 49 (84%) of these incidents or 91 (88%) cases were caused by native foods containing type E toxin.

Reviews (Hauschild, 1993; Austin and Dodds, 2000) of foodborne botulism worldwide during various time periods showed that type E outbreaks continued to occur in northern Japan, Greenland, Scandinavia, the former Soviet Union, and Iran. Fish and marine mammals were consistently the main implicated food types with few exceptions.

From 11 type E outbreaks reported in Greenland between 1984 and 1989, seven were associated with seal meat, and three with sheep meat (Hauschild, 1993). Similar to Alaska and the Canadian Arctic regions, traditional fish processes involving long storage practices have often been incriminated in other countries. For instance, in Iran, about 90% of the cases recorded for 1972-74 were due to the consumption of traditional salt-cured fish eggs called *ashbal* (Austin and Dodds, 2000). In northern Japan, a total of 97 outbreaks were reported between 1951 and 1987, of which the great majority were linked to the consumption of *izushi*. Little data has been published on the epidemiology of type E botulism in the former U.S.S.R. after 1964 and the incidence of the disease in Inuit populations of Siberia has not been reported. In Africa, type E botulism occurs rarely, but has been observed in Egypt and Madagascar, where salted and ungutted fish (*faseikh*) and locally manufactured bologna were implicated, respectively (Austin and Dodds, 2000).

1.4.5. Preventive measures against botulism in the Arctic

Immunization of native populations with botulinum toxoid has been proposed (Stuart *et al.*, 1970; Hauschild and Gauvreau, 1985) as a preventive measure, but was not retained due to the large number of persons at risk and the sporadic occurrence of the disease (Wainwright *et al.*, 1988). Currently, there is no licensed vaccine available for any botulinum toxin types (Sobel, 2005). Cooking has also been proposed as an effective means to both destroy heat-sensitive type E spores and heat-labile botulinum toxins in traditional marine products (Dolman, 1974), but this procedure has the disadvantages of significantly altering the original taste of products and eliminating a traditional dietary culture that has been existing for centuries.

Education and improved clinical management of botulism cases have been the most efficient measures used in the North. The preparation procedures of various traditional marine mammal and fish products have been recognized as hazardous and were repeatedly discouraged among the native populations through education (Bowmer and Wilkinson, 1976). Early recognition of botulism and efficient management of cases have clearly contributed to the decrease in fatality rates observed over the years in the Canadian Arctic and Alaska (Hauschild and Gauvreau, 1985; Wainwright *et al.*, 1988). However, the number of reported botulism outbreaks and number of cases has steadily increased in the Canadian Arctic and Alaska during the period from 1971 to 1984, but not in the coastal regions of British Columbia, where education about the risks associated with fermented salmon eggs apparently has made an impact (Hauschild and Gauvreau, 1985; Wainwright *et al.*, 1988). In Alaska, one common approach has been to educate people on how to reduce contamination of foods with spores and prevent toxin production. This approach did not appear to achieve significant success due to the ubiquitous nature of *C. botulinum* type E and the current hunting and food preparation practices (Castrodale, 2005). The use of plastic bags or sealed containers in the aging process of fish and marine mammals was recognized as a hazardous practice and was discouraged (Eisenberg and Bender, 1976a). Traditional methods that promote slow aging of native foods at low temperature were encouraged over methods employing modern-type containers placed above ground or inside a shed which enable foods to age more rapidly at higher temperatures (Shaffer *et al.*, 1990). The CDC, in collaboration with the Alaska State Department of Health Social Services, developed educational materials describing five food safety steps to protect families from botulism, including recommended methods for aging native foods (Sobel *et al.* 2004; Castrodale, 2005). These methods consist of using a traditional grass-lined hole in the ground to allow air

circulation around foods and storing food preparations at temperatures below 3°C during the aging process to prevent toxin production. In addition to these safety measures, it is also recommended to boil aged or fermented native foods prior to consumption to destroy any botulinum toxins that could have inadvertently been produced during aging.

A series of recommendations suggested by *igunaq* producers originating from Nunavik was gathered by Weetaluktuk (1997) during a survey on traditional and contemporary food preservation practices. Similar to Alaska, in Nunavik it was recommended to avoid: i) the use of plastic bags; ii) stagnant air around foods during aging and; iii) preparing *igunaq* during the warmer months, particularly in Ungava Bay region where the incidence of botulism is high. The presence of gas bubbles in the liquid phase was found to be an indication of bad preparation and it was recommended that any suspect preparations should be discarded. More recently, Suppa and Proulx (2002) recommended that walrus hunting activities in Ungava Bay be carried out during the fall rather than mid-July or early August. In comparison, the incidence of human botulism related to walrus consumption is rare in the Hudson Bay and Hudson Strait regions of Nunavik, where most of the walrus hunt is done during the fall. Also, approximately 70% of botulism outbreaks recorded in Canada between 1971 and 1984, which primarily affected the native populations of the Arctic regions, occurred during the period from May to October, with July being the peak month (Hauschild and Gauvreau, 1985).

1.5 Detection of *Clostridium botulinum* type E and BoNT/E

1.5.1. Media and growth conditions

The concentration of *C. botulinum* spores in soil or sediment is usually low, and with the presence of competitive anaerobes (Matches and Liston, 1974; Smith, 1975a), it becomes difficult to isolate the organism without enrichment. Many studies have reported the isolation of *C. botulinum* using various amounts of soil or sediment, selective pre-treatment, enrichment media, incubation temperature and time, but very few of them have compared the sensitivity of these different procedures. Bott *et al.* (1968) compared the sensitivity of different enrichment media by culturing mud samples spiked with $\leq 10^3$ spores and found that the beef heart infusion-cooked meat medium of Johannsen (1963) produced levels of BoNT/E that were 10-fold higher than the Reinforced Clostridial Medium, Trypticase-peptone-glucose, fish infusion, and others. Johannsen (1963) cultured sediment and shoreline samples in broth incubated for 6 days at 30°C, allowing detection of *C. botulinum* type E in 388 of 650 (60%) samples tested. The recovery rate of *C. botulinum* from environmental samples was shown to be significantly influenced by the composition of the medium (Smith, 1975b). Use of medium composed of Trypticase, yeast extract, and glucose, with and without trypsin, allowed the growth and toxin production of *C. botulinum* type A despite the presence of inhibiting *C. perfringens*, but not when soil samples were cultured in cooked meat medium (CMM).

Bott *et al.* (1968) found a higher percentage of positive enrichment cultures of mud samples when they were incubated at 25°C rather than 30°C, and no significant differences were found between 20 and 25°C. Gunnison *et al.* (1935) found that growth of *C. botulinum* type E was equally rapid at room temperature as at 37°C, but toxin production was more constant at room temperature than at 37°C. Notermans *et al.*

(1979) have recommended the use of two temperatures to recover multiple types of *C. botulinum* from mud samples. The best recovery rate for type E was achieved at 20°C. The incubation of cultures is usually performed under anaerobic conditions either in a vacuum jar or a jar filled with 80% N_2, 10% CO_2 and 10% H_2 (Johannsen, 1963; Notermans *et al.*, 1979).

According to Bott *et al.* (1968), increasing the sample size does not necessarily improve the sensitivity. The authors observed a better recovery rate of *C. botulinum* type E from mud samples by using 1-g amounts in 10-mL volumes of broth, than using 30-g amounts in 50-mL broth. Due to the low level of spores in parts of eviscerated whitefish chubs, Pace *et al.*, (1967) recommended the culturing of the entire mass for maximum detection of *C. botulinum.* Although spores of *C. botulinum* type E are known to be heat-sensitive, some researchers have used a heat pre-treatment prior to cultivation to select for spore-formers and reduce the background flora (Pasricha and Panja, 1940; Bott *et al.*, 1968; Rouhbakhsh-Khaleghdoust, 1975; Notermans *et al.*, 1979). The use of heat-treatment at 70°C for 20 min in CMM appeared as good as the non-heated treatment for the recovery of type E and other serotypes from naturally contaminated mud samples, regardless of incubation temperature (Notermans *et al.*, 1979). Bott *et al.* (1968) found that heating at 60°C for 30 min did not improve the detection of *C. botulinum* type E in mud samples. However, Pace *et al.* (1967) obtained a greater number of positive samples when two sets of treatment were used for the enrichment cultures of fish samples, i.e., one culture was nonheat-shocked and another was heat-shocked at 60°C for 15 min. Spores of *C. botulinum* type E can also be selected by immersion of specimens in 50% ethanol for 1 h before enrichment (Austin and Dodds, 2000), or after enrichment for isolation purposes, by mixing the enrichment culture with equal volume of absolute ethanol (Fantasia and Duran, 1969). At such

concentration, the ethanol was found germicidal for vegetative cells, without adversely affecting spores of *C. botulinum* type E (Johnston *et al.*, 1964).

1.5.2. Inhibition by background flora

The demonstration of *C. botulinum* in soil, sediment, or food is usually performed by cultivation of samples and detection of botulinum toxin in culture supernatants. The growth and toxin formation of *C. botulinum* type E in cultures could be inhibited by organisms present in the environment that have common morphological and physiological properties (Kautter *et al.*, 1966). These nontoxigenic type E-like organisms which have been isolated from fish viscera and mud collected from the Great Lakes, have been found to produce a bacteriocin-like substance called boticin E. Boticin E specifically affects the growth of *C. botulinum* type E and not strains of serotypes A, B, and F, by killing vegetative cells and inhibiting spore germination (Kautter *et al.*, 1966). Another study also reported the possible isolation of non-toxic variants of *C. botulinum* type E from fish intestines which were morphologically and culturally identical and displayed serological similarities to *C. botulinum* type E (Cann *et al.*, 1965a). The presence of organisms that inhibit the growth of type A strains have also been demonstrated in soil, and include various strains of *Clostridium sporogenes*, *Clostridium perfringens* and *Bacillus cereus* (Smith, 1975). Additional experiments with *C. perfringens* showed that the organism could interfere with the growth of other serotypes within *C. botulinum* group I and group II, including type E. The isolation of *C. botulinum* type E from fish intestinal samples was also found to be difficult due to the presence of several other clostridial species, including *C. welchii*, *C. sporogenes*, *C. tetani*, *C. bifermentans* and others (Cann *et al.*, 1965a). To reduce the number of false-negative sediment cultures due to inhibitory activity against *C. botulinum* type E, Bott *et*

al. (1968) considered only samples for which a duplicate enrichment control showed toxicity at a level of 10^2 spores. About 19% of the enrichment controls of river sediments were non-toxic, requiring in some instances the addition of 10^6 spores to the enrichment broth to overcome the inhibition contained in one gram of sediment.

1.5.3. Tryptic activation and toxin assays

The *in vivo* mouse bioassay is the standard method for the detection and quantification of botulinum toxin (Sobel, 2005). The toxicity, or biological activity of toxin, is expressed in terms of mouse intraperitoneal lethal doses and can detect as low as 5 to 10 pg of BoNT per mL (Kautter *et al.*, 1984). Although the mouse bioassay is the most sensitive and reliable test to detect BoNT in suspect foods or clinical specimens, it is expensive, time-consuming, and can take up to 72 h for completion. This has led to the development of several *in vitro* assays (Doellgast *et al.*, 1993; Witcome *et al.*, 1999; Ferreira *et al.*, 2003; Barr *et al.*, 2005; Cadieux *et al.*, 2005; Kalb *et al.*, 2006). Detection of BoNT by the mouse bioassay involves the inoculation of serum and extracts of food, feces, and gastric liquid, with and without neutralized antitoxin antibodies, into mice. Inocula of suspected cases of type E botulism are trypsinized prior to injection to potentiate the effect of type E neurotoxin.

Bott *et al.* (1968) did not recommend trypsinisation of inoculum from samples producing low toxin titers in enrichment broth. Ward *et al.* (1967) recommended to test samples with, and without, trypsinization since tryptic activation could destroy weak positive samples rather than potentiate them, but this procedure is time consuming for a large-scale survey. Sugiyama *et al.* (1972) observed that the addition of trypsin (0.2%) to type E cultures before incubation significantly increased the MPN count, possibly because of the inactivation of boticin E by trypsin (Kautter *et al.*, 1966). In a study

described by Bulatova *et al.* (1969), the prevalence of *C. botulinum* type E in soil samples increased from 36 to 74% when 0.1% trypsin was added into the medium before incubation, while others found that there was no value of adding trypsin to the medium (Kautter *et al.*, 1966; Huss, 1980).

1.6. Molecular subtyping of *Clostridium botulinum*

1.6.1. Pulsed-field gel electrophoresis

Genotyping methods, which analyze the genetic profile of microbial strains, are extensively used in molecular epidemiology and have several advantages over phenotypic methods (Cohen *et al.*, 2001). These methods have been applied in outbreak investigations to determine the role of the environment and track disease transmission. Pulsed-field gel electrophoresis (PFGE) has been considered the gold standard method for subtyping bacteria and is based on a comparison of DNA restriction patterns of whole bacterial genomes (Struelens, 1998; Lukinmaa *et al.*, 2004). Cells are embedded in agarose to protect DNA from mechanical breakage while allowing for the free flow of solutions for lysis and digestion during the DNA isolation steps (Birren and Lai, 1993). Digestion of cellular proteins is performed under conditions that prevent degradation of the chromosomal DNA by endogenous enzymes. The entire genome, estimated to be 3.9 Mb in size for *C. botulinum* group II (Hielm *et al.*, 1998a) , is then cleaved with a low-frequency restriction endonuclease into large DNA fragments of typically 10 to 700 kb in size (Struelens, 1998). The selection of restriction endonuclease is usually based on the GC content of the genome, whereby restriction endonucleases with GC-rich recognition sites are generally suitable for AT-rich genomes (Birren and Lai, 1993). The GC content of *C. botulinum* DNA is between 26 to 28% (Hobbs, 1976). The separation of large DNA fragments is then accomplished by periodically alternating the electric field at a fixed reorientation angle during electrophoresis using a clamped homogenous electric field electrophoresis systems (CHEF).

PFGE was initially used as a molecular tool to study the biodiversity of *C. botulinum* type E (group II) in sediments, fish and fishery products from Finland (Hielm

et al., 1998a-c; Hyytiä *et al.*, 1999b). The results of these studies as well as those on the diversity of *C. botulinum* group I strains recently published by Nevas *et al.* (2005a), are presented in a preceding section. The usefulness of PFGE as an epidemiological tool was recently demonstrated during investigations of infant and foodborne botulism incidents (Lindström *et al.*, 2004; Johnson *et al.*, 2005; Nevas *et al.*, 2005b). Korkeala *et al.* (1998) were the first to use PFGE during an outbreak investigation involving a commercial whitefish product, however, due to failure to recover a type E isolate from the clinical specimens they were unable to link the food source to the patients. Lindström *et al.* (2004) later traced the food source of the first domestic case of botulism in Finland by comparing the PFGE patterns of isolates recovered from suspect whitefish eggs eaten as a meal condiment, with those isolated from the gastric content and feces of the patient. Interestingly, the authors simultaneously recovered a type B strain from the gastric content of the patient, but this isolate had no clinical significance since type B neurotoxin was not detected in any of the investigated specimens, and its PFGE pattern was clearly different from the outbreak strain that belonged to group II. Franciosa *et al.* (2004) used PFGE for the molecular characterization of group I *C. botulinum* types A, Ab (DNA harboring a BoNT/B gene which is expressed), and A(B) (DNA harboring a BoNT/B gene which is unexpressed) strains, and found that PFGE clusters correlated well with BoNT/A gene cluster types by PCR. More recently, PFGE was used in the investigation of two infant botulism incidents, one involving household dust and the other potentially associated with the consumption of commercial infant formula (Johnson *et al.*, 2005; Nevas *et al.*, 2005b). The incident associated with household dust was unique, as the PFGE patterns of spores of *C. botulinum* type B recovered from a vacuum cleaner were identical to that of the intestinal isolate from an infant that apparently died from sudden infant death syndrome. This lead the authors to

suggest that airborne spores present in household environments may cause infant botulism (Nevas *et al.*, 2005b).

1.6.2. PCR-based DNA typing methods

Randomly amplified polymorphic DNA (RAPD) is a PCR procedure that amplifies an unspecified DNA sequence using a single arbitrary primer which anneals two complementary binding sites separated by close distance (Tyler *et al.*, 1997). Amplification is conducted at low temperature, which allows for multiple mismatches (Venugopal *et al.*, 1993). The source of polymorphism between RAPD patterns of strains of the same species could be related to various genetic events or differences that have been well described by Lamboy (1994) and others (Berg *et al.*, 1994). The conditions of amplification must be rigorously constant and well optimized to obviate analytical variation. Variation in Mg^{2+} concentration, primer/DNA template ratio, annealing temperature, or even the use of a different type of thermocycler can cause variability in RAPD patterns from run to run (Meunier and Grimont, 1993; Muralidharan and Wakeland, 1993; Tyler *et al.*, 1997).

A limited number of studies have used RAPD PCR for typing strains of *C. botulinum* group I and group II (Hyytiä *et al.*, 1999a,b). The discriminatory power of the RAPD technique was not similar for group I and group II strains. Using the same primers (OPJ-6 and OPJ-13), strains of *C. botulinum* group II were differentiated at the strain level, while strains belonging to group I could only be differentiated at the serotype level by RAPD analysis. The authors suggested that the lack of discrimination for group I strains could be attributed to either a low genetic diversity among group I strains or an inadequate primer selection. Interestingly, a serotype-specific fragment of

about 1300 bp was observed for all type E strains with primer OPJ-13 (Hyytiä *et al.*, 1999a).

Repetitive element sequence-based PCR (rep-PCR) is also a PCR-based DNA method that has been used for subtyping strains of *C. botulinum* group I and group II (Hyytiä *et al.*, 1999a). As opposed to RAPD PCR, the rep-PCR technique uses two opposing primers that target specific repeated motifs of the genome and amplify sequences of spacer fragments that separates the motifs under high stringency conditions (Struelens, 1998). These repeated motifs are highly conserved and are present in various copy numbers and locations throughout the bacterial genome depending on the species or strain (Farber, 1996). Several highly conserved intergenic repetitive short sequences of 33 to 154 bp have been used as a target for PCR amplification such as the repetitive extragenic palindromic (REP) elements, the enterobacterial repetitive intergenic consensus (ERIC), or the BOX element (Tyler *et al.*, 1997). The technique is known to be reproducible, but has not been found as discriminatory as RAPD for differentiating strains of *C. botulinum* group I and group II using different REP and BOX primers (Hyytiä *et al.*, 1999a). The presence of small number of generated fragments with REP primers (REP1R-Dt and REP2R-Dt) lead the authors to suggest that the genomes of *C. botulinum* strains do not contain large copy numbers of REP sequences. Nevertheless, the use of REP primers in this study allowed the differentiation of *C. botulinum* group I strains at the serotype level and down to the strain level for types B and E strains belonging to group II. In comparison, the use of primer BOXAIR could only differentiate strains of *C. botulinum* at the group level, while primer RW3A did not yield any DNA amplified fragments that could be detected on agarose gels, even with the use of a low annealing temperature during PCR amplification (Hyytiä *et al.*, 1999a).

Amplified fragment length polymorphism (AFLP) is a relatively recent PCR-based DNA typing method for the molecular characterization of genomic DNA (Lukinmaa *et al.*, 2004). The technique was developed by Vos *et al.* (1995) and later applied for epidemiological typing of outbreak strains of clostridial species such as *C. perfringens* (McLauchlin *et al.*, 2000) and *C. difficile* (Klaassen *et al.*, 2002). The AFLP procedure is relatively easy to perform, reproducible, and less time consuming than PFGE, allowing for the characterization of genomic DNA within 24 h (Klaassen *et al.*, 2002). In brief, the whole-genome DNA is simultaneously digested with a set of two restriction endonucleases and the restricted fragments are tagged with specific adapters. The labelled fragments are amplified with selective primers complementary to the adapters and adjacent nucleotides and the fragments are separated by electrophoresis (Struelens, 1998; Lukinmaa *et al.*, 2004). Keto-Timonen *et al.* (2005) recently applied the AFLP technique to *C. botulinum* and showed that it was able to clearly differentiate group I from group II strains, which is in agreement with previous studies that demonstrated that these two groups are phylogenetically distant (Hutson *et al.*, 1993; Collins and East, 1998). The authors showed that AFLP could reproducibly differentiate strains of *C. botulinum* at the strain level, including those untypeable by PFGE due to extensive DNA degradation. However, due to variation in lane or background intensities, the authors recommended the use of an internal reference strain to determine the cutoff value that defines the AFLP type, which is based on the similarity level among replicates of AFLP patterns of the reference strain.

1.6.3. Ribotyping

Ribotyping is a genotyping technique that uses restriction fragments of ribosomal RNA genes for differentiation of organisms. The rRNA genes are highly conserved and

most prokaryotic genomes contain multiple operon copy numbers varying from one to 15 copies located at different positions on the chromosome, which allow interspecies and intraspecies differentiation (Klappenbach *et al.*, 2001; Lukinmaa *et al.*, 2004). Various ribotyping methods have been developed for different bacterial pathogens and have been reviewed by Farber (1996). In brief, chromosomal DNA is cleaved with a specific restriction endonuclease and DNA fragments are separated by agarose gel electrophoresis. The DNA fragments are then transferred to a nylon or nitrocellulose membrane, hybridized to a labelled cDNA probe from *Escherichia coli* 16S and 23S rRNA and detected by colorimetry. The technique is relatively labour intensive and requires analytical skills to achieve reliable results (Dalsgaard *et al.*, 1999). The technique was automated in a stand-alone ribotyping instrument, the RiboPrinter, which can process all the steps from cell lysis to image analysis within 8 h, as compared to 4 to 5 days for manual ribotyping methods (Bruce, 1996). The use of ribotyping as a molecular tool in outbreak investigations of botulism has not been reported yet, but the technique has been assessed for the differentiation of *C. botulinum* group I and II strains. Using manual ribotyping, Hielm *et al.* (1999) were able to clearly differentiate strains of *C. botulinum* group I from strains of group II using either EcoRI or HindIII restriction enzymes. The authors suggested that ribotyping was as discriminatory as PFGE and that a cut-off value between 30 to 40% Dice similarity is indicative of species divergence in ribotyping. In contrast, Skinner *et al.* (2000) found that certain type B strains from group I and group II could not be distinguished by automated ribotyping when using the restriction enzyme EcoRI. This apparent discrepancy with regards to the discriminatory power of ribotyping has been discussed by Hielm *et al.* (2001).

1.6.4. Fingerprinting analysis

DNA fragment patterns can be classified into groups or clusters based on visual examination. PFGE patterns with only a one or two fragment difference (consistent with a single genetic event) can be further classified into a subcluster according to the criteria established by Tenover *et al.* (1995) for the interpretation of PFGE patterns in clinical microbiology. The PFGE pattern of an outbreak strain are expected to be indistinguishable from the PFGE patterns of epidemiologically linked strains of clinical or food origin, but random genetic events such as point mutations, or insertions and deletions of DNA, can alter the PFGE patterns during the course of an outbreak and complicate the interpretation. There are four categories of relatedness that have been proposed by Tenover *et al.* (1995) for the comparison of PFGE patterns of isolated strains during an outbreak investigation: i) indistinguishable (no fragment difference from the outbreak pattern), ii) closely related to the outbreak pattern (one genetic event, two to three fragments difference from the outbreak pattern); iii) possibly related to the outbreak pattern (two genetic events, four to six fragments difference from the outbreak pattern); and iv) unrelated (≥3 genetic events, ≥7 fragments difference from the outbreak pattern). Using the same criteria, Kristjánsson *et al.* (1994) classified both PFGE patterns and ribotypes of *C. difficile* strains in subgroups and any patterns that differed by multiple changes not consistent with a single genetic event, were assigned to different groups.

Changes in RAPD fingerprint patterns are not interpreted in relation to genetic events such as those described for PFGE. Differences of a single faint band between two isolates are not significant as they are frequently not reproducible. For these reasons, Samore *et al.* (1996) have not used a subgroup classification in the assignment of groups of arbitrarily-primed polymerase chain reaction (AP-PCR) patterns of *C.*

difficile. Due to the lack of reproducibility, the authors have limited the analysis to PCR products of the same amplification set electrophoresed in the same gel to obtain consistent interpretations. Fragments that differed in intensity, but were indistinguishable in size, were considered the same. Cohen *et al.* (2001) have recommended the use of uniform criteria for the interpretation of DNA fragment patterns, which was found particularly critical for AP-PCR. For instance, if faint bands are not considered on a given run, they should not be analyzed in any subsequent run. In some cases, the resolution of the entire fragment pattern may be poor in one gel and hence all faint bands must be weighed accordingly. Silva *et al.* (1994) grouped AP-PCR fragment patterns of *C. difficile* if they had a general common fragment pattern and then they were sub-divided if they displayed unique fragment patterns in addition to the general pattern. Hyytiä *et al.* (1999a) included the faint fragments from RAPD fingerprints of *C. botulinum* group I and group II in the analysis, only if they were detected reproducibly from two isolates of the same strain. RAPD fingerprints were all examined visually and assigned into different subtypes if a difference of two or more fragments was observed.

Regardless of typing methods, Cohen *et al.* (2001) have used 90% similarity between DNA fragment patterns as the cutoff point for determining if two isolates of *C. difficile* are highly related or clonal. Hielm *et al.* (1998b) had clustered PFGE patterns yielded from SmaI (or XmaI) digestions of *C. botulinum* group II DNA at a similarity level of 76% based on three fragment differences. Similarly, Hyytiä *et al.* (1999b) used a three-band difference as a basis for clustering SmaI (or XmaI) and XhoI PFGE patterns of *C. botulinum* type E at 96% and 90% similarity, respectively.

1.7. Rationale and hypothesis of this study

The sources and contamination routes of botulinum spores in marine mammal meat are unknown. Following the findings of spores of *C. botulinum* type E in the coastline environment and marine species of Alaska (Miller *et al.*, 1972; Miller, 1975), it was proposed that contamination of marine mammal meat would occur from direct exposure to exogenous spores from the environment or endogenous spores originating from the intestinal tracts of animals during the butchering process. The risk of contamination of marine mammal meat possibly depends on the distribution and levels of spores encountered in shoreline areas of various coastal villages where marine mammals are commonly butchered. The link between botulism outbreaks and environmental sources of *C. botulinum* contamination has not as yet been demonstrated by molecular epidemiological tools.

The modes and risks of contamination of marine mammal meat from potential environmental sources of *C. botulinum* type E throughout the entire butchering and meat preparation process have not been assessed under field conditions. It is believed that meat from harvested marine mammals may become contaminated with spores when the hunters place the meat directly on coastal rocks or with spores from the intestinal contents, if handling precautions are not taken during evisceration or when the intestines are emptied with bare hands, which may lead to various cross-contamination scenarios. It is expected that the risk of contamination from direct contact with coastal sediments, particularly at low tide in areas such as a bay, or the mouth of river rich in organic matter, would be higher than contamination from salt water or from direct contact with shoreline rocks above the water line. Identification of high-risk handling practices in the field or the critical points where contamination is likely to occur, would lead to the development of control measures that could minimize environmental contamination

with spores, and likely reduce the probability of toxigenesis in aged marine mammal products.

Although we know that environmental spores of *C. botulinum* that naturally contaminate raw marine mammal meat may germinate, outgrow, multiply, and produce toxin in traditional aged products, little is known about the conditions or factors that lead to these events. The safe preparation of aged marine mammal products called *igunaq* requires knowledge and experience, but under certain conditions, the *igunaq* can turn toxic. It is believed by the Inuit that the traditional aging methods are safer than the modern methods which use various containers and storage locations to age raw meat into *igunaq*. Based on the experience of the *igunaq* producers, the use of plastic bags as containers, high temperature, lack of fat, and the absence of air circulation around the preparations are potential factors that may lead to the production of poisonous *igunaq* (Weetaluktuk, 1997). The potential of each risk factor or their combined effects to induce the production of botulinum toxin in various *igunaq* preparations has not yet been determined under laboratory conditions.

Aged marine mammal products are produced and consumed in most Northern villages of Nunavik. However, most of the reported *C. botulinum* type E outbreaks in this region occur in only three villages of southern Ungava Bay (Dolman, 1974; Hauschild and Gauvreau, 1985; Austin, 1996; Austin *et al.*, 1997; Austin *et al.*, 1999). Our hypothesis is that the contrasting and very different incidence rates of type E botulism observed among villages of Nunavik depend on the levels of spores naturally found in the environment where the marine mammals are butchered. We are also testing the hypothesis that the probability of toxigenesis in *igunaq* is not influenced by aging methods, whether traditional or modern. The probability of toxigenesis in meat products can be estimated by conducting inoculation studies, with the knowledge of the natural

contamination level of raw meat and the number of spores required for a single spore to become toxic in a specific product (Hauschild, 1982).

1.8. Objectives

This research project has been designed to identify the sources of *C. botulinum* contamination in seal meat and to determine the factors that influence its growth and toxin production in traditional *igunaq* preparations. Results of these studies will be integrated and analyzed to establish the risk of botulism under multiple conditions. Specifically, the objectives were:

a) Identify potential sources of contamination of seal meat with *C. botulinum* type E. This will be achieved by conducting field surveys on the distribution, levels, and genetic diversity of type E strains in seals harvested in Nunavik and in the coastline environment where they are commonly butchered.

b) Identify the routes of contamination of seal meat with spores of *C. botulinum* type E by conducting interviews with igunag producers, field observations of the butchering and meat preparation process, and microbial source tracking studies.

c) The third objective was to study the molecular epidemiology of foodborne type E botulism in the Canadian Arctic and determine the frequency and distribution of outbreak subtypes.

d) Determine the factors that influence the lag phase of *C. botulinum* type E toxigenesis in aged seal meat preparations by conducting inoculation studies using different levels of spore inoculum, storage temperature, atmosphere conditions and preparation methods.

e) Develop a risk profile of human type E botulism from eating aged marine mammals in Nunavik and identify knowledge gaps for a quantitative risk assessment.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Enrichment media and growth conditions

Prior to testing field samples, two pre-treatments and three media were tested to determine which combination of pre-treatment/medium was the most sensitive and reliable to detect low levels of spores in an environmental matrix. Cooked meat medium (CMM) and special peptone-peptone-glucose-yeast extract (SPGY), two common broths used at the BRS for enrichment cultures and toxin production (Austin and Blanchfield, 1996), were tested along with SPGY-containing trypsin (SPGYT). Trypsin (0.1%) was added to cultures prior to incubation to prevent potential inhibition by nontoxigenic *C. botulinum* type E-like microorganisms producing boticin E, a bacteriocin that kills vegetative cells and inhibits spore germination of *C. botulinum* group II strains (Kautter *et al.*, 1966; Sugiyama *et al.*, 1972). CMM was prepared in dilution bottles by adding 7.8 g of CMM (Oxoid Company, Nepean, Ontario, Canada) to 60 mL of double-distilled water. SPGY broth was prepared in a large flask with 5% special peptone (Oxoid LTD, Basingstoke, England), 0.5% peptone (Bacto™, Becton, Dickinson and Company, Sparks, Maryland, USA), 2% yeast extract (Bacto™, Becton, Dickinson and Company), 0.4% glucose, and 0.1% sodium thioglycollate, with the pH adjusted to 7.2. All broths were autoclaved and stored in an anaerobic chamber with an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 until use.

A pool of seawater samples (1450 mL) was prepared by combining 50 mL of each of the 29 shoreline water samples collected along the Nunavik coast during the field survey of fall 2000. From a freshly-prepared stock suspension of 1000 spores/mL containing four laboratory strains of *C. botulinum* type E (E Russ, 8550, Gordon, and Bennett), two working suspensions were prepared in concentrations of 0.1 and 1 spore per mL, respectively. The stock suspension was prepared using the following volumes from botulinum spore suspensions (spores/mL) stored at –86°C: 4.2 µL of 6×10^6 E

Russ, 22.7 μL of 1.1×10^6 Gordon, 5.8 μL of 4.3×10^6 Bennett, and 6.6 μL of 3.8×10^6 8550, each corresponding to a total of 2.5×10^4 botulinum spores, which were combined and suspended in 100 mL of the pooled seawater. The stock suspension (100 μL) was plated in duplicate onto McClung Toabe agar medium at the time of inoculation to verify its concentration. Lipase-positive colonies were counted under fluorescent light and the stock concentration was expressed as the number of colony forming unit (cfu) per mL.

Duplicate seawater samples (100 mL) were spiked with 10 or 100 type E spores and subjected or not (untreated) to an alcohol pre-treatment selective for spore-forming bacteria. The alcohol treatment consisted of immersing (or mixing) a sample in 50% ethanol for 1 h at room temperature. Samples were then filtered through a 0.22 μM nitrocellulose membrane (Millipore Canada LTD., Nepean, Ontario) to adsorb the spores and the membrane was incubated. All cultures were incubated at room temperature for 7 days in an anaerobic chamber with an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 and then assessed for toxicity using the mouse bioassay.

All enrichment broth techniques were able to detect *C. botulinum* spores in samples inoculated with 100 spores, regardless of the pre-treatment or medium (Table 2.1). However, duplicate samples inoculated with 10 spores were both positive for the alcohol-treated samples cultured in SPGYT and for the untreated samples cultured in CMM and SPGY, while only one sample of each duplicate cultured using the other combinations of pre-treatment and medium was positive. Thus, none of the pre-treatments and media tested were conclusively better at either inoculation level. SPGYT was generally preferred over CMM as it can be prepared in large volumes, is easily dispensed in culture bottles, and can be used with trypsin to counteract the inhibiting

Table 2.1. Sensitivity of CMM, SPGY, and SPGYT with and without 50% ethanol pre-treatment for the recovery of *C. botulinum* type E spores in seawater

Spore loads	CMM[a]		SPGY[b]		SPGYT[c]	
	Untreated	**50% ethanol**	**Untreated**	**50% ethanol**	**Untreated**	**50% ethanol**
10	2/2[d]	1/2	2/2	1/2	1/2	2/2
100	2/2	2/2	2/2	2/2	2/2	2/2

[a]CMM: cooked meat medium.
[b]SPGY: special peptone-peptone-glucose-yeast extract.
[c]SPGYT: special peptone-peptone-glucose-yeast extract plus trypsin.
[d]Number of positive samples/total number of samples.

effect of boticin E if the latter is ever produced in cultures of environmental samples originating from the Canadian Arctic.

2.2. Toxin assays and serotyping

Toxicity of cultures was assessed using the standardized mouse bioassay performed at Health Canada (Austin and Blanchfield, 1996). All 250-mL culture bottles were centrifuged at 18,500 × *g* (4°C) for 10 min to separate the liquid from the solid phase. Approximately 10 mL of supernatant was aspirated and filtered-sterilized through a 0.45μm syringe filter (Whatman®, Clifton, New Jersey) into 15-mL polystyrene tubes and kept refrigerated until tested by the mouse bioassay. To minimize the number of mice required for the assay, supernatants from three cultures were combined into a composite of equal volumes and diluted 1:2 (or 1:6 for an individual sample) with gelatin phosphate buffer (GPB). The inoculum was trypsinized just prior to inoculation by incubating the inoculum with 0.1 volume of 1.4% trypsin (1:250) at 37°C for 1 h to allow for the activation of type E botulinum toxin. The trypsinized inoculum was injected intraperitoneally (using 0.75 mL of inoculum) into two CF1 mice (Charles River Canada, Saint-Constant, Quebec), which were then closely monitored for clinical signs of botulism over a three-day period. Mice showing typical signs of botulism such as a pinched waist and laboured breathing, were euthanized using carbon dioxide gas.

Confirmation of the *C. botulinum* type E serotype was performed by colony blot immunoassay and by seroneutralization test using monovalent botulism antitoxin E (Aventis, Toronto, Ontario). Toxic broth culture was streaked on *Clostridium botulinum* Isolation (CBI) agar for the detection of colony producing BoNT/E by colony blot immunoassay using affinity-purified antibodies (see complete procedure below). In the

event of a negative type E colony blot immunoassay, or when mice were not protected with antitoxin E, the toxic supernatant was successively neutralized with monovalent antitoxin B (in-house rabbit botulism antitoxin B) and A (Serum antibotulinique A Pasteur, Institut Pasteur, Paris, France). The in-house polyclonal rabbit antitoxin B was produced according to the method of Harlow and Lane (1988), using purified botulinum toxoid type B (Wako Pure Chemicals Industries, Chuo-Ku, Osaka, Japan) and aluminium hydroxide adjuvant (Alhydrogel™, Cedarlane Laboratories Limited, Hornby, Ontario, Canada). Regardless of the monovalent antitoxin type, neutralization of toxic supernatants was performed by incubating 0.75 mL of culture supernatant with 0.1 volume of antitoxin at room temperature for 45 min, after which one mouse each was injected with and without neutralized inoculum and observed for signs of botulism.

2.3. Isolation of *Clostridium botulinum* type E

2.3.1 Back-up enrichment

From each positive culture supernatant tested by the mouse bioassay, approximately 2 to 4 mL of the corresponding enrichment broth was transferred into a CMM culture. Tubes were incubated at least seven days at room temperature in the anaerobic chamber for sporulation, and then kept at 4°C for long-term storage until the strain was isolated and purified (Figure 2.1). The selection of CMM as a sporulation medium was based on the examination of different enrichment broth cultures inoculated with 100 spores of a cocktail composed of four laboratory type E strains (E Russ, 8550, Gordon, and Bennett) and incubated as previously described in section 2.1. After being gently mixed, a loopful of culture suspension was transferred onto a slide and examined at 400× magnification under a phase-contrast microscope (Zeiss West Germany, Carl Zeiss Canada Ltd., Toronto, Ontario). The proportion of spores among vegetative cells

Figure 2.1. Isolation procedure for *C. botulinum* type E in environmental samples. Each sample was subjected to three cultivation procedures involving three pre-treatments (50% ethanol, heated at 60°C for 20 min, untreated) and then cultured in special peptone-peptone-glucose-yeast extract (SPGY) broth containing 0.1% trypsin (SPGYT) for 7 days at room temperature under anaerobic atmosphere (10% H_2, 10% CO_2, and 80% N_2). The culture supernatants were then assayed for BoNT/E using the mouse bioassay. An inoculum of each positive supernatant was transferred into a cooked meat medium (CMM) to allow production of spores. This enrichment broth was used as a back-up when no isolates were recovered during the isolation procedure. A positive SPGYT broth culture was streaked on a selective medium, *Clostridium botulinum* Isolation (CBI) agar, for detection of colonies producing BoNT/E by colony blot immunoassay using affinity-purified antibodies followed by IgG-AP and visualized with BCIP/NBT. Three well-separated positive colonies were picked and streaked onto CBI plates and assayed for BoNT/E by colony blot immunoassay. Each isolate was then purified using two sets of non-selective egg-yolk McClung Toabe agar (MT) before being confirmed by seroneutralization (using monovalent botulism antitoxin E) following enrichment in CMM. The entire isolation procedure required a minimum of 34 days to complete.

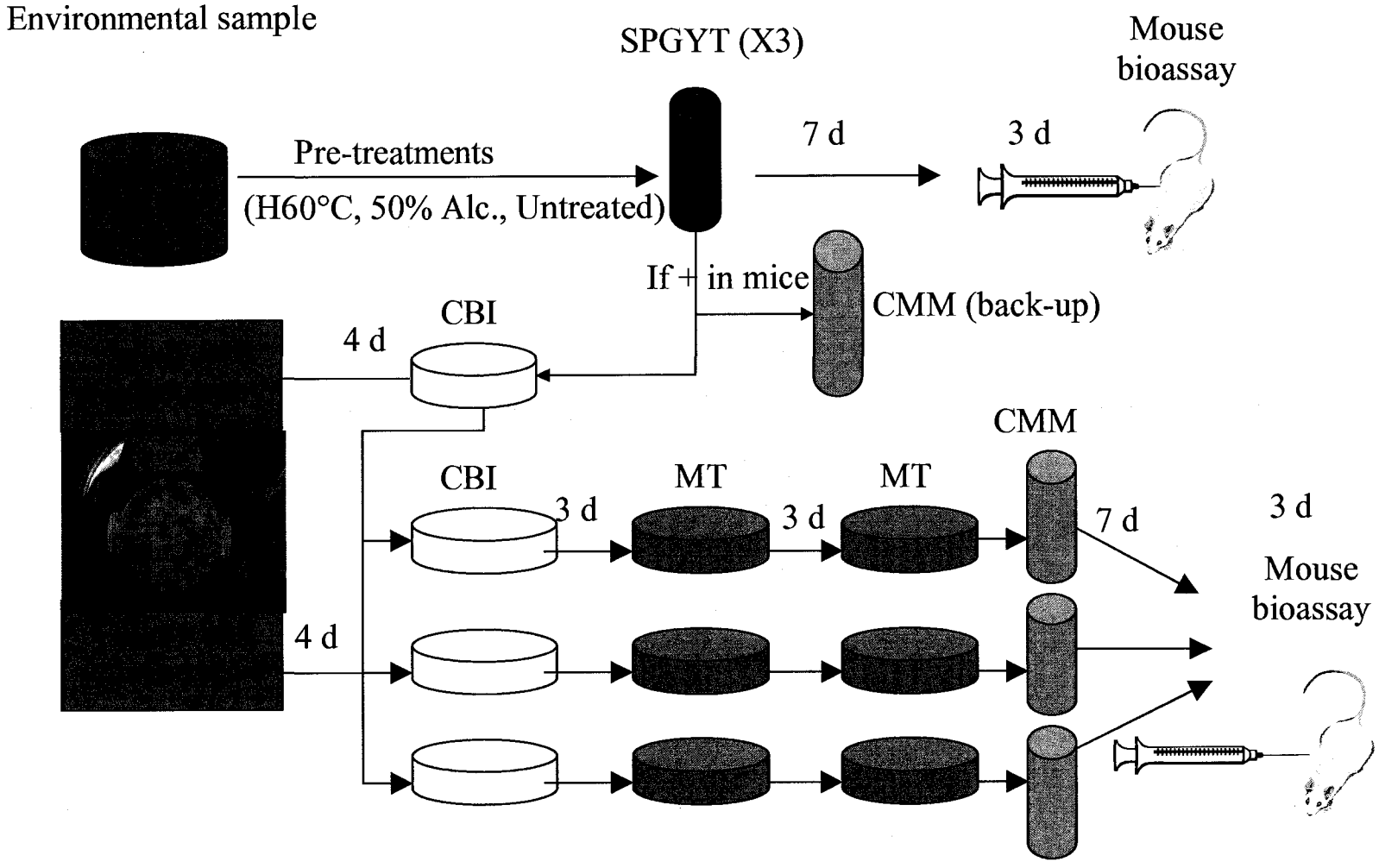

Environmental sample
SPGYT (X3)
Mouse bioassay
Pre-treatments
(H60°C, 50% Alc., Untreated)
7 d
3 d
If + in mice
CMM (back-up)
CBI
4 d
CMM
CBI
MT
MT
3 d
3 d
7 d
3 d
Mouse bioassay
4 d

Table 2.2. Proportion of spores among vegetative cells in enrichment broth cultures of seawater inoculated with 10^2 spores of *C. botulinum* type E

Sample	CMM[a]		SPGY[b]		SPGYT[c]	
ID	Untreated	50% ethanol	Untreated	50% ethanol	Untreated	50% ethanol
A	70%	90%	<10%	<5%	<5%	<10%
B	70%	50%	<10%	<5%	<5%	<10%

[a]CMM: cooked meat medium.
[b]SPGY: special peptone-peptone-glucose-yeast extract.
[c]SPGYT: special peptone-peptone-glucose-yeast extract plus trypsin.

present in the mixed cultures was estimated by examining at least three fields per slide. CMM with and without alcohol pre-treatment had the highest sporulation levels, i.e., 70% on average, whereas the other enrichment broth techniques yielded less than 10% of spores per field examined (Table 2.2). Thus, a CMM culture was inoculated with all positive SPGYT cultures to avoid losing viable strains required for isolation and typing.

2.3.2. Colony blot immunoassay

Isolation of *C. botulinum* type E from inoculated CMM or SPGYT broth was performed using colony blot immunoasssay based on the method of Goodnough *et al.* (1993). Detection of type E botulinum toxin is achieved by using an affinity-purified rabbit antibody produced in our laboratory. The production and the affinity purification of the anti-toxin E antibodies have been recently reported by Cadieux *et al.* (2005) and are briefly described in the slot blot immunoassay section (Chapter 3). A loopful of enrichment broth culture was initially streaked onto a selective CBI medium (Dezfulian *et al.*, 1981) prepared with the following ingredients and antibiotics: 7.5% McClung Toabe agar base (Bacto™, Becton, Dickinson and Company), 0.5% yeast extract (Bacto™, Becton, Dickinson and Company), 5% egg yolk suspension (one egg yolk dissolved in 0.85% saline), 1% D-cycloserine (Sigma®, Sigma-Aldrich Canada Ltd., Oakville, Ontario), 1.9% sulfamethoxazole (Sigma®, Sigma-Aldrich Canada Ltd.), and 0.1% trimethoprim lactate (Sigma®, Sigma-Aldrich Canada Ltd.). A second CBI plate was streaked using four to six streaks from the master CBI plate and both were incubated anaerobically for 3-4 days at room temperature to allow for toxin production.

A nitrocellulose membrane (82 mm diameter and 0.45 μm pore size) (Protran®, Schleicher & Schuell, Keene, New Hampshire, USA), was carefully applied to the surface of the second CBI plate containing well isolated colonies and less background

flora. The membrane was rinsed with distilled water to remove any adhering colony material after being in contact with surface colonies for 1 h in the anaerobic chamber at room temperature to capture proteins. The remaining protein-binding sites on the membrane were blocked with 2% skim milk powder dissolved in TBS (0.05 M Tris-HCl, 0.2 M NaCl, pH 7.4) for 1 h on a rocker at room temperature to avoid nonspecific binding with primary antibodies and detection reagents in subsequent steps. The blocking solution was then discarded and replaced with 1% skim milk-TBS containing affinity-purified rabbit antibodies against type E botulinum neurotoxin diluted to 1:200. The primary antibody solution was incubated at room temperature for 1 h with rocking, and unbound antibodies were removed by three 5-min washes in 25 mL TBS.

The membrane was then incubated for 30 min (rocking at room temperature) with goat anti-rabbit alkaline phosphatase labelled immunoglobulin G antibodies (IgG-AP) (Cedarlane laboratories Ltd, Hornby, Ontario) diluted to 1:4000 in 1% skim milk-TBS. The secondary antibody solution was then discarded and the membrane was rinsed three times as previously described to remove unbound antibodies. The membrane was incubated in alkaline phosphatase buffer (0.1 M Tris, 0.09 M NaCl, 0.15 M $MgCl_2 \cdot 6H_2O$, pH: 9.5) for 5 min to remove residual TBS. The development buffer was discarded and the membrane was immersed in the substrate solution containing 4 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4.1 mM nitrobluetetrazolium (NBT) in alkaline phosphatase buffer for 5-10 min, with rocking until the background colour began to appear. The reaction was stopped by rinsing the membrane with distilled water. Blots were then air-dried on a large paper filter in the dark. Positive blots were characterized by the presence of dark purple dots of various sizes, which were clearly distinguished from the background colour (Figure 2.2).

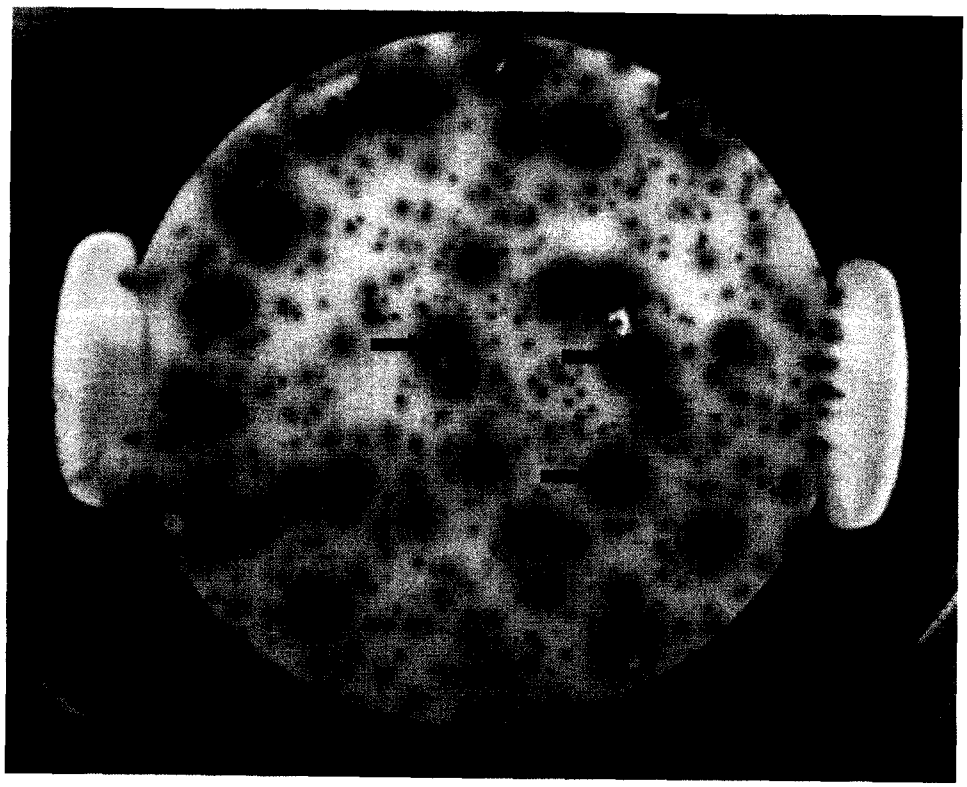


Figure 2.2. Colony immunoblot of BoNT/E adsorbed on nitrocellulose membrane. Colonies were grown on a CBI plate and toxin was detected using affinity-purified antibodies followed by IgG-AP and visualized with BCIP/NBT. Arrows indicate three colonies out of 38 which presumably had produced BoNT/E.

2.3.3. Purification and long-term storage of pure cultures

One well isolated colony identified by colony blot immunoassay was streaked onto two CBI plates and grown at room temperature for 3-4 days in the anaerobic chamber. Another colony blot immunoassay was then performed to clearly separate positive colonies from any potential contamination transferred during colony picking and hence avoiding accidental purification of lipase-positive non-toxic bacteria. A positive colony identified with the second blot was streaked onto a non-selective egg-yolk medium (McClung Toabe agar), which was then incubated anaerobically for 3-4 days at room temperature. A second purification was performed similarly on McClung Toabe before the purity of the isolate was confirmed by assessing its toxicity in mice. Following confirmation, fresh colonies produced on three McClung Toabe plates were picked and stored in Microbank™ (Pro-Lab Diagnostics, Richmond Hill, Canada) vials at -86°C, according to the manufacturer's instructions.

CHAPTER 3

SOURCES, LEVELS AND DISTRIBUTION OF *CLOSTRIDIUM BOTULINUM* TYPE E IN THE ARCTIC ENVIRONMENT

3.1. Introduction

The geographical distribution of *C. botulinum* type E has been extensively studied in the coastal waters of Alaska, Greenland, and Scandinavian countries (Miller, 1975; Huss, 1980; Hielm *et al.*, 1998c) but not along the Canadian Arctic coastline. Bacterial contamination of marine mammal meat from the coastal environment could occur during butchering, particularly when tissues are cut and placed in direct contact with shoreline soil or rocks. In Alaska, spores of *C. botulinum* type E are widely distributed along the coast and were isolated from beach soil near locations where botulism outbreaks had been documented (Miller, 1975). Shoreline water collected along these beaches also contained spores, which could contaminate meat if the water was used to rinse the meat.

Another potential source of spores includes the marine mammal itself. Fish and shellfish are known to be contaminated with *C. botulinum* type E spores (Craig *et al.*, 1968; Houghtby and Kaysner, 1969; Miller, 1975). Consumption of fish and shellfish by marine mammals would lead to contamination of the intestinal tract with *C. botulinum*. Miller (1975) has reported the presence of *C. botulinum* type E spores in one beluga whale harvested in Alaska. Therefore, bacterial contamination of marine mammal meat with intestinal materials could represent a source of contamination for *C. botulinum* type E if handling precautions are not taken during evisceration. No studies have yet examined the skin of marine mammals as a potential source for contamination of *igunaq* preparations with *C. botulinum* type E. The skin of walrus and seals harvested in Nunavik is commonly used as a chamber for aging meat and fat into *igunaq* (Weetaluktuk, 1997), and could potentially introduce spores.

Identification of environmental and endogenous sources of *C. botulinum* type E spores are necessary to assess the contamination risks and determine the transmission

routes occurring at the butchering sites of regions where foodborne botulism remains a public health problem. The objectives of this study were i) to determine the geographical distribution and levels of *C. botulinum* type E in the coastal environment of Nunavik where botulism is endemic and ii) identify shoreline areas at risk for butchering marine mammals. The prevalence of type E spores in harvested seals was also investigated as a potential endogenous source of bacterial contamination. Preliminary results from the field survey showed a distinct cluster of shoreline butchering sites located in southern Ungava Bay that tested positive for *C. botulinum* type E. This area receives discharges from large rivers draining areas of large land masses. To determine the influence of the freshwater and terrestrial environments on the occurrence of *C. botulinum* type E in the coastal environment, the Koksoak River, which is the largest freshwater river flowing into the south end of Ungava Bay, was also included in the survey.

3.2 Materials and Methods

Study areas. Nunavik is situated north of the 55th parallel in the Arctic region of Quebec. It is a vast territory of 507 000 km^2 bordered by Hudson Bay to the west, Hudson Strait to the north, and Ungava Bay and Labrador to the east (Figure 3.1). The traditional fishing and hunting activities are practiced by native populations (primarily Inuit) of 14 isolated Northern villages scattered on the Hudson and Ungava Bay coasts. The local Hunting, Fishing, Trapping Associations (HFTAs) from each village of Nunavik participated in the location of traditional sites used for butchering bearded seals (*Erignathus barbatus*) and ringed seals (*Phoca hispida*) during the summer and fall. HFTA representatives were asked to mark on 1:250000 maps, the two most frequently used butchering sites on the Nunavik coastline. The sites were visited between October 2 and 12, 2000 using the Department of Fisheries and Oceans's helicopter, starting from Kuujjuaraapik in the south-eastern Hudson Bay, and ending in Kuujjuaq, south of Ungava Bay. The selection of the butchering area was based on the type of ground surface present on the shoreline, its proximity to water, and ease of access by canoes.

Field surveys were also conducted in the environment of the Koksoak River during the summers of 2001 and 2002 near the village of Kuujjuaq, where more than 60% of the botulism cases have been documented in Nunavik since 1964 (BRS, unpublished data). The marine portion of the river extends to Kuujjuaq, approximately 50 km inland from southern Ungava Bay. The area in which there is no tide effect is located 85 km from the mouth of the river. The freshwater portion of the river extends from the 50 kilometer to the 136.5 kilometre mark, where the Caniapiscau and Larch rivers merge.

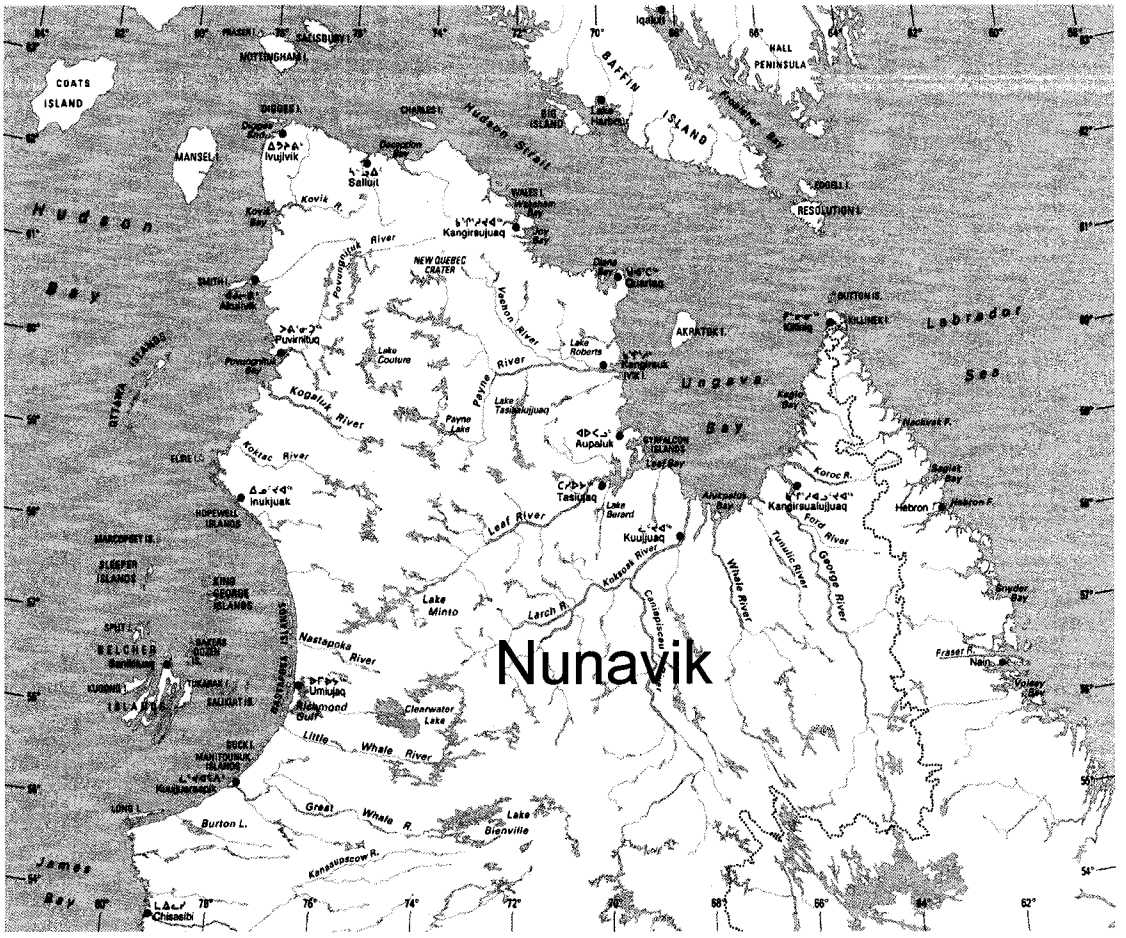


Figure 3.1. Map of Nunavik, Northern Quebec. The region bordered by Hudson Bay to the west, Hudson Strait to the north, and Ungava Bay and Labrador to the east. The estimated population of Nunavik is 10,000 inhabitants, primarily Inuit, distributed in 14 Northern villages along the Hudson and Ungava coasts. Source of map: Cartography Services, Makivik corporation, available at http://www.makivik.org.

Sediment samples were arbitrarily collected in the river and its tributary, downstream and upstream of Kuujjuuaq, for a median distance between sampling sites of 9 km. Waters and inland areas near sampling sites located upstream of Kuujjuaq were also investigated for *C. botulinum* type E.

Location of butchering sites and collection of environmental data. Sampling sites of the survey conducted along the coastline of Nunavik were recorded in latitude-longitude using the helicopter's global positioning system (GPS) (Appendix A; Table A1), while the sampling sites from the Koksoak River were geographically positioned with a portable Ensign GPS™ (Trimble Navigation Ltd, Sunnyvale, California, USA) (Appendix A; Table A2). Tide level was recorded as low, medium or high, and as descending or increasing, based on visual assessment. Water salinity was measured with a hand refractometer (Atago model 2442-W05, Atago Co., Ltd, Bellevue, Washington, USA) calibrated with sterile double-distilled water. Observation of high turbidity in water samples collected at low tide was noted. Substrate type characterizing each shoreline soil and sediment sample was classified as follows: mud, sand, pebbles or cobbles (Appendix A; Table A1). The type of butchering site was recorded as large flat coastal rock or pebble beach, and the presence of slime on their surfaces was also noted in a field book.

Sample collection in the coastal environment. From each butchering site, a square meter area was delineated on a large flat rock or beach surface and gently swabbed in a cross-hatched pattern using two sterile cellulose sponges (3.8 × 7.5 cm) (bio-spo™, Solar biologicals Inc., Ottawa, Ontario) pre-moistened with 10 mL of buffered peptone water. Sponges were put in labelled Whirl-pack™ (Fisher Scientific Ltd, Montreal,

Quebec) bags and kept along with other sample types in coolers with cold packs during the field trip. Near the water line, a shoreline soil sample of about 300 to 500 g and approximately 10 cm deep, was collected using a metal scoop or simply by pushing the 500-mL jar into the ground. Within two feet from the shore, seawater was collected 10 cm under the surface into a 2-L polyethylene wide-mouth bottle using a sweeping motion to avoid potential contamination with gloves. Whenever possible, a sediment core was collected with a hand corer (Wildco® model 2424-A15, Wildlife Supply Company, Saginaw, Michigan, USA) at a depth of approximately 1.5 meters. To avoid cross-contamination between butchering sites, the metal scoop was cleaned with distilled-deionized water and rinsed with 70% ethanol, and sampling gloves discarded. All environmental samples were put in large self-sealing bags to avoid external contamination, sealed and stored in coolers with cold packs pending shipment to the laboratories of Health Canada in Ottawa, where they were kept at 4°C until tested.

Sample collection in the Koksoak River and its tributaries. Sediment samples were arbitrarily collected every 5-10 km from the mouth to the fork of the river (136.5 km) using a Petite Ponar® (Wildlife Supplier Company, Buffalo, New York, USA) grab sediment sampler. Perpendicular to the surface, the grab sampler was lowered slowly until it rested on the bed. The self-closing sampler closes its jaws when the rope slackens. The sampler was then slowly retrieved to minimise the turbulence effect and a plastic tray was put beneath the sampler just as it broke the water surface. The jaws were then gently opened and a sample of 300 to 500 g was transferred from the grab into a 500-mL polyethylene wide-mouth container using a large spatula. For sediments located in small tributaries, samples were collected by simply pushing a 500-mL polyethylene jar into the bed until it filled up. In addition to sediment samples,

terrestrial soil samples of dry earth of 300 to 500 g were collected at locations approximately 25 to 100 meters inland from the main river or its tributaries using a large spatula. A small number of freshwater and seawater samples were also collected according to the described procedure for coastline waters. Any instruments used repeatedly for sample collection were cleaned with deionized-distilled water and rinsed with 70% ethanol between samples, except for the grab sampler and the large tray which were only rinsed in the river.

Collection of intestinal contents from harvested seals. Hunters originating from eight villages of Nunavik participated in the collection of the intestinal tracts and biological data of harvested ringed seals and bearded seals. With the use of sampling kits provided by the Nunavik Research Centre (NRC) in Kuujjuaq, hunters initially (1999 survey) collected the entire intestinal tract of bearded seals and ringed seals, of which four segments of approximately 50 cm from the small intestine and one segment of approximately 25 cm from the recto-colon were dissected at the NRC and assessed by Health Canada for the presence of *C. botulinum* spores. For the 2001 and 2002 samplings, the sampling protocol was modified to include the collection of the entire intestinal contents to increase the probability of detection. Using laboratory gloves, hunters were asked to gather the entire intestinal content into the rectum using their fingers until the content reached a pre-tied end of the intestine near the anus. The other end of the intestine was then tied up with a string to make a small intestinal bag and then removed. The intestinal segments were put into small plastic bags and their contents later transferred to a suitable plastic container at the NRC, where they were kept refrigerated or frozen at -18°C until they were sent to the laboratories of Health Canada.

Collection of skin samples from harvested seals. Through different sampling programs, the NRC staff collected and kept frozen at -20°C a body skin sample of approximately 12.5 × 12.5 cm from a limited number of marine mammals in order to have them tested for the presence of *C. botulinum* spores along with the intestinal samples collected during the 1999 survey. For the 2001 and 2002 samplings, the sampling procedure was standardized and instructions were given to the hunters along with those for collecting the intestinal contents. A 5-cm wide strip of abdominal skin from the navel to the tail was excised by the hunters, put into a plastic bag and sent along with the intestinal samples in cool conditions to the NRC, where they were kept at refrigerated temperature until they were sent to Health Canada's laboratories. Upon reception, samples were stored at 4°C until cultured within two weeks or frozen at -20°C if testing was delayed.

Analysis of freshwater and seawater. The laboratory procedure for detection of *C. botulinum* in seawater essentially involves concentration of spores by filtration, culture of filters in enrichment broth, and verification of toxicity using the mouse bioassay. To reduce the background flora, all water samples were initially cultured with and without an alcohol pre-treatment. A sample volume of 200 mL of water was tested for each treatment. Samples which were culture negative with both of these techniques were then re-cultured using a heat pre-treatment in broth culture. The sample volume was increased to 1000 mL for this last cultivation, which brought the maximum volume tested to 1.4 L per sample. Each 2-L water bottle was well shaken manually for 5-10 seconds prior to pouring approximately 100 mL of seawater into two sterile 250-mL culture bottles. For alcohol-treated samples, 80 g of 95% ethanol (specific gravity:

0.80057) was added to each sample of seawater, mixed vigorously, and left at room temperature for 1 h. Samples were filtered through a manifold system using a 0.22 μM nitrocellulose filter (Millipore Canada LTD.), followed by a rinse of the water holders with sterile double-distilled water to detach any adherent spores. Membrane filters were then aseptically transferred to their bottles and filled with 60 mL of SPGY broth containing 0.1% trypsin (1:250) (SPGYT). The heat-treated samples were processed as previously described for the untreated samples, except that they were heated in SPGY broth at 60°C for 20 min; trypsin was added (4.3 mL of freshly-prepared 1.4% trypsin solution) only when samples had cooled down.

Analysis of surface swabs of coastal rocks. The two cellulose sponges used to swab the surface of the coastal rocks of a typical butchering site were aseptically cut in half with scissors, transferred into sterile 250-mL bottles, and cultured with and without alcohol pre-treatment in SPGYT broth. Sixty mL of SPGY broth was added to one set of half sponges and then trypsinized and incubated as previously described. The other set of half-sponges was immersed in 100 mL of 50% ethanol solution diluted with sterile gelatin-phosphate-buffer (GPB) (0.2% gelatin, 0.4% Na_2HPO_4, pH 6.2) and were left for 1 h at room temperature. The alcohol fraction was filtered through a 0.22 μm membrane filter according to the method used for water samples. Instead of 60 mL, filters and corresponding sponges were cultured in 150 mL of SPGYT broth to dilute the residual alcohol absorbed by the sponge (maximum ethanol concentration estimated in SPGYT broth: 2.6%).

Analysis of sediment, shoreline and terrestrial soil. Most shoreline, sediment and terrestrial soil samples collected during the field surveys were initially cultured in

SPGYT with and without an alcohol pre-treatment. All samples that were culture-negative with both of these techniques were re-cultured using a heat pre-treatment in broth culture at 60°C for 20 min. A sample size of 50 g was used for each treatment, which brought the maximum amount tested per sample to 150 g. As the initial batch of alcohol-treated samples all tested negative, including the positive controls, an additional washing step with GPB (after the alcohol fraction was filtered) was found necessary to reduce the inhibitory effects of residual alcohol.

Analysis of intestinal content samples. The intestinal content samples collected by hunters were similarly cultured, but with some modifications. Each sample was weighed in a double-stomacher bag and stomached with sterile GPB in an approximate 1:1 ratio for 4 min and then transferred into a sterile 250-mL culture bottle. The homogenate was centrifuged at 18,500 × *g* (4°C) for 20 min and the supernatant subsequently discarded. The pellet was divided into three parts and each part was subjected to the three different pre-treatments as earlier described, except that CMM was used instead of SPGYT.

Analysis of seal skin samples. The attached fat from the seal skin was dissected off each skin sample using curved scissors and then cut into three pieces of equivalent size. Pieces were roughly measured for length and width and transferred into individual 250-mL culture bottles and weighed. The skin samples were subjected to three pre-treatments and cultured in SPGYT broth accordingly. Occasionally, up to 140 mL of SPGYT broth was required to ensure that the entire piece was immersed in the enrichment broth.

Field and laboratory quality control. A sample of seawater, shoreline soil, and a swab of a large coastal rock were collected from a selected butchering site in each region (Hudson Bay, Hudson Strait and Ungava Bay) of Nunavik for quality control purposes. All environmental samples were spiked in the field with a known number of *C. botulinum* type E spores to assess the effects of storage and transport on the quality of the samples, the analytical performance of the laboratory procedure, and to test for potential inhibition arising from antagonistic organisms naturally present in the environment. A spore suspension composed of the same laboratory type E strains used for the enrichment broth study (Gordon, E Russ, 8550, and Bennett) was prepared as previously described, except that the spores from the high concentration stocks were suspended in sterile GPB and diluted to make a working spore suspension of 5 spores/μL that was carried into the field in a 2-mL vial under cool conditions. An inoculum of 500 spores was dispensed onto each control bacterial sponge (after a typical coastal rock was swabbed) and into the control shoreline soil samples, whereas each of the 2-L seawater control samples was spiked with 2×10^3 spores for a final concentration of 1 spore per mL. Quality control samples were kept under the same conditions as the non-spiked samples, from field through transport, and during storage in Health Canada's laboratories.

A separate stock suspension was prepared with the same type E strains used for the field quality control samples and diluted to make a working spore suspension of 100 spores per mL. The suspension was used to spike samples of 100 mL of GPB with 10 spores used as the internal quality control samples. Two inoculated GPB controls were filtered through a 0.22 μM nitrocellulose membrane, and cultured with and without alcohol pre-treatment, for each batch of 18 seawater samples tested. A similar spore suspension was also freshly prepared for spiking 15 intestinal and skin control samples

that were cultured according to the described procedures for these sample types. All spore suspensions used for spiking controls were verified by plate counts as previously described.

Quantification of *C. botulinum* type E in environmental samples. All positive sediment, shoreline and terrestrial soil samples from the Koksoak River area and the coastal environment of Nunavik were tested to determine the levels of spores, using a 5-tube most probable number (MPN) procedure combined with toxin assays. Three decimally decreasing amounts (30 g, 3 g, 0.3 g) and five culture tubes for each amount, for a total of 15 tubes per environmental sample, were cultured and assayed for type E botulinum toxin. Each sample was weighed aseptically in a 50-mL conical tube and pre-heated in 30 mL of SPGY broth at 60°C for 20 min. All cultures were then incubated in the anaerobic chamber for 7 days at room temperature to allow for toxin production. Samples were centrifuged at 2,550 × *g* for 10 min and approximately 10 mL of the clear supernatant was aspirated and filter-sterilized through a 0.45 μM filter disc into a 15-mL polystyrene. All samples were stored at –20°C until analysis. Toxicity of culture supernatants was determined by the slot blot immunoassay and confirmed by the mouse bioassay. The mouse bioassay was performed as described in the previous section. To reduce the number of mice only one mouse and two mice were used, respectively, to confirm the positive and negative results obtained by the slot blot immunoassay. The MPN of organisms causing toxicity per gram of sample was determined with the MPN Calculator VB6 version (developed by Mike Curiale and available at http://www.i2workout.com/mcuriale/mpn/index.html), which is a programmed calculator that can compute MPN values with 95% confidence intervals for any combination of results.

Slot blot immunoassay. Sixty untrypsinized culture supernatants were thawed overnight to perform two slot blots on the next day according the method of Cadieux *et al.* (2005), with minor modifications. A set of 30 tubes (1.5 mL) was labelled for each slot blot and two hydrophobic polyvinylidene fluoride (PVDF) membranes used for protein blot (Westran®, Schleicher & Schuell, Keene, New Hampshire, USA) were cut to the appropriate size. Samples were diluted 1:10 with GPB by adding 100 µL of pre-vortexed supernatant in 900 µL of GPB to allow adequate filtration of culture supernatants through the slot blot. A first set of samples was diluted, briefly vortexed, and centrifuged at 16,000 × *g* for 15 min. The remaining samples were diluted, but centrifuged only prior to slot blotting. The membrane was pre-wetted in methanol and soaked in transfer buffer (25 mM Tris, 192 mM Glycine, 20% [v/v] methanol) containing 0.05% sodium dodecyl sulphate (SDS) for 5 min. The membrane was placed inside the slot blot manifold (Hoefer Scientific Instruments, San Francisco, California, United States) and 0.5 mL of harvested supernatant was delivered in each well and filtered by vacuum. To minimize nonspecific binding, the blots were blocked for 1 h with 2% skim milk dissolved in Tris-buffered-saline (0.05 M Tris-HCl, 0.2 M NaCl, pH 7.4) containing 0.1% Tween-20 (TBST). Blots were incubated with affinity-purified rabbit antibodies against type E botulinum neurotoxin diluted 1:200 (100 µL/25 mL) in TBST containing 1% skim milk for 1 h at room temperature and left overnight in the same solution at 4°C. The production of polyclonal rabbit antiserum type E was done according to the method of Harlow and Lane (1988) using purified botulinum toxoid type E (Wako Pure Chemicals Industries, Chuo-Ku, Osaka, Japan) and aluminium hydroxide adjuvant (Alhydrogel™, Cedarlane Laboratories Limited, Hornby, Ontario, Canada). The antiserum was affinity-purified through a sulfhydryl-affinity column

(Pierce, Rockford, Illinois, U.S.A.), coupled with type E toxoid according to the manufacturer's instructions (Cadieux *et al.*, 2005). Blots were washed three times for 5 min each with TBST and incubated with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) diluted 1:5000 in TBST for 1 h at room temperature, followed by three similar washes with TBST.

A detection solution from an ECL Plus Western Blotting kit (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) was prepared and added to each blot according to the manufacturer's instructions. The excess of detection solution was removed using an adsorbent paper and detection of chemiluminescence from blots was immediately performed using a Kodak Imagestation 440 (Eastman Kodak Company, Rochester, New York, USA) equipped with a full-frame-capture charge coupled device (CCD) camera. The images or band intensities were quantitatively analyzed with the Kodak ID Image Analysis Software™ (Eastman Kodak Company, Rochester, New York, USA). Quantification was facilitated by incorporating serial dilutions of a *C. botulinum* type E internal standard made from a culture supernatant of the E Russ strain with known toxicity levels, which was also used as a positive control. Samples showing chemiluminescence (net intensity) greater than 4 mouse lethal dose (MLD) were considered positive. Neutralization of toxin supernatant was performed on a selected number of negative samples by slot blot but positive by mouse bioassay to confirm the presence of type E botulinum neurotoxin in MPN samples.

Statistical analysis. Comparison of spore counts between sediments samples collected from different regions of Nunavik or between different sampling areas of the Kokosak

River was performed using the Mann-Whitney test (StatTools, Palisade Corporation, Ithaca, NY). The significance level was set at $p<0.05$.

3.3. Results

Description of butchering sites. A minimum of two sites per Northern village for a total of 29 butchering sites, was investigated in the coastal environment of Nunavik for the presence of *C. botulinum* type E spores. The locations of the butchering sites varied greatly between villages. Some sites were located near the village on a beach where boats are docked or on a river bank accessible by vehicles, while others were situated away from the village, on a coastal island or along the shoreline of the mainland. Large and flat coastal rock characterized 72% of the investigated butchering sites (Figure 3.2). Gravel beaches composed of pebbles and cobbles, were also used as butchering sites by the villages of Inukjuak, Puvirnituq, Salluit and Aupaluk.

No butchering activities were observed during the survey, but the presence of marine mammal remains such as oil and bones was noted in a few sites. The surface of the rocks was coated with slime for two butchering sites, one near the main beach of Ivujivik (SP423424), and the other located at the mouth of the Koksoak River (SP457458). The tide level was high for 65% (11/17) of the butchering sites sampled along the coastline of Hudson Bay and Hudson Strait, while up to 80% (8/10) of the sites from Ungava Bay were sampled at low tide. The salinity of shoreline water along the coast of the three regions of Nunavik varied from 1.2 to 3.2%, except for two sites located in the mouth of the Puvirnituq River, where both water samples were of a low salinity, i.e., ≤0.1%. Most of the shoreline soil collected in close proximity to the butchering sites was composed of sand and/or pebbles, except for three sites located in the southern part of Ungava Bay, where the samples were muddy in texture, indicating a higher water level and organic material (Appendix A; Table A1).

Figure 3.2. Sample collection of skin and intestinal samples from harvested seals and environmental samples from traditional butchering sites. Panel A shows the collection of tidal water trapped in a depression of a coastal rock while panel B shows the collection of a mud sample at low tide near a butchering site. Panel C shows the collection of marine sediment using a hand corer. Panel D shows the swabbing of the surface (approx. 1 m^2) of a typical costal rock used for butchering seals while panel E shows the skin area incised from the navel to the tail and collected by Inuit hunters. Panel F shows a hunter removing the intestinal tract from the abdominal cavity of a bearded seal.

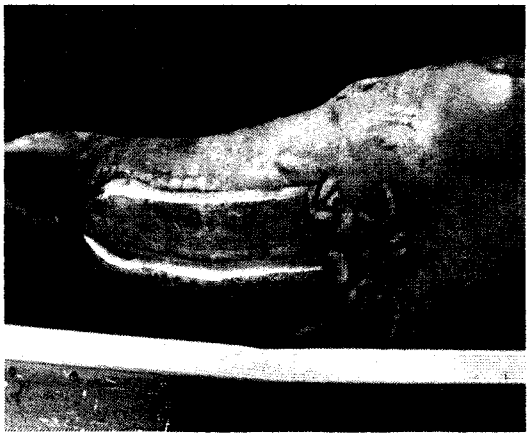
A

B

C

D

E



F

Potential inhibition by background flora. All cultures of field and laboratory quality control samples (n=38) tested positive for botulinum neurotoxin, indicating that the spores remained viable following transport and storage from the field to the laboratory, and that the enrichment broth techniques were sensitive and reliably detected low levels of spores (Appendix A; Table A3). The background flora of the environmental samples did not prevent the growth and toxin production of *C. botulinum* type E under these conditions. However, two of the 12 skin samples spiked with low levels of *C. botulinum* type E spores did not produce toxicity in mice (Appendix A; Table A4). The lack of toxin production from these quality control samples could have been due to the presence of a competing background flora producing inhibitors. As for the environmental samples, *C. botulinum* type E spores from the spiked seal intestinal content samples were able to outgrow and produce botulinum toxin at levels detected by the mouse bioassay, despite the presence of the background flora.

Sensitivity and specificity of the slot blot immunoassay. Up to 870 environmental samples were analyzed to estimate the concentration of spores in 58 different sediment and soil samples using a 5-tube MPN procedure. Detection of botulinum toxin in culture supernatants was performed by both the mouse bioassay and the slot blot immunoassay (Figure 3.3). The sensitivity and specificity of the rapid chemiluminescent assay was compared to the gold-standard mouse bioassay for the detection of type E botulinum toxin (Table 3.1). Supernatants that did not totally filter through the slot blot and showed a low net intensity of the blot, were excluded from the analysis, decreasing the total number of samples for the cross-table analysis to 841.

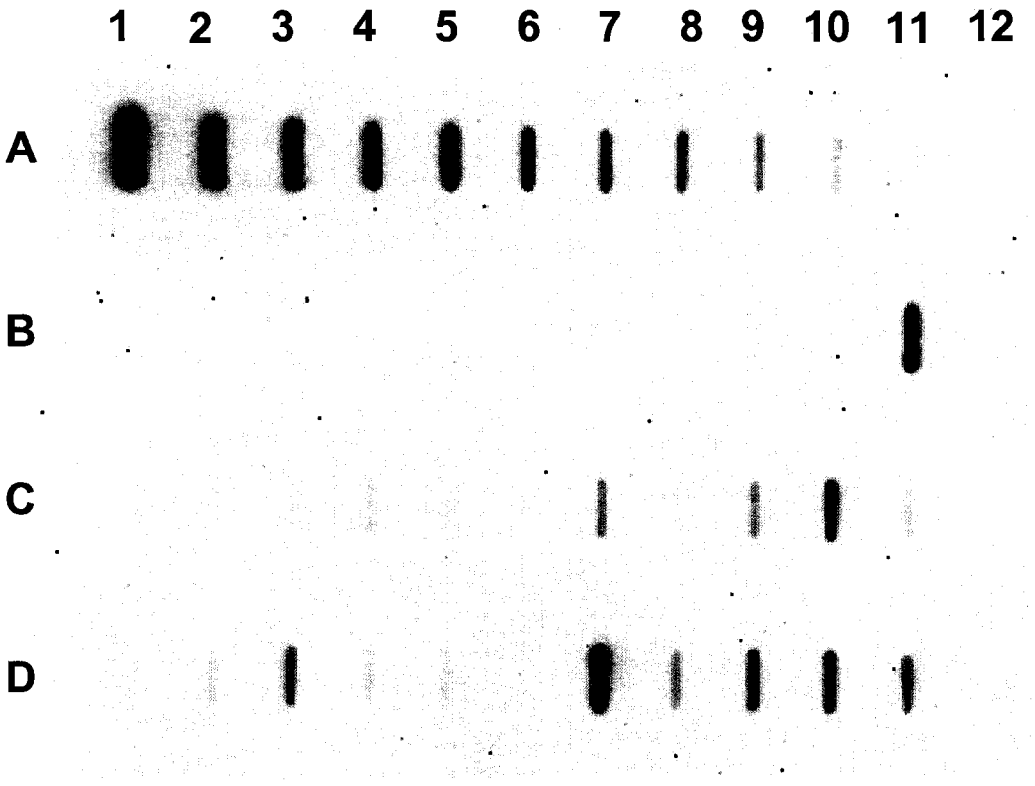


Figure 3.3. Slot blot detection of BoNT/E in culture supernatants of environmental samples. Two sets of 5-tube MPN samples using three decimal amounts of freshwater sediments from tributaries (KR-18, KR-22) of the Koksoak River were analyzed. Culture supernatants were diluted 1:10 in GPB and blotted onto PVDF membrane and type E botulinum neurotoxin detected by chemiluminescence using affinity-purified polyclonal rabbit antiserum E and horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G as secondary antibody. Row A: columns 1-11, 2-fold serial dilution of type E botulinum toxin internal standard, from 512 to 0.5 MLD, and column 12, GPB; Row B: columns 1-5, 5 X 0.3 g KR-18 samples, and columns 7-11, 5 X 0.3 g KR-22 samples; Row C: columns 1-5, 5 X 3 g KR-18 samples, and columns 7-11, 5 X 3 g KR-22 samples; Row D: columns 1-5, 5 X 30 g KR-18 samples, and columns 7-11, 5 X 30 g KR-22 samples.

Table 3.1. Sensitivity and specificity of the slot blot immunoassay compared to the mouse bioassay

		Mouse bioassay		
	Test results	Positive	Negative	Total
Slot blot	Positive	139	11	150
Immunoassay	Negative	191	500	691
	Total	330	511	841

Sensitivity = 139/(139+191) X100 = 42.1%

Specificity = 500/(500+11) X 100 = 97.9%

Using 4 MLD as the detection limit, the specificity of the slot blot immunoassay was 97.9% with a false-positive rate of 7.3%, but its sensitivity was poor, i.e., 42.1%, with a high false- negative rate of 27.6%. By decreasing the limit of detection to 2 MLD, the sensitivity of the immunoassay increased to 57%, but the false-negative rate remained relatively high at 22.6%. Due to the high false-negative rate observed with the slot blot, the estimation of the MPN counts in environmental samples was done based on the mouse bioassay results.

Distribution and levels of *C. botulinum* type E in the coastal environment of Nunavik. Estimation of the prevalence and levels of *C. botulinum* type E in the coastal environment of Nunavik was based on 35 coastal rock, 37 seawater, and 49 shoreline soil samples, collected during the preliminary study conducted in 1999, the Nunavik survey of October 2000, and the field survey on the investigation of transmission routes of *C. botulinum* type E in July 2001 (Chapter 5). Overall, the prevalence of *C. botulinum* type E spores in shoreline soil was 0, 50, and 87.5% for the Hudson Strait, Hudson Bay, and Ungava Bay regions, respectively (Table 3.2). Figure 3.4 shows the two clusters of positive shoreline soil samples located in the Ungava Bay and southern part of the Hudson Bay. Similarly, all *C. botulinum* type E positive coastal rock and seawater samples were predominantly distributed along the shoreline of Ungava Bay with a prevalence of 18.8 and 28.8%, respectively, followed by Hudson Bay with only a 9.1 and 0% prevalence, respectively. Interestingly, no spores were detected from any samples collected in the Hudson Strait.

The level of *C. botulinum* type E spores was highly variable in shoreline soil samples. The median concentration was significantly ($p<0.05$) higher in shoreline soil samples from Ungava Bay with a spore count of 23 spores/kg, as compared to a median

of 5 spores/kg for samples collected from Hudson Bay. The highest levels of spores, ranging from 270 to

Table 3.2. Prevalence and levels of *C. botulinum* type E in the coastal environment of Nunavik

	Percent prevalence			Shoreline soil	
				MPN spores per kg	
Region	**Seawater**	**Coastal rocks**	**Shoreline soil[c]**	**Median**	**Range**
Hudson Bay	0 (0/11)[b]	9.1 (1/11)	50 (7/14)	5	<6 – 15
Hudson Strait	0 (0/8)	0 (0/8)	0 (0/19)	NA[a]	NA
Ungava Bay	27.8 (5/18)	18.8 (3/16)	87.5 (14/16)	23	<6 – 1800

[a]NA: not applied.
[b]( ): number of positive samples/total number of samples.
[c]Includes two shoreline soil samples containing strains of *C. botulinum* group I; a type A strain isolated from a shoreline soil from Kuujjuaraapik (Hudson Bay) and a type B strain from Kangiqsualujjuaq (Ungava Bay).

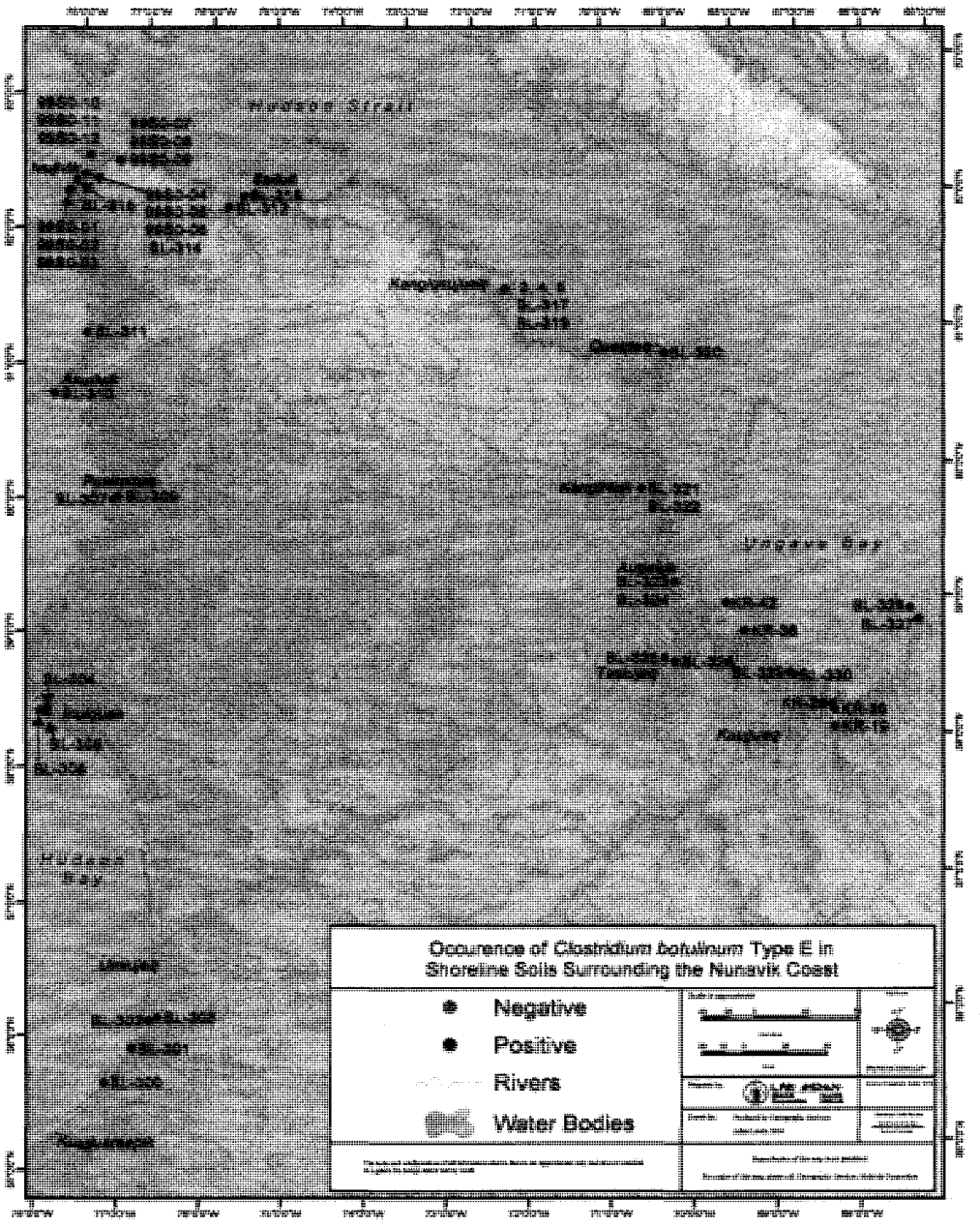


Figure 3.4. Distribution of *C. botulinum* type E in shoreline soil collected in proximity of butchering sites commonly used by hunters of Nunavik. The distribution of positive samples is predominantly located along the shorelines of the Ungava Bay and southern Hudson Bay.

1800 spores/kg, were found in four mud samples collected at the mouths of the Whale River (KR-29 and KR-359) and the Koksoak River (BL-329 and KR-44). All five positive seawater samples were also collected from the mouth of these two rivers at low or rising tide.

Two of the 29 positive environmental cultures from which the isolation of *C. botulinum* was successful, were not type E and did not belong to *C. botulinum* group II. One strain of *C. botulinum* type A, and one strain of type B were isolated from shoreline soil samples collected near the villages of Kuujjuaraapik (BL-300, Hudson Bay) and Kangiqsualujjuaq (BL-327, Ungava Bay), and were considered as the first two group I strains isolated in Nunavik.

Collection of sediment cores was possible in only one site from Salluit and one from Kangiqsujuaq, and both samples were found negative for *C. botulinum* type E. The coastal sediment of the Hudson Bay and Hudson Strait was generally too hard and compact to manually core samples. In some cases, the sediment bed was also not accessible because of the high tide level at the time of sampling.

Distribution and levels of *C. botulinum* type E in the Koksoak River. Ninety-four percent of freshwater (15/16) and 100% of marine (9/9) sediments from the Koksoak River (Figure 3.5) contained *C. botulinum* type E spores (Table 3.3). Unexpectedly, all terrestrial soil samples (11/11) collected within 25 to 100 meters from small tributaries upstream of Kuujjuaq, were also culture-positive. The concentration of spores was highly variable ranging from 5 to >5400 spores per kg in sediment and soil samples of the Koksoak River environment. The median concentration of spores in marine sediment (270 spores/kg) of the river was significantly ($p<0.05$) higher than the median concentration in freshwater

Table 3.3. Prevalence and levels of *C. botulinum* type Ein the Koksoak River environment

Area	Percent prevalence	MPN spores per kg	
		Median	Range
Marine sediment	100 (9/9)[a]	270	7 - >5400
Freshwater sediment	93.8 (15/16)	35	<6 - 1400
Terrestrial soil	100 (11/11)	90	<6 - 5400

[a]( ): number of positive samples/total number of samples.

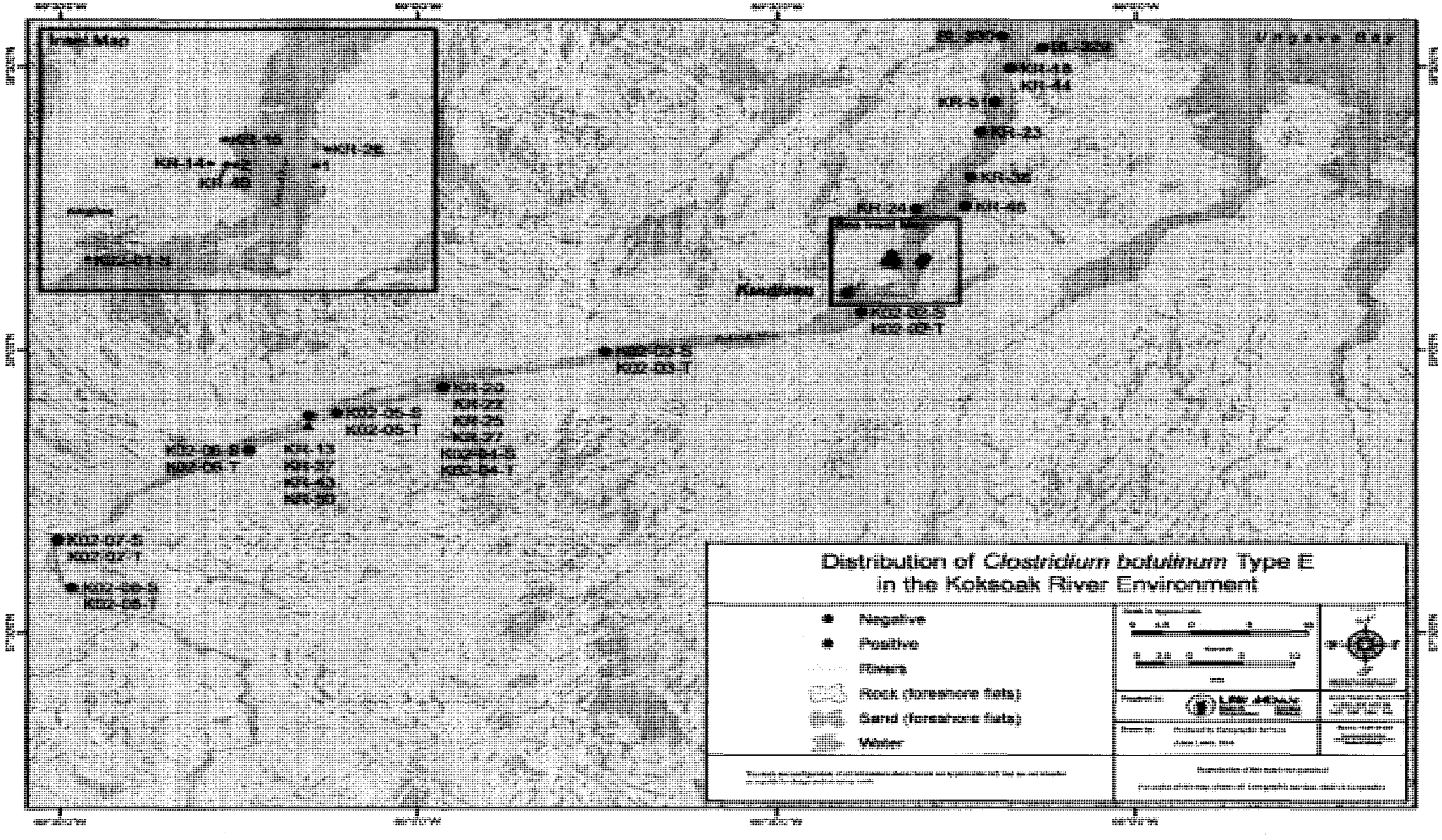


Figure 3.5. Distribution of *C. botulinum* type E in freshwater and marine sediments of the Koksoak River and in terrestrial soil surrounding its tributaries. Sediments were arbitrarily collected in the river with a median distance of 9 km between sampling sites. Terrestrial soil samples were collected within 25 to 100 m from selected tributaries downstream of Kuujjuaq.

sediment (35 spores/kg), but not (p>0.05) from the median concentration of spores in terrestrial soil (90 spores/kg). The data indicate that *C. botulinum* type E is abundant and widely distributed in the aquatic environment of the river, and can also be encountered in inland soil surrounding the tributaries (Figure 3.4).

Prevalence of *C. botulinum* type E in seals harvested in Nunavik. By combining data from the preliminary study in 1999 and from the field surveys conducted in 2001 and 2002, a total of 90 intestinal contents and 72 abdominal skin samples from harvested ringed seals and bearded seals were collected by hunters and cultured for *C. botulinum*. Most of the samples were from harvested ringed seals with only 8.3% (6/72) and 10% (9/90) of skin and intestinal samples, respectively, originated from bearded seals. The participation rate of some villages of Ungava Bay region was low for 2001 and 2002, despite the distribution of sampling kits in all villages, and coordination of the sampling program at the regional level.

The total number of skin and intestinal samples received was representative of three regions of Nunavik. Overall, the prevalence of spores of *C. botulinum* in intestinal tracts and skin samples were very low with only 4.4% (4/90) and 1.4% (1/72), respectively, being positive (Table 3.4). Two cultures of seal intestinal contents contained botulinum isolates producing type A and type E botulinum neurotoxins within the same sample, while the others were positive for type E neurotoxin. The type A isolates were recovered from a bearded seal and a ringed seal harvested near the villages of Kuujjuaq (Ungava Bay) and Kangiqsujuaq (Hudson Strait), respectively. The single positive skin sample ringed seal harvested in Inukjuak (Hudson Bay) contained *C. botulinum* type B toxin. These data indicate that serotypes A and B known to be pathogenic to humans, may be encountered in the environment and carried by seals along the Hudson and Ungava coasts of Nunavik.

Table 3.4. Prevalence of *C. botulinum* type E in seals harvested in Nunavik

Region	Percent prevalence[b]	
	Intestines	Skin
Hudson Bay	0 (0/27)[a]	5.3 (1/19)
Hudson Strait	2.8 (1/36)	0 (0/36)
Ungava Bay	11.1 (3/27)	0 (0/17)
All regions	4.4 (4/90)	1.4 (1/72)

[a]( ): number of positive samples/ total number of samples.
[b]Two intestinal tracts had both a type A and a type E isolate, and one skin sample harboured a type B isolate belonging to *C. botulinum* group I.

3.4. Discussion

Identification of the sources and distribution patterns of *C. botulinum* type E in areas affected by endemic botulism was the first step of the risk assessment process to determine the role of marine mammals and the environment in the epidemiology of human botulism in Nunavik. Harvested seals and pre-selected sites for seal butchering were surveyed on the coastal environment of Nunavik for the occurrence and levels of spores of *C. botulinum* type E. The use of large amount of shoreline soil enriched into SPGYT broth combined with pre-treatments was effective for the detection of type E spores in the coastal environment of Nunavik. Cultures of field and laboratory quality control samples were all positive for type E spores, indicating that growth was not affected by bacteriocins or other inhibiting factors produced by the background microflora. However, quantification of spores in environmental samples using the slot blot assay was not as sensitive as the mouse bioassay. The sensitivity of the slot blot was 42.1%, with a high false-negative rate of 27.6%. This contrasted with the study of Cadieux *et al.* (2005) who reported false-negative rates of 7.5 and 3.8% from enrichment cultures derived from inoculated fish and naturally-contaminated environmental samples, respectively. The main difference between the two studies was that in our study, to overcome filtration problems, the concentration of the culture supernatant that was 10 times more dilute, which increased the detection limit from 4 to 40 MLD. Thus, it is possible that many environmental samples contained less than 40 MLD and could not be detected by slot blot, but were detected by the mouse bioassay because the latter did not require a dilution of inoculum. Bulatova *et al.* (1969) found that of 134 shoreline soil samples from Lake Balkhash, Kazakhstan, up to 76% contained 4-5 MLD of BoNT/E. Such a low toxicity level was attributed to the antagonistic effect of the background flora

Based on environmental studies conducted in Northwest Alaska, beach shores used as butchering sites for marine mammals have been identified as potential sources for food contamination with *C. botulinum* spores (Miller *et al.*, 1972). Inuit hunters of Nunavik usually cut and butcher seals near the hunting area where they can find a suitable flat coastal rock, but in other cases, hunters prefer to use specific sites for post-harvesting activities. For example, hunters from Umiujaq like to butcher seals on the shoreline of small bays of the Richmond Gulf, while hunters from Puvirnituq and Aupaluk mainly butcher their seals on a beach close to their villages. The use of gravel beaches was also observed in Inukjuak and Salluit. These sites provide ample opportunities for contact with sediment particles present on the shore when meat tissues are placed on the ground by hunters during butchering.

Of the 30 positive environmental samples collected in the Nunavik coastline (Table 3.2), 22 (73.3%) were from Ungava Bay region where most botulism outbreaks (90.8%) occurred in Nunavik between 1964 and 2004 (Dolman, 1974; Hauschild and Gauvreau, 1985; Botulism Reference Service [unpublished data]). Up to 80.3% of these outbreaks were from the villages situated in the southern part of the bay (i.e., Tasiujaq, Kuujjuaq, and Kangiqsualujjuaq), where the concentrations of *C. botulinum* type E spores in shoreline soil reached levels up to 1800 spores per kg. The geographical distribution of type E spores in the coastline environment follows the spatial distribution of botulism outbreaks in Nunavik, suggesting that the environment plays a significant role in the epidemiology of the disease. In Alaska, the presence of *C. botulinum* type E was also demonstrated along the coastline of villages where botulism outbreaks were reported (Miller, 1975). Interestingly, two shoreline soil, two intestinal tracts and one skin sample from seals harvested in different geographical regions of Nunavik harboured spores of types A and B belonging to *C. botulinum* group I. These serotypes

have been previously isolated in sediments of the Canadian Atlantic seaboard (Laycock and Longard, 1972) and in soil of several provinces of Canada (Dubovsky and Meyer, 1922). However, they were not associated with previous botulism outbreaks in Nunavik, likely because they cannot grow well at cool temperatures, the minimum growth temperature being 10 and 3°C for *C. botulinum* group I and II strains, respectively (Austin, 2001). In the Arctic, most of the outbreaks have been associated with type E botulism, but on several occasions, they have been attributed to types A and type B in Alaska (Barrett *et al.*, 1977; Wainwright *et al.*, 1988).

Possible sources of type E botulinum spores in the coastline environment of Alaska have been associated with the deposition of organic waste, such as the butchering of marine mammals on the beach, or effluents discharged from crab, fish or shrimp canneries (Miller, 1975). The intestinal contents of marine mammals were also identified as a potential source of botulinum contamination of the butchering sites following the finding of *C. botulinum* type E in the colonic contents of one beluga whale out of 44 marine mammals tested (Miller, 1975). The prevalence of spores in the intestinal contents of 90 seals harvested in Nunavik was also low, with only one bearded seal and three ringed seals carrying the organism, despite the fact that culture of both the small and large intestinal contents were cultured to increase spore recovery. The prevalence of type E spores on the surface of abdominal skin samples was even lower at 1.4%, but this prevalence was likely underestimated as the total surface area cultured was relatively small, being on average 136.5 ± 58.7 cm^2. In addition, some of the spiked skin samples used as quality control samples were culture-negative, which indicated that the growth of *C. botulinum* type E could be inhibited by the background flora of the skin. Thus, the risk of contamination of seal meat with *C. botulinum* spores from these animal sources appears lower than the shoreline environment. However, repeated use of

butchering sites would promote accumulation of these endogenous spores on the shoreline, particularly from the intestines that are usually emptied on the shore and kept for consumption.

The presence of botulinum spores along the coast could result from the butchering of fish and marine mammals on the shore, but also from the passive accumulation of spores from the drainage of the watershed. The origin of *C. botulinum* type E spores in the coastal environment of Nunavik was investigated by sampling the Koksoak River, a large river flowing into the southern part of the Ungava Bay, where the highest concentrations of spores were detected along the Nunavik coastline. Up to 96% of sediment samples collected over the entire length of the river (136.5 km), which included several tributaries upstream and downstream of Kuujjuaq, contained *C. botulinum* type E spores. The Koksoak River has the highest prevalence rate of *C. botulinum* type E spores ever reported in an aquatic environment in Canada. The highest concentration of spores was measured in the marine sediment of the river, with more than 5400 spores per kg found in one sampling site. These high levels may result from a higher deposition rate in the marine portion of the river, rather than from an active multiplication of a resident population of *C. botulinum* type E. The low water temperature of the Koksoak River during the summer (July; 5°C) and the short warm seasons in the Arctic regions likely would not support active multiplication of the organisms in sediments, as was proposed for Green Bay, Lake Michigan, to explain the presence of unusually high concentrations of spores (up to 36,000 spores/g) detected in the mud (Sugiyama *et al.*, 1970). Huss (1980) suggested that the presence of spores in the Arctic zones was the result of the decomposition of stranded marine mammals on the shoreline that allowed for the growth of *C. botulinum* and its release in high numbers in the environment. However, the finding of stranded or dead marine mammals

is not common in Nunavik and such incidents would not explain the geographical distribution pattern of *C. botulinum* type E observed in this region.

Spores of *C. botulinum* type E may originate from the large southern watershed rich in vegetation, where spores may germinate and grow in decaying material. The spores may flow from the headwaters through the tributaries and rivers, accumulate downstream in cold waters, and disperse along the coast by current and tide effects. The concentrations of spores in terrestrial soil samples collected near the tributaries were not significantly different from those measured in marine sediments, suggesting that *C. botulinum* type E may be of terrestrial origin. The high prevalence of spores observed in terrestrial soils supports the terrigenous sedimentation hypothesis of Johanssen (1963), which proposed that spores of *C. botulinum* type E are carried from large land masses by heavy rains into watercourses leading the organisms to the sea, rather than being a truly aquatic organism as proposed by Bott *et al.* (1968) and Huss (1980). Whether the coastal type E spores were of terrestrial or aquatic origin is beyond the scope of this study, but our results indicate that the organism is widely distributed in the Koksoak River and its surrounding terrestrial soil, and may easily reach the coastal environment either through runoff waters or the river flow, and thus contaminate the shoreline at high tide. The influence of the freshwater environment was previously demonstrated by Craig *et al.* (1968), who showed that salmon caught in the Columbia River in Oregon harboured the organism more frequently in their viscera than those captured in ocean waters. The risk of contamination of seal meat would therefore be higher on butchering sites located along rivers than those lining the mainland and the offshore islands away from freshwater sources. This was supported by the findings that the highest concentrations of spores were found in shoreline soil samples collected from butchering sites located at the mouth of large rivers, such as the Koksoak and Whale rivers, and

that the presence of spores on coastal rocks and in seawater samples were mainly detected from these butchering sites.

In summary, spores of *C. botulinum* type E were predominantly found in shoreline soil collected near butchering sites along the coasts of southern Ungava Bay and southern Hudson Bay. The occurrence of type E spores was observed in all samples collected from three butchering sites along the mouths of the Koksoak and Whale rivers, which flow into southern Ungava Bay and where the highest concentrations of spores were observed among all butchering sites investigated along the coast of Nunavik. The risk of contamination of marine mammal meat from environmental sources of *C. botulinum* type E during butchering would be higher for animals that are butchered along southern Ungava Bay coast, particularly within the mouths of large rivers, due to the high levels of naturally occurring spores in these areas. Although much less prevalent, the endogenous spores from the seal itself, either from skin or the intestinal materials, may pose a risk for contamination of seal meat if handling precautions are not followed. Thus, multiple sources of *C. botulinum* may be involved in the contamination of marine mammals during butchering. Further studies are needed to determine the high-risk handling practices leading to the contamination of raw seal meat from the environmental and endogenous sources of *C. botulinum* type E using a combination of field observation and microbial source tracking studies.

CHAPTER 4

MEAT HANDLING PRACTICES AND RISKS OF CONTAMINATION WITH *CLOSTRIDIUM BOTULINUM* TYPE E IN THE COASTAL ENVIRONMENT

4.1 Introduction

The coastal environment was identified as a potential source of contamination of marine mammal meat by Miller *et al.* (1972), who showed that 74% of beach soil samples of western Alaska where marine mammals are usually butchered contained *C. botulinum* type E spores. This was further supported by field observations that butchering of marine mammals along shoreline areas provides ample opportunity for contamination of meat by beach soil (Miller *et al.*, 1972). The food handling steps and the various points for contamination and growth of bacterial pathogens from harvesting of raw foods to consumption have been previously described in a general model (Himelbloom, 1998). However, the handling practices that lead to the contamination of marine mammal meat from sources of *C. botulinum* type E during butchering have not been thoroughly examined under field conditions.

The objectives of the study were to assess potential sources of bacterial contamination during processing of seals in shoreline areas and to identify various meat handling practices that may contribute to the contamination of seal meat with *C. botulinum* type E spores at the butchering site. To achieve these objectives, a series of interviews on the potential risks of contamination of raw seal meat during butchering were conducted with hunters that currently age seal meat into *igunaq*, a traditional food delicacy frequently involved in previous type E outbreaks in Nunavik. To assess on-site the different risks, a number of field observations of seal butchering activities were then carried out in southern Ungava Bay where botulism is endemic. Based on the results of these surveys, control measures were developed to reduce the risks of bacterial contamination at the butchering site; these are summarized under the section recommendations.

4.2 Materials and Methods

Survey with seal *igunaq* producers from Nunavik

Questionnaire design and survey sampling. A series of interviews were conducted with hunters from five Northern villages of Nunavik to assess the potential risks of contamination of seal meat with *C. botulinum* type E spores during butchering and preparation of a traditional country food called *igunaq*, which means in Inuktitut "aged meat". Five Northern villages were selected based on the prevalence of botulism and their geographic locations. Kuujjuaq, Kangiqsualujjuaq, and Tasiujaq, three villages located in southern Ungava Bay and previously afflicted by several episodes of type E botulism (Dolman, 1974; Hauschild and Gauvreau, 1985; Austin, 1996; Austin *et al.*, 1997, 1999) constituted the affected villages. Kangiqsujuaq and Quaqtaq, two villages located on the coast of the Hudson Strait, were selected as control villages, as both had only one recorded botulism incident in 1967 and 1996, respectively.

A list of potential *igunaq* producers was provided to the designated Inuit interviewer from Kuujjuaq by each local HFTAs. Up to five hunters known to produce aged seal meat in each village were contacted and interviewed. The questionnaire (Appendix B) comprised five sections containing a set of questions of three types: i) yes or no; ii) multiple choice; and iii) written response. Section A was designed to generate information on the production and consumption of *igunaq* at the individual level, and more limited at the population level. Questions in section B pertained to the characterization of butchering sites frequently used by hunters and section C assessed the potential risks of microbial contamination of seal meat from the environment and processed animals. Section D evaluated the risks of microbial contamination of seal meat during the aging process and section E was developed to generate information on current preparation methods used by producers. This chapter analyzed results from

Sections B, C, and D of the interviews. Section A is reported under Chapter 9 on the risk profile of type E botulism in Nunavik and section E was used for development of inoculation studies described in Chapter 8.

Validation of questionnaire and interviews. The questionnaire was tested in Kuujjuaq between April 25 and May 2, 2001 with five experienced producers of seal *igunaq* to allow adjustments and standardization of the questions by the interviewer. All questions and discussions were conducted in Inuktitut and recorded on a standard tape recorder. Following these initial interviews, the trained Inuit interviewer from Kuujjuaq proceeded to the interviews of four *igunaq* producers from Kangiqsualujjauaq between May 2 and 31, 2001, and five from Kangiqsujaq between December 12 and 13 of the same year. Three producers from Tasiujaq were then interviewed between February 12 and 15, 2002, followed by three others from Quaqtaq, between June 21 and 27, 2002.

Analysis of interviews. Each questionnaire was examined after each interview to verify if all questions were asked and if the responses were adequate. When needed, the interviewer listened to the recorded tape and completed any missing information, or clarified a response with the producer at a later time. Data were entered into an Excel spreadsheet and examined to ensure that all questions were asked by the interviewer and adequately answered. Whenever possible, in the case of an incomplete response or the absence of a response to a question, the interviewer contacted the producer by telephone and completed the appropriate sections of the questionnaire.

Field observations of the butchering process

Seal hunting and location of butchering sites. A seal hunting trip was conducted at Diana Bay (N 59°06'.807', W 68°56'.246') in southern Ungava Bay on July 18, 2001, to assess the potential risks of contamination of seal meat with *C. botulinum* type E from environmental and animal sources and to determine high-risk meat handling practices. The campsite of Diana Bay was selected as it is commonly used for butchering, preparing, and aging seal meat into *igunaq*. Diana Bay is a small bay located near the mouth of the Dancelou River and situated east of the Northern village of Tasiujaq. The field team, comprised of one *igunaq* producer from Kuujjuaq, a wildlife technician, and a researcher from the NRC, reached the campsite by freighter canoe from Kuujjuaq after an overnight stay at a camp near the mouth of the Koksoak River. Only one ringed seal (seal No. 1) was harvested within five days. Due its small size, the animal was pulled out of the water, placed in a large plastic container, and carried onto a large flat rock located in front of the cabin for butchering. According to the hunter, this flat rock is only covered by seawater once a month during the period of high tides.

On July 22, the field team returned to Kuujjuaq to replenish the gas tanks. Instead of returning to Diana Bay on July 24, the *igunaq* producer made a suggestion to change the hunting area to Big Island (N 58°17'.589', W 67°40'.046'), located at the mouth of the Whale River, and east of the Koksoak River. Three bearded seals were harvested within three days in the river and butchered in two different shoreline areas of Big Island. The first two bearded seals (seals No. 2 and No. 3) were butchered on similar large flat rocks at high tide, just below the campsite. Since the tide was elevated in both cases, the animals could be pulled out of the water onto the coastal rocks without any contact with the sediment present at low tide. The third bearded seal (seal No. 4) was not harpooned after being shot and rapidly sank to the bottom of the river. The bearded

seal was found at low tide on the following morning (July 26) laying on the river bed. The animal was attached to a float so it could be located at high tide and brought to the shoreline by boat. However, due to the strong wind prevailing at high tide, we were forced to anchor in a small bay of Big Island away from the shoreline until the end of the falling tide. Thus, instead of being butchered immediately after killing, the seal No. 4 was butchered approximately 24 h after it was killed. At low tide, the hunter cut and butchered seal No. 4 where it was laid in the mud and its skin was used as a tarp, since no one could lift or pull the heavy animal up to a flat rock. The hunter carefully cut and collected all meat and fat tissues, which were immediately transferred into a plastic pail placed inside the boat.

Observation of handling meat practices. The handling practices of the hunters were observed to determine if they posed a risk for contamination of meat of harvested seals with *C. botulinum* type E spores. A risk for contamination was considered significant during the butchering, if any field practices allowed the meat or fat tissues to come into contact with potential environmental sources of *C. botulinum* type E such as coastal rocks, shoreline soil, and seawater, or from endogenous sources like intestinal materials and skin of seals. These practices were recorded in a field book and, whenever possible, filmed with a video recorder.

4.3. Results

Survey and sample description. Twenty producers of seal *igunaq* from five Northern villages of Nunavik were interviewed for a total length of 12.5 h. Sixty-five percent of the interviews were conducted in the three villages of southern Ungava Bay and 35% in the two control villages located in Hudson Strait region. Nineteen of the producers were men (95%), with an average age of 62 years (range: 41-84 years) and one was a 66 year old woman. Most of them (79%) had more than 30 years of experience in the preparation and production of seal *igunaq*, while others (11%) had less than 2 years (Figure 4.1). Eighty-five percent of the interviewed producers actively make seal *igunaq* and 53% produced *igunaq* using meat from other species including walrus, beluga whale, and caribou. Thus, two *igunaq* producers from Kangiqsujuaq and one from Tasiujaq were not active producers of seal *igunaq*. In spite of this discrepancy in the survey, these interviews were kept in the analysis under the assumption that the sources and routes of contamination of *C. botulinum* type E spores in raw meat were similar among species of marine mammals. None of the producers were currently producing *igunaq* from fish, but two from Kuujjuaq acknowledged that they had made fish *igunaq* or seal *igunaq* with fish flesh many years ago. Figure 4.2 shows the proportion of various types of *igunaq* produced by region and suggests that the production in the Hudson Strait is more diversified with higher proportions of producers making walrus, whale and seal *igunaq*, while several producers from Ungava Bay predominantly favour the seal *igunaq* production.

Characteristics of butchering sites. Up to 70% (14/20) of the *igunaq* producers used specific sites for butchering their seals. These sites are commonly used by other hunters of the same village and can be located along the shoreline of various coastal

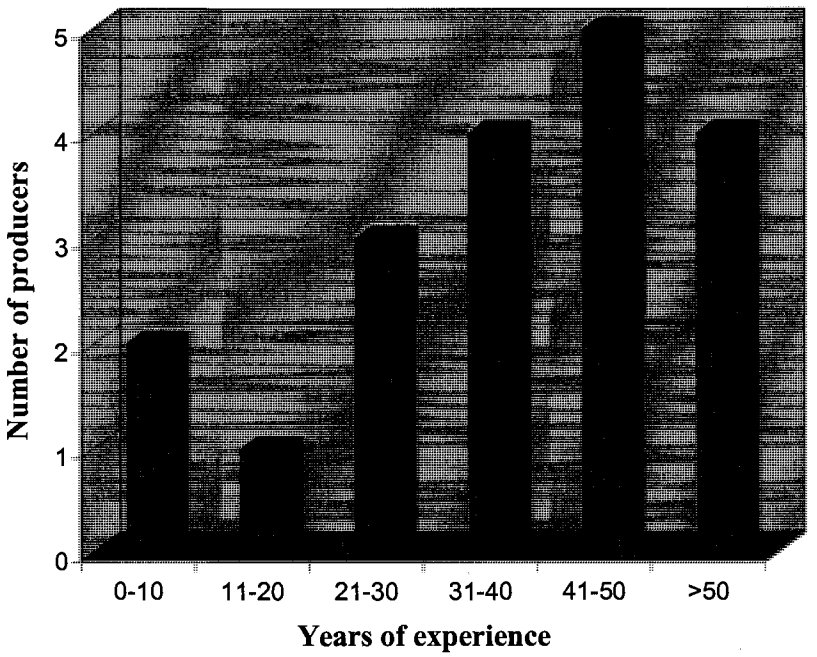


Figure 4.1. Years of experience of producers of seal *igunaq* interviewed during the survey

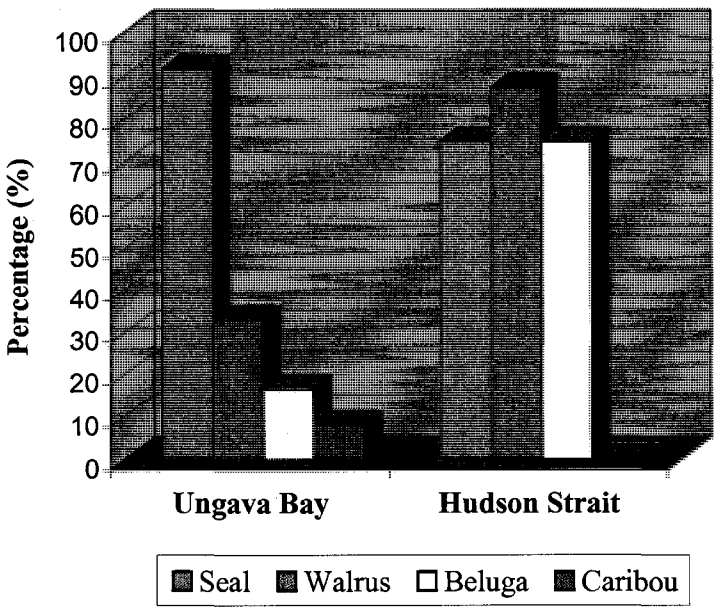


Figure 4.2. Production of various types of *igunaq* in Ungava Bay and Hudson Strait regions

environments such as the mouth of large rivers, peninsulas, and bays of the mainland and offshore islands. The large and flat coastal rocks present along the shoreline of a site are preferably used by most producers for butchering their seals, but some of them also use ice floes or gravel beaches as butchering sites. The use of a gravel beach was reported by four producers originating from Kuujjuaq, Tasiujaq, and Kangiqsualujjuaq. All butchering sites from Ungava Bay and Hudson Bay regions are covered with seawater at high tide, and hence 89% (16/18) of producers reported that their sites were either harbouring slime and/or were contaminated with sediment particles at low tide, such as mud, sand, and small gravel.

High-risk meat handling practices. All meat and fat tissues from seals No. 1, 2, and 3 were directly placed onto the surface of the coastal rocks aside from the animals (Figure 4.3A-C). According to all interviewed producers, this handling practice should not result in the contamination of tissues with shoreline soil. However, three producers from Kuujjuaq reported that the back skin of the seals could occasionally become contaminated with shoreline soil when the animal is pulled on the shore. For every seal butchered on coastal rocks during the field study, sediment particles were visually detected on the surface of several pieces of meat and fat tissues. Instead of being butchered on a flat coastal rock at high tide, due to hard weather conditions bearded seal No. 4 was butchered in the mud at low tide (Figure 4.3D). Despite the precautions taken by the producer during butchering, it was very difficult to avoid contamination from occurring because of the proximity of the carcass to the marine sediment.

Sediment particles as well as seawater could accumulate in depressions of coastal rocks following the receding tide, and become a potential source of bacterial contamination (Figure 4.3B). During butchering, some of the meat from seal No. 2 was

found to be in direct contact with tidal water present in these depressions. To wash off the blood covering the meat after the butchering process, a pail was filled with seawater from a large depression and poured over the carcass of seal No. 3 cut in large pieces (Figure 4.3C). This practice was commonly used by all interviewed *igunaq* producers. All producers rinse the skin and plastic containers used for carrying meat and fat tissues with seawater. The high-risk handling practices observed in the field and reported during the survey can be seen in Table 4.1. A large number (17/19) of producers of *igunaq* reported that they carry flippers, meat and fat pieces from the field to the camp in the same container. During transport by boat, this practice allows direct contact of meat with the microbial flora of skin attached to the flippers. The other producers either carry the flippers separately or only with pieces of fat. Four producers reported that the pieces of meat and fat could come into contact with the inner surface of the boat during transport. During the field survey, all pieces of fat and meat butchered for the production of *igunaq* were transported together in plastic pails (Figure 4.3C), while the flippers were placed in separate containers with the remaining meat and fat.

Only 10% (2/20) of the interviewed producers stated that during evisceration, the contents of the intestinal tract may accidentally fall into the carcass following the detachment of the rectum from the anus. These producers would use seawater to rinse off the fecal material from the carcass if such an event were to occur. Prior to detaching the rectum from the anus, the hunter manually pushes upward the fecal material from the rectum into the colon, thus preventing the leakage of fecal material into the abdominal cavity. All producers usually wash their hands after emptying the intestinal tract, but this hygienic practice was not followed during the butchering of the seal No. 1. As it was observed during the field study, 60% (13/19) of surveyed producers avoided transporting the intestines with meat and preferred to use a separate plastic bag

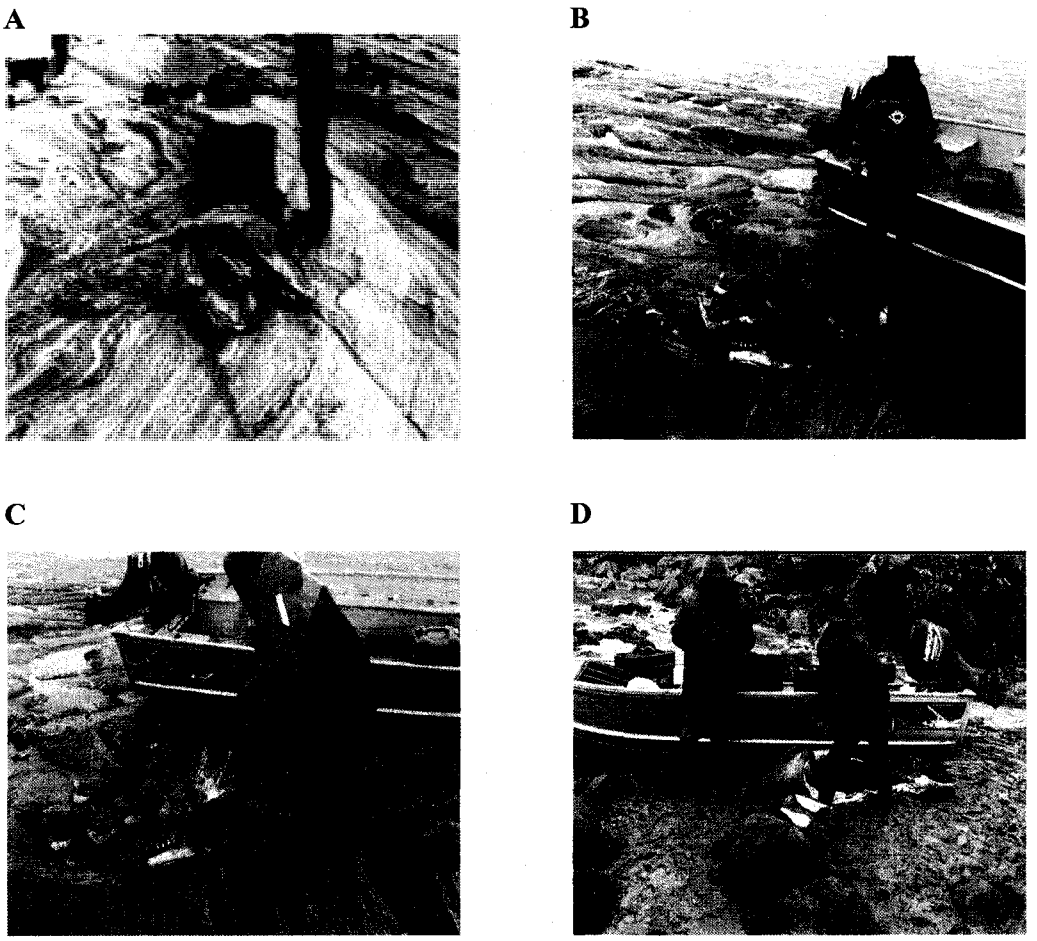


Figure 4.3 High-risk meat handling practices observed during butchering of seals harvested in southern Ungava Bay. Panel A: Meat parts hooked and pulled across rocks over a long distance from the water line and pilled up. Note the use of skin hide to protect the meat from adverse weather conditions and birds. Panel B: Butchering site near residual seawater left by the previous tide. Note the presence of fat tissues soaking in seawater. Panel C: Rinsing of seal meat with seawater after butchering to clear off blood. Panel D: Butchering of bearded seal in mud bed containing 1800 spores per kg.

Table 4.1. Meat handling practices that pose a high risk for contamination of seal meat with *C. botulinum* type E identified during the survey with 20 producers of *igunaq* and assessed in the field during the butchering of four seals

Sources	High-risk handling practices	Survey	Field observation
Coastal rocks	Repeated use of specific butchering sites	70% (14/20)	75% (3/4)
	Placing meat and fat tissues on coastal rock surfaces	100% (20/20)	100% (3/3)
	Pulling heavy pieces of meat across rocks	NA[b]	66.7% (2/3)
Shoreline soil	Cutting seal on a pebble beach	21% (4/19)	0% (0/4)
	Cutting seals in the mud at low tide	NA	25% (1/4)
Seawater	Rinsing carcasses and butchered meat and fat tissues with seawater	100% (20/20)	33.3% (1/3)
	Rinsing meat carriers with seawater	100% (20/20)	50% (2/4)
	Rinsing the skin with seawater	100% (20/20)	0% (0/4)
Intestinal contents	Leakage of fecal material into the abdominal cavity during evisceration	10% (2/20)[a]	0% (0/4)

Table 4.1 (Continued). Handling meat practices that pose a high risk for contamination of seal meat with *C. botulinum* type E identified during the survey with 20 producers of *igunaq* and assessed in the field during the butchering of four seals

Sources	High-risk handling practices	Survey	Field observation
Intestinal contents	Not washing hands after emptying the intestines of their contents	0% (0/20)	25% (1/4)
	Transport of intestines with meat in the same container	68% (13/19)	0% (0/4)
Skin	Transport of flippers with meat in the same container	90% (17/19)	100% (4/4)
	Covering meat and fat tissues with skin hide to protect from adverse weather conditions and birds	NA	66.7% (2/3)
	Use of inverted skin as container for aging	100% (16/16)	NA

[a]Two *igunaq* producers stated that accidental leakage of fecal material occurs occasionally.
[b]NA: not applied.

or a bucket to carry the intestines. The remaining 40% carry the intestines with meat and fat. This practice may pose a risk for contamination of seal meat with the intestinal flora from the opened intestinal segments.

4.4 Discussion

These surveys showed that using current meat handling practices during butchering, contamination of raw seal meat with spores of *C. botulinum* type E may occur through several routes. Some of these practices were found more hazardous than others, beginning with the selection of the butchering site. Most hunters preferably selected a large and flat rock in the coastal environment to butcher their seal. However, these coastal rocks are only accessible at high tide, and under certain circumstances, the hunters may be forced to butcher the animal onto a sediment bed below the water line in areas rich in spores. Spores of *C. botulinum* type E were detected in low tide water and in sediment surrounding seal No. 4 with a concentration of 1800 spores/kg. Although the hunter was very careful not to contaminate the meat during butchering, it was extremely difficult to prevent splashing of mud onto the meat under these conditions. The practice of butchering seals in the mud is probably not common among hunters, but it should be discouraged due to the high risk of meat contamination.

With the exception of seal No. 4 butchered in the mud, the other three seals were processed on a flat coastal rock at high tide preventing contact with shoreline soil exposed during low tide. The risk of contamination of seal meat from environmental sources may be less when the animal is butchered on a flat coastal rock than when on a pebble beach or a shoreline area covered by a mud substrate. The beach soil of the coastline environment of Alaska was previously identified as a potential source of *C. botulinum* type E, but the processing routes leading to the contamination of raw meat were not described (Miller *et al.*, 1972). The first high-risk meat handling practice observed in the field consisted of placing all the meat and fat tissues used for the preparation of *igunaq* onto the surface of coastal rocks while performing the butchering. Although the coastal rocks appeared visually clean, the surface may harbour viable

spores left by the tide water. This practice was used by all interviewed producers of *igunaq*. The butchered tissues are placed into a plastic container only at the end of the butchering process, allowing ample contact of tissues with spores potentially present on the surface of the rocks or from residual tide water enclosed in depressions of the rocks. In other circumstances, the meat was piled up on the surface of coastal rocks away from the water line, but the meat had to be carried by hooking and dragging large pieces of meat across the rocks. This favours contact between residual spores on the surface of the rocks and the meat. While the spores were not detected on the surface of the coastal rocks of the three butchering sites visited at high tide during the field survey, suggesting that under these conditions the levels of spores encountered on the surface of coastal rocks is probably low, this practice should be avoided.

Contamination of seal meat from seawater containing spores of *C. botulinum* type E was previously suspected when spores were detected in coastline water from western Alaska near areas where marine mammals are usually butchered (Miller, 1975). Rinsing seal meat or immersing the entire carcass in seawater to remove blood or to provide meat with a salty taste could lead to the contamination of meat with spores present in seawater. This practice was used by all interviewed *igunaq* producers and was observed once in the field at Whale River where meat from seal No. 3 was rinsed with seawater taken from the depression of a coastal rock. In this case, spores of *C. botulinum* type E were not detected in the rinse water. The presence of spores was demonstrated in low tide water near the butchering area where seal No. 4 was processed, but the shoreline water was not used to rinse the meat or to clean any containers used to carry meat. Cross-contamination events involving seawater may occur when plastic containers, used to carry meat, are rinsed with seawater. This practice was observed once in the field and was used by all interviewed producers of *igunaq*.

According to the survey conducted with the producers of *igunaq*, accidental contamination of seal meat with intestinal material does not occur frequently during evisceration. No evidence of leakage of the intestinal materials, into the carcass or beside the four butchered seals was observed during the field observations. Hand washing is also important to prevent cross-contamination of meat from intestinal materials as the butcher's hands could be contaminated following the emptying of intestines that could take place before meat butchering. This hygienic measure was not followed during butchering of seal No. 1, as was usually practiced by most surveyed *igunaq* producers. In this case, spores of *C. botulium* type E were not detected in the intestinal tract of seal No. 1, but they were present in the intestines of bearded seal No. 2. The intestines of the four seals were transported separately from the meat during the field survey, but 32% of *igunaq* producers stated that they occasionally transport the intestines and the meat in the same container. Although the prevalence of spores in the intestinal tracts of seals and other marine mammals (Miller, 1975) is low, on certain occasions these meat handling practices may lead to cross-contamination events.

4.5 Recommendations

In summary, several high-risk meat handling practices for contamination of seal meat with endogenous and environmental sources of *C. botulinum* type E were identified through the survey and confirmed by field observations, allowing the development of the following control measures to reduce the risk of contamination of seal meat during butchering:

a) Avoid selecting a butchering site located at or near the mouth of a river. These areas are contaminated with high levels of spores of *C. botulinum* type E.

b) Do not butcher seals in the mud or on a sediment bed at low tide, particularly in Ungava Bay where the spore concentration is high. Instead, select butchering sites such as large and flat rocks along the shoreline of the mainland or islands at high tide, whenever possible.

c) Use a physical barrier such as a tarp to avoid contact with bacterial spores from shoreline soil and rocks. The use of a tarp will reduce the exposure of the carcass to spores of *C. botulinum* type E naturally present on the surface of the coastal rocks and in shoreline soil and water.

d) Put all butchered meat and fat into a clean (not rinsed with shoreline water) plastic container immediately after cutting. Placing all butchered meat and fat tissues inside a clean plastic container immediately after cutting, will reduce exposure of these raw materials to spores of *C. botulinum* type E naturally present on the surface of the coastal rocks and in shoreline soil and water.

e) Avoid rinsing any plastic containers used for carrying the butchered meat and fat tissues with shoreline water. This will reduce the risk of contamination of the

container with *C. botulinum* type E naturally present in seawater, and consequently, reduce the risk of contamination of meat and fat.

f) Do not rinse meat or any parts of the carcass with shoreline water after butchering. This will reduce the exposure of the carcass and its parts to spores of *C. botulinum* type E naturally present in shoreline water.

g) Transport the intestines and the flippers of harvested seals separately from the butchered meat and fat. This will reduce the risk of contamination of meat and fat with spores of *C. botulinum* type E occasionally present in the intestinal material and on the surface of the skin of harvested seals.

CHAPTER 5

COMPARISON OF DNA FIGERPRINTING METHODS FOR TYPING EPIDEMIOLOGICALLY RELATED ISOLATES OF *CLOSTRIDIUM BOTULINUM* TYPE E

Portions of this chapter were published in:

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5.1 Introduction

Fingerprinting methods such as pulsed-field gel electrophoresis (PFGE) (Hielm *et al.*, 1998a,b) and randomly amplified polymorphic DNA (RAPD) typing (Hyytiä *et al.*, 1999b) have been used to study the genetic relatedness of *C. botulinum* type E strains isolated from fish, fishery products and sediments. More recently, PFGE has been applied to type strains of *C. botulinum* group I and II involved in sporadic cases (Lindström *et al.* 2004; Johnson *et al.*, 2005; Nevas *et al.*, 2005a). The usefulness of PFGE in epidemiological studies or during a foodborne outbreak investigation involving *C. botulinum* group II remains unknown, in part because of the low incidence of botulism outbreaks yielding isolates from clinical, food, and environmental sources (Korkeala *et al.*, 1998).

Type E strains belonging to *C. botulinum* group II have been previously reported to be more difficult to type by PFGE due to degradation of DNA during the procedure (Hielm *et al.*, 1998a). The untypeable group II strains presumably produced high levels of extracellular DNases causing DNA degradation during its isolation (Hielm *et al.*, 1998a). The use of a large number of cells generated in broth culture, combined with a formaldehyde fixation step of harvested cells, resolved the problem of smearing observed during PFGE of many *C. botulinum* group II strains (Hielm *et al.*, 1998a). However, it was estimated that about 10% of type E strains could not be typed by PFGE in spite of the formaldehyde step (Hielm *et al.*, 1998a; Hielm *et al.*, 2001) and that RAPD (Hyytiä *et al.*, 1999b) and manual ribotyping (Hielm *et al.*, 1999) were suitable alternative typing methods.

The problem of DNA degradation affecting the typeability of PFGE is not exclusive to *C. botulinum*, but also occurs in other clostridial species including *C. perfringens* (Maslanka *et al.*, 1999), *C. sporogenes* (Lin and Johnson, 1995), *C. difficile*

(Kristjéánsson *et al.*, 1994) as well as several Gram-negative bacteria (Römling and Tümmler, 2000; Inglis *et al.*, 2002; Silbert *et al.*, 2003). The addition of thiourea in the running buffer resolved the smearing problem in some *C. difficile* isolates (Corkill *et al.*, 2000), but in other studies required additional modifications of the PFGE protocol to reduce DNA degradation (Fawley and Wilcox, 2002; Alonso *et al.*, 2005). Klaassen *et al.* (2002) concluded that amplified fragment length polymorphism (AFLP), which is not affected by DNA degradation, was a fail-safe alternative method to PFGE. AFLP was recently used for the characterization of *C. botulinum* group I and II strains and the technique was also able to type all strains, including those from group II (Keto-Timonen *et al.*, 2005). While AFLP is a useful procedure, standardized PFGE is used by Centers for Disease Control and Prevention (CDC and Public Health Agency of Canada in PulseNet to type pathogens of public health importance (*Salmonella*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Shigella*, *Vibrio cholerae*, and *Yersinia pestis*).

The objective of this study was to compare the performance of PFGE with RAPD and automated ribotyping, using epidemiologically related clinical and food isolates. In this study, we compared each method in terms of typeability, reproducibility, discriminatory power, and epidemiological concordance, and evaluated whether the addition of thiourea in the running buffer could improve the typeability of *C. botulinum* group II strains using PFGE.

5.2 Materials and Methods

Bacterial strains and growth media. Thirty strains of *C. botulinum* group II from the culture collection of the Botulism Reference Service for Canada, and nine strains of *C. botulinum* type E recently isolated from the Arctic environment were used in this study. The origin, source and date of isolation of all strains are described in Table 5.1. Nine environmental isolates and 12 food or clinical isolates of *C. botulinum* type E showing smearing during PFGE, were selected to evaluate the protective effect of thiourea. Eight clinical and three food isolates associated with four type E botulism outbreaks in the Canadian Arctic were included to evaluate the ability of each typing method to link outbreak isolates. The outbreaks were not related and occurred in three separate Northern villages at different time periods between July 1995 and May 2000. In addition to these isolates, 16 group II *C. botulinum* type B, E and F strains of various origins were included to assess the discriminatory power of each typing method.

These isolates were stored at -86°C on Microbank™ beads (Pro-Lab Diagnostics) and were routinely grown on McClung Toabe Agar Base (Bacto™, Becton, Dickinson and Company) containing 0.5% yeast extract (Bacto™, Becton, Dickinson and Company) and 5% egg yolk suspension (two egg yolks dissolved in 100 ml of 0.85% saline solution) for 2-3 days in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at room temperature. For PFGE and RAPD analysis only, one whole colony from each isolate was transferred into 8 ml of SPGY broth for overnight growth using the same temperature and anaerobic atmosphere as described above.

Table 5.1. List of *C. botulinum* group II strains used for the comparison of PFGE with RAPD and automated ribotyping

Strain ID	Origin	Location	Year of isolation	Source[a]
Serotype B				
2B	Marine sediment	Pacific coast, USA	1960s	Eklund/Solomon[b]
17B	Marine sediment	Pacific coast, USA	1960s	Eklund/Solomon
DB-2	Marine sediment	Pacific coast, USA	1968	Eklund/Solomon
II60-15B	Feces	British Columbia, Canada	1982	BRS[c]
KAP-B-3	Kapchunka	California, USA	1981	Solomon
KAP-B-8	Kapchunka	California, USA	1981	Solomon
Serotype E				
Eruss	Sturgeon intestine	Russia	1935	Gunnison/NR
8550	Perch intestine	France	1951	Prevot[g]/NR
Gordon	Clinical specimen	Quebec, Canada	1975	Gauvreau[d]
Bennett	Gastric liquid	Happy Valley, Canada	1976	BRS
FE9507EEA	Feces	Kangiqsualujjuaq, Canada	1995	BRS

Table 5.1 (Continued). List of *C. botulinum* group II strains used for the comparison of PFGE with RAPD and automated ribotyping

Strain ID	Origin	Location	Year of isolation	Source[a]
MI9507E	*Misiraq*	Kangiqsualujjuaq, Canada	1995	BRS
F9508EMA	Feces	Kuujjuaq, Canada	1995	BRS
VI9508E	Seal meat	Kuujjuaq, Canada	1995	BRS
FE9508EPB	Feces	Tasiujaq, Canada	1995	BRS
S9510E	Seal meat	Kuujjuaq, Canada	1995	BRS
GA9709EHS	Gastric liquid	Kangiqsualujjuaq, Canada	1998	BRS
GA9709ENS	Gastric liquid	Kangiqsualujjuaq, Canada	1998	BRS
GA9709EJA	Gastric liquid	Kangiqsualujjuaq, Canada	1998	BRS
FE9709ELB	Feces	Kangiqsualujjuaq, Canada	1998	BRS
FE9709EBB	Feces	Kangiqsualujjuaq, Canada	1998	BRS
FE9909ERG	Feces	Inuvik, Canada	1999	BRS
FE0005EJT	Feces	Inuvik, Canada	2000	BRS
MU0005EJT	*Muktuk*	Inuvik, Canada	2000	BRS

Table 5.1 (Continued). List of *C. botulinum* group II strains used for the comparison of PFGE with RAPD and automated ribotyping

Strain ID	Origin	Location	Year of isolation	Source[a]
SW280E	Saltwater	Koksoak River, Canada	2001	This study[e]
SO309E2	Shoreline soil	Hudson Bay, Canada	2001	This study
SO325E1	Shoreline soil	Ungava Bay, Canada	2001	This study
SO326E1	Shoreline soil	Ungava Bay, Canada	2001	This study
SO329E1	Shoreline soil	Ungava Bay, Canada	2001	This study
SP455456E2	Coastal rock	Ungava Bay, Canada	2001	This study
SOKR-23E1	Marine sediment	Koksoak River, Canada	2002	This study
SOKR-29E1	Shoreline soil	Ungava Bay, Canada	2002	This study
SOKR-37E2	Freshwater sediment	Koksoak R. trib., Canada	2002	This study
Serotype F				
70F	Marine sediment	California coast, USA	1960s	Eklund/Solomon
190F	Marine sediment	California coast, USA	1960s	Eklund/Solomon
202F	Marine sediment	California coast, USA	1965	Eklund/Solomon

Table 5.1 (Continued). List of *C. botulinum* group II strains used for the comparison of PFGE with RAPD and automated ribotyping

Strain ID	Origin	Location	Year of isolation	Source[a]
205F	Marine sediment	California coast, USA	1960s	Eklund/Solomon
610F	Salmon	Columbia R., Oregon, USA	1966	Craig/Solomon
19501F	Marine sediment	Oregon coast, USA	1960s	Eklund/Crowther[f]

[a]First name is the initial source and the second is the donor source; NR: no records of the donor source found.
[b]Solomon H, U. S. Food and Drug Administration, Washington D.C., USA.
[c]Botulism Reference Service for Canada, Health Canada, Ottawa, ON, Canada.
[d]Gauvreau L, Laboratoire de microbiologie, Centre hospitalier de l'Université Laval, Ste-Foy, QC, Canada.
[e]Ecological survey of *Clostridium botulinum* type E in Nunavik, Canada.
[f]Crowther J. S., Unilever Research, Sharnbrook, Bedford, England.
[g]Prévot, A.R., and Huet, M. (1951). Bull. Acad. Natl. Med. *135*, 432-435.

Under these growth conditions, the optical density was 1.2 ± 0.2 at 540 nm of absorbance, corresponding to 10^6 to 10^7 CFU per ml of SPGY broth.

PFGE analysis. The PFGE procedure combined the method of Hielm *et al.* (1998a) and the short DNA preparation protocol of Sperner *et al.* (1999), with some modifications. Cultures grown overnight in 8 mL of SPGY were chilled on ice and cells were harvested by centrifugation at 14,500 × *g* for 10 min at 4°C. Cells were suspended in 960 µL of PIV (10 mM Tris, 1 M NaCl, pH 7.5) and fixed with formaldehyde (4% vol/vol) to reduce the endogenous DNase activity. Cells were washed twice in 1 mL of PIV, harvested by centrifugation at 16,000 × *g* for 5 min, and resuspended in 1 mL of lysis buffer (6 mM Tris, 1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, and 0.5% sodium lauryl sarcosine, pH 7.6) containing RNase A (20 µg/ml), lysozyme (1 mg/ml), and mutanolysin (20 U/ml), which were added to the suspensions just prior to embedding cells in 2% low-melting-point agarose (Seakem® Gold Agarose, BMA, Rockland, Maine). The agarose plugs made from the mixture of equal volumes (500 µL) of cell suspension and 2% low-melting-point agarose were incubated in 3 mL of the same lysis solution at 37°C for 2 h with gentle shaking, then placed in 2 mL of ESP solution (0.5 M EDTA and 10% sodium lauryl sarcosine, pH 8.0) containing 150 µg proteinase K per ml for 2 h at 50°C with gentle shaking. Plugs were washed five times in 15 mL of TE buffer (10 mM Tris and 1 mM EDTA, pH 7.6) at 37°C for 15 min and stored in TE at 4°C until use.

The DNA embedded in agarose was digested with a high concentration of restriction enzymes to reduce the incubation time. Two plugs were restricted with either 200 U of SmaI (Roche Diagnostics GmbH, Mannheim, Germany) or 200 U of XhoI (Roche Diagnostics GmbH) in 150 µL of the corresponding enzyme buffer, and

incubated at 25°C for 2 h or at 37°C for 2 h, respectively. Restriction fragments were separated in a 1% low-melting agarose (Seakem® Gold Agarose, BMA) gel in 0.5× Tris-borate-EDTA (45 mM Tris-borate, 1 mM EDTA) (Sigma®, St. Louis, Missouri), with and without 50 μM thiourea, in a contour-clamped homogeneous electric field CHEF-MAPPER apparatus (Bio-Rad Laboratories, Hercules, California) set at 6.0 V/cm and a temperature of 10°C for 16 h using initial and final pulse times of 1 and 18 s, respectively. A low range PFGE molecular weight marker (New England BioLabs, Ipswich, Massachusetts) was used as a fragment size marker. The gels were stained using ethidium bromide (1 mg/L) for 30 min, destained in distilled water for 60 min, and photographed using a Gel-Doc 1000 system (Bio-Rad Laboratories). Two isolates per strain were grown and tested at different times by PFGE with both restriction enzymes for quality control and to assess the reproducibility of the procedure.

Automated ribotyping. Ribotyping analyses were conducted with the automated RiboPrinter® System (DuPont Qualicon, Wilmington, Delaware, USA) operated with proprietary preparations and membrane processing packs which include reagents for cell lysis, deproteination, restriction digestion, and hybridization. A complete description of the RiboPrinter® system from cell lysis to image analysis was previously reported (Bruce *et al.*, 1995; Bruce, 1996). Two restriction enzymes, EcoRI (Qualicon™) and ClaI (Roche Diagnostics GmbH) were selected based on previous reports (Hielm *et al.*, 1999; Skinner *et al.*, 2000), while PstI (Qualicon™) was tested with *C. botulinum* for the first time. The temperature and time for restriction digestion was set at 37°C for 120 min for ClaI, while the standard protocols for EcoRI and PstI were followed according the manufacturer's instructions. Replicate samples of group II strains were also included within runs for quality control.

RAPD analysis. DNA isolation and RAPD analysis were essentially performed as described by Hyytiä *et al.* (1999a) with minor modifications. Cells in 8 mL of SPGY broths were harvested at 1,500 × *g* and 4°C for 30 min and washed in 5 mL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5), and resuspended in 500 µL of TE containing lysozyme (8.3 mg/ml) and mutanolysin (167 IU/ml) following low-speed centrifugation. Lysis was initially performed by incubating the cell suspensions at 37°C with gentle shaking for 2 h, and then continued for 1 h at 60°C with the addition of proteinase K (50 µg/ml), EDTA (9.5 mM), NaCl (0.24 M), and sodium dodecyl sulphate (0.8%). A phenol-chloroform-isoamyl alcohol (25:24:1) extraction was then performed to remove proteins from the cell lysate (1:1 v/v) and DNA was recovered in the aqueous phase at 16,000 × *g* for 5 min. A subsequent extraction was performed with phenol-chloroform-isoamyl alcohol (25:24:1) to remove any residual proteins and DNA was precipitated with two volumes of 95% ice-cold ethanol at -20°C overnight. The solution was centrifuged at 16,000 × *g* for 20 min, air-dried, and the precipitate resuspended in TE. To remove any RNA present in the extract, RNase A (166 µg/ml) was first heat-treated to remove any DNases, added into the solution and then incubated with gentle shaking at 37°C for 40 min. For better precipitation, sodium chloride (0.21 M) was added prior to performing a third phenol-chloroform-isoamyl alcohol (25:24:1) extraction. DNA was again precipitated with two volumes of 95% ice-cold ethanol maintained for 1 h at -20°C, followed by a 70% ethanol wash. The purified DNA was resuspended in 100 µL of Tris buffer (10 mM Tris, pH 7.5), quantified with a UV-160A spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and diluted to a final concentration of 5 ng/µl.

RAPD analysis was performed using Ready-To-Go™ RAPD Analysis Beads (Amersham Biosciences, Piscataway, New Jersey) which are coated with the required

PCR reagents at defined concentrations, except for the primer. Sample preparation was performed according to the manufacturer's instructions using 10 ng genomic DNA and 25 pmol of a single primer mixed with a RAPD bead in a 25 µl reaction volume. Two previously tested arbitrary primers (Hyytiä *et al.*, 1999; Hyytiä *et al.*, 1999), OPJ-6 (5′-TCGTTCCGCA) and OPJ-13 (5′-CCACACTACC) (Operon Biotechnologies, Inc., Huntsville, Alabama, USA) were used with the following cycling parameters: the mixture was subjected to an initial denaturing step at 95°C for 5 min, followed by 45 cycles of 1 min at 95°C, 1 min at 36°C, 2 min at 72°C, and a final extension step for 5 min at 72°C using a Tpersonal thermocycler (Biometra®, Goettingen, Germany). The amplified products were separated by electrophoresis in a 2% agarose gel using 1× Tris-Borate EDTA (0.09 M Tris, 0.09 M boric acid, 2 mM EDTA) buffer containing 0.5 µg/ml of ethidium bromide at 100 V for 2 h (Mini Gel Electrophoresis System Gel X_L^{plus}, Labnet International Inc., Woodbridge, New Jersey). Two positive controls (*E. coli* strains provided in the Ready-To-Go™ RAPD kit) and a negative control (reaction tube containing PCR reagents, a primer but no DNA) were used for each set of samples. The DNA molecular weight marker VI (154-2176 bp) (Boehringer Mannheim Gmbh, Mannheim, Germany) was used as the size marker. The reproducibility of the method was tested by analyzing two isolates for each strain cultured and tested at different times.

Fingerprint analysis. Comparison of DNA fragment patterns and cluster analysis were performed using BioNumerics version 3.5 software (Applied Maths, Kortrijk, Belgium). The level of similarity between DNA fragment patterns was estimated using the Dice coefficient correlation as determined with the following equation: $S_D = [2n_{AB}/\ n_A + n_B] \times 100$, where n_{AB} is the number of common fragments and $n_A + n_B$

is the total number of fragments in profiles *A* and *B*. Cluster analysis of fingerprints was performed with the unweighted pair group method using arithmetic averages (UPGMA). The optimisation and position tolerance were both set at 1% for all pairwise and cluster analysis, based on the analysis of DNA fragment patterns of duplicate samples tested by PFGE and automated ribotyping. Low fragment sizes (<23.1 kb) generated by SmaI digestions were ignored in the analysis because they were generally much fainter and present in most PFGE patterns of *C. botulinum* type E isolates. The Simpson's index of diversity was used to evaluate the discriminatory ability of the typing methods (Hunter and Gaston, 1988). The index of discrimination is based on the probability that two unrelated strains sampled consecutively from the test population will be the same type and can be calculated with the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

where N is the total number of strains in the sample population, s is the total number of types, and n_j is the number of strains belonging to the jth type.

5.3 Results

PFGE analysis. Of the 39 *C. botulinum* group II isolates analyzed with the modified PFGE protocol, only one type B strain (DB-2) could not be typed due to low DNA yield available for restriction. To overcome this problem, cells from three separate cultures of DB-2 were then combined and fixed with formaldehyde directly in SPGY broth at room temperature and harvested at a lower centrifugation speed. These modifications increased DNA yield and allowed for the typing of DB-2. All DNA of *C. botulinum* group II strains was successfully cleaved with SmaI and XhoI, but the resolution of DNA macrorestriction patterns of several type E strains was thiourea-dependant. When thiourea was not included in the electrophoresis buffer, smearing of the PFGE lane was observed with specific type E strains only and not with type B or F strains belonging to group II. For most of these type E strains, DNA smearing occurred between the markers at 9.4 and 294 kb (Figure 5.1A). Adding thiourea (50 μM) to the running buffer resolved (Figure 5.1B) the smearing of the 21 untypeable type E isolates and did not alter the DNA macrorestriction patterns of the two type E strains included as controls (i.e., typeable without thiourea). Mock digestion without enzymes was performed with embedded DNA from six untypeable type E isolates. After an overnight incubation at 37°C followed by PFGE with inclusion of thiourea, the DNA remained intact, suggesting that the degradation occurs during electrophoresis when thiourea is not added and not during DNA isolation (data not shown). However, elimination of the formaldehyde-fixation step during DNA isolation of 12 group II strains caused smearing in PFGE lanes, regardless of serotype and inclusion of thiourea in the running buffer (Figure 5.2). Pairwise analysis of replicates showed that PFGE was reproducible and only on rare occasions did some replicates differ by the presence or absence of a faint band.

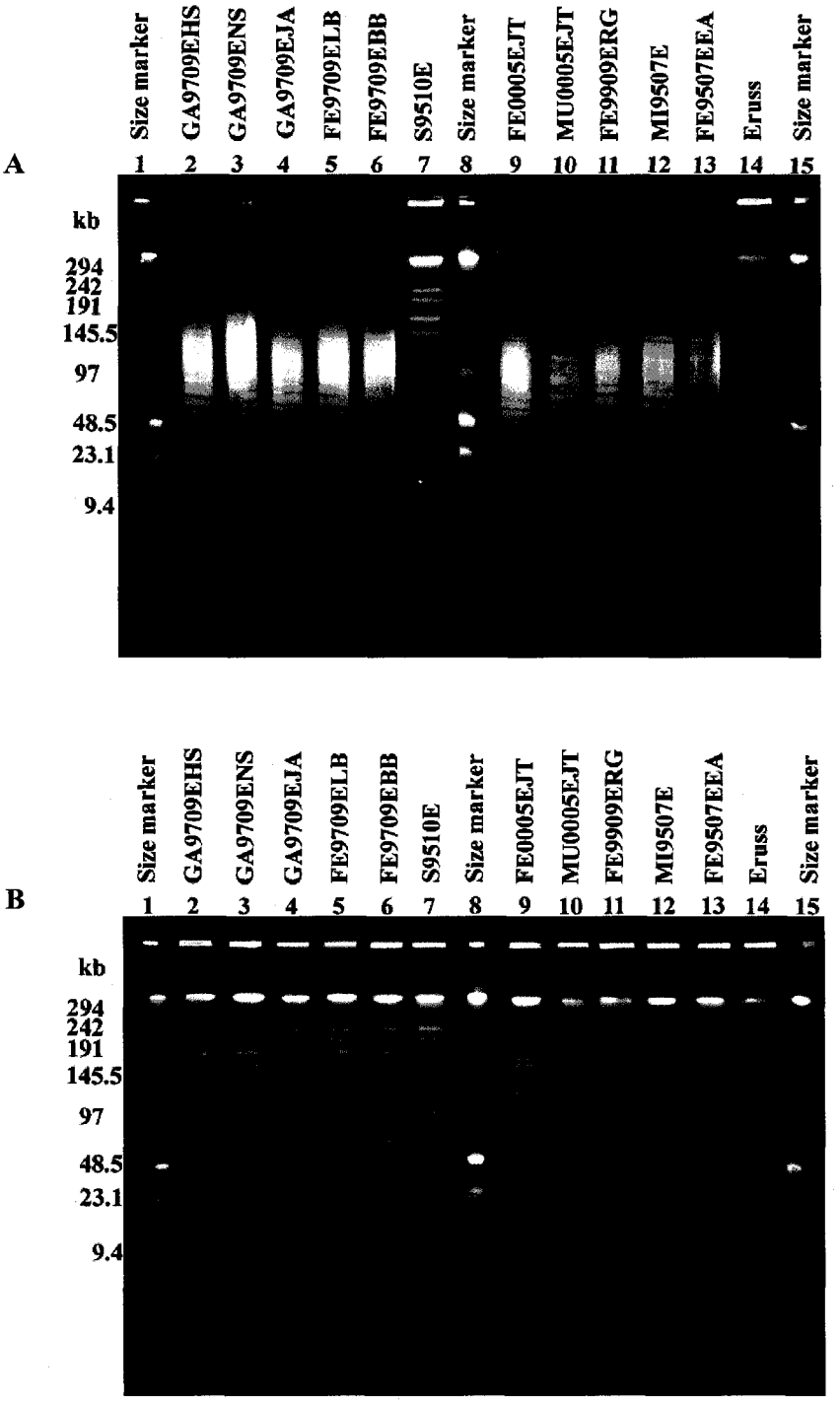


Figure 5.1. PFGE patterns of SmaI digests of food and clinical isolates of *C. botulinum* type E originating from the Canadian Arctic run without (A) and with (B) 50 µM thiourea in the running buffer. Lanes 1, 8, and 15, low PFGE molecular weight marker; lanes 2-6, outbreak 3 strains; lanes 9-10, outbreak 4 strains; lane 11, epidemiologically unrelated clinical isolate yielding identical PFGE pattern to that of strains in lanes 9-10; lanes 12 and 13, outbreak 1 strains; lanes 7 and 14, control type E strains typeable without thiourea.

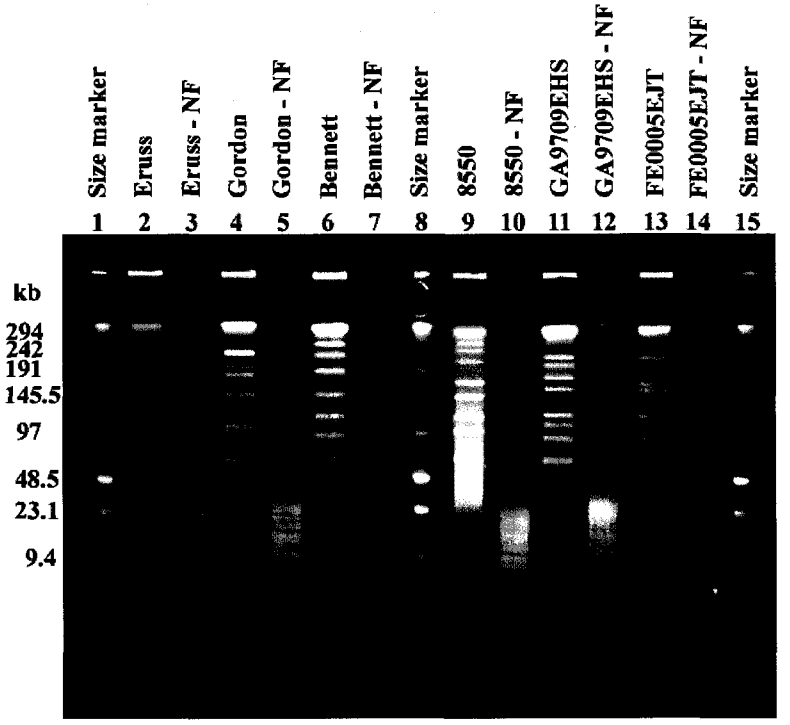


Figure 5.2. PFGE analysis using SmaI digests of six *C. botulinum* type E strains analyzed with and without a formaldehyde fixation step. Lanes 1, 8, and 15, low PFGE molecular weight marker; lanes 2, 4, 6, 9, 11, and 13, strains fixed with formaldehyde; lanes 3, 5, 7, 10, 12, and 14, same strains without formaldehyde fixation. NF: no formaldehyde.

Cluster analysis of SmaI and XhoI PFGE patterns generated dendrograms characterized by a large cluster of type E strains, which diverged from a cluster composed of type B and type F strains at 61% and 55% similarity coefficient, respectively (Figures 5.3-5.4). Both SmaI and XhoI restriction digestions allowed differentiation of 31 epidemiologically unrelated strains of *C. botulinum* group II at the strain level, producing 18 and 23 different pulsotypes, respectively. Despite the relatively low number of fragments (5 to 11) produced, the restriction enzyme XhoI was very discriminatory as evidenced by its high discrimination index (D.I.) of 0.983 (Table 5.2).

The analysis of the epidemiologically related strains demonstrated that PFGE was able to group strains from four type E botulism outbreaks into clusters representing four different pulsotypes. PFGE fingerprints of all related food and clinical isolates within each outbreak were indistinguishable using either SmaI or XhoI, indicating that these pulsotypes represent distinct outbreak strains unique to each incident (Figures 5.3-5.4). PFGE analyses also revealed the high genetic relatedness among isolates originating from a common geographic region or aquatic environment. For instance, SP455456E2, a type E isolate recovered in 2001 from the surface of a coastal rock used for seal butchering possessed PFGE patterns that were indistinguishable from that of isolate SO329E1, a type E strain isolated from a shoreline soil sample collected near the same coastal rock and at the same time. A type F isolate (19501F) originating from marine sediment collected at a depth of 1,326 meters 100 km off the coast of Oregon in 1965 (Eklund and Poysky, 1965) also had identical PFGE patterns to that of isolate 610F, which was recovered from a salmon captured in the Columbia River in Oregon State in 1966 (Craig and Pilcher, 1966).

Table 5.2. Discriminatory ability of PFGE, RAPD, and automated ribotyping for the typing of *C. botulinum* group II strains

Typing methods	Number of			Discrimination index[b]
	Strains[a]	Types	Fragments	
PFGE				
*Sma*I[c]	31	18	7-10	0.951
*Xho*I	31	23	5-11	0.983
RAPD				
OPJ-13	31	28	3-16	0.994[d]
Ribotyping				
*Eco*RI	25	2	1-2	0.280

[a]Strains that were epidemiologically related to an outbreak strain were not included.
[b]Numerical index of the discriminatory ability of typing systems (Hunter and Gaston, 1998).
[c]Fragments below 23.1 kb were not included.
[d]The value of the discrimination index is overestimated due to the low reproducibility of the RAPD technique which had an average coefficient of similarity of 80.5% for duplicate samples with primer OPJ-13.

Figure 5.3. Dendrogram revealing the genetic relatedness of 11 epidemiologically related type E strains originating from four different outbreaks analyzed among 39 *C. botulinum* group II isolates and based on their SmaI PFGE patterns. The epidemiologically-related strains from each outbreak (outbreak No. 1, 2, 3 and 4 in colour) yielded an identical PFGE pattern. The similarity (%) among strains was determined using the Dice Coefficient and the clustering was performed by UPGMA.

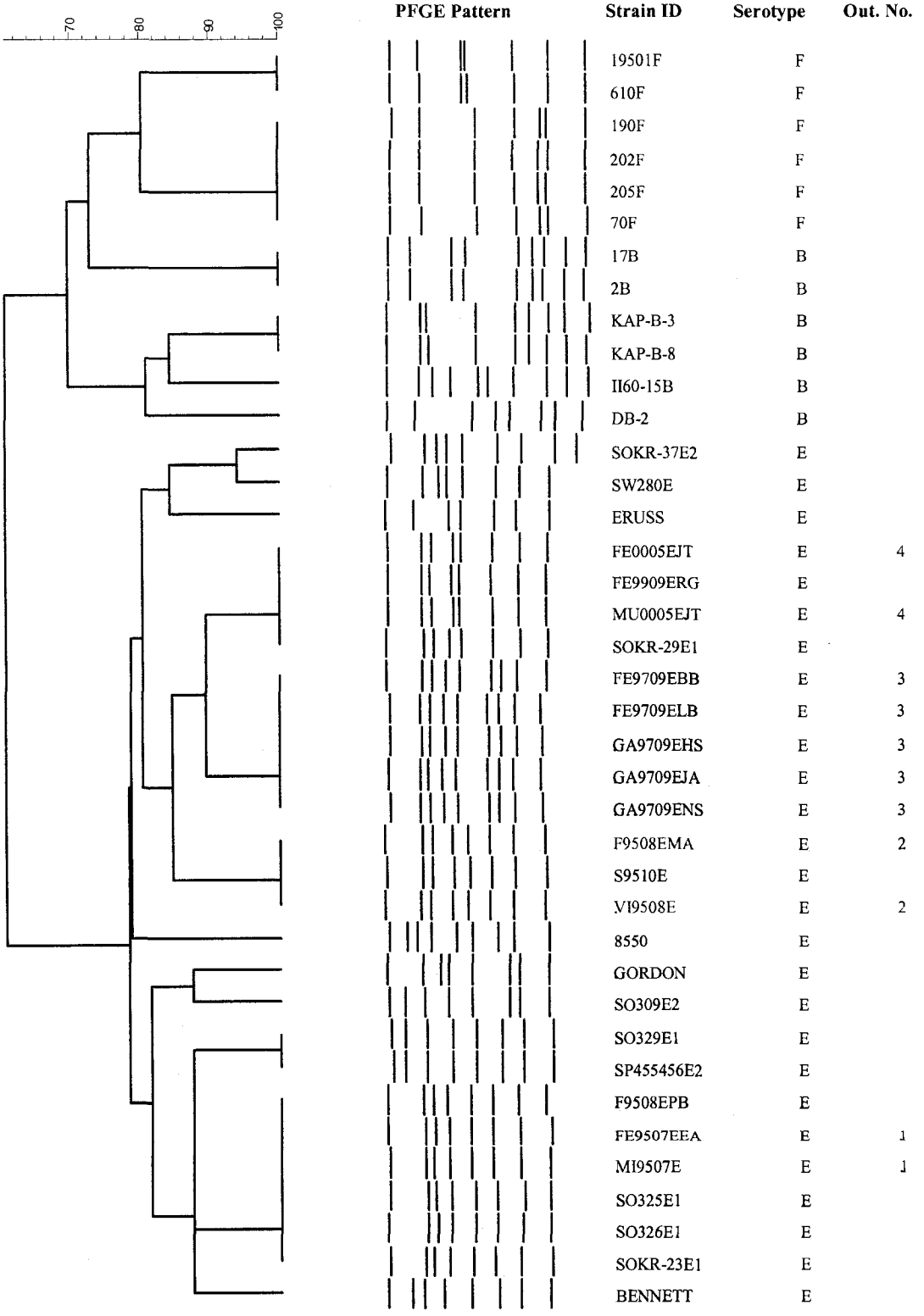
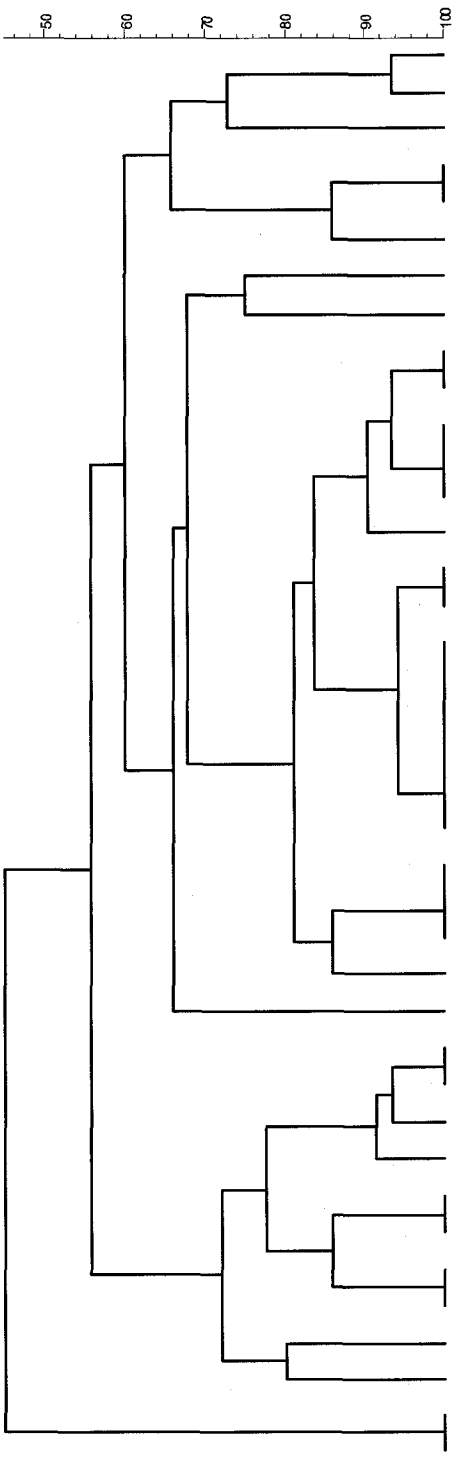

70
80
90
100
PFGE Pattern
Strain ID
Serotype
Out. No.
19501F F
610F F
190F F
202F F
205F F
70F F
17B B
2B B
KAP-B-3 B
KAP-B-8 B
II60-15B B
DB-2 B
SOKR-37E2 E
SW280E E
ERUSS E
FE0005EJT E 4
FE9909ERG E
MU0005EJT E 4
SOKR-29E1 E
FE9709EBB E 3
FE9709ELB E 3
GA9709EHS E 3
GA9709EJA E 3
GA9709ENS E 3
F9508EMA E 2
S9510E E
VI9508E E 2
8550 E
GORDON E
SO309E2 E
SO329E1 E
SP455456E2 E
F9508EPB E
FE9507EEA E 1
MI9507E E 1
SO325E1 E
SO326E1 E
SOKR-23E1 E
BENNETT E

Figure 5.4. Dendrogram revealing the genetic relatedness of 11 epidemiologically related type E strains originating from four different outbreaks analyzed among 39 *C. botulinum* group II isolates and based on their XhoI PFGE patterns. The epidemiologically-related strains from each outbreak (outbreak No. 1, 2, 3 and 4 in colour) yielded an identical PFGE pattern. The similarity (%) among strains was determined using the Dice Coefficient and the clustering was performed by UPGMA.



PFGE Pattern	Strain ID	Serotype	Out. No.
	SOKR-37E2	E	
	SW280E	E	
	8550	E	
	F9508EMA	E	2
	VI9508E	E	2
	S9510E	E	
	BENNETT	E	
	SO309E2	E	
	SO329E1	E	
	SP455456E2	E	
	FE0005EJT	E	4
	FE9909ERG	E	
	MU0005EJT	E	4
	ERUSS	E	
	F9508EPB	E	
	SO325E1	E	
	FE9709EBB	E	3
	FE9709ELB	E	3
	GA9709EHS	E	3
	GA9709EJA	E	3
	GA9709ENS	E	3
	SOKR-29E1	E	
	FE9507EEA	E	1
	MI9507E	E	1
	SOKR-23E1	E	
	SO326E1	E	
	GORDON	E	
	202F	F	
	70F	F	
	190F	F	
	205F	F	
	19501F	F	
	610F	F	
	KAP-B-3	B	
	KAP-B-8	B	
	II60-15B	B	
	DB-2	B	
	17B	B	
	2B	B	

Automated ribotyping analysis. Of the three enzymes tested on group II strains of *C. botulinum*, only EcoRI was able to cleave and generate DNA fragment patterns of good resolution. The EcoRI ribotypes were usually characterized by only two fragments, one intensely-stained and one faint fragment, both located in the lower size-range (~3.2 and 2.1 kb, respectively) (Figure 5.5). All replicate samples (n=7) included were consistently reproducible. Many PstI and ClaI digestions of the 14 and 32 group II strains, respectively, did not produce ribopatterns, regardless of serotype. Of nine PstI (56%) and 16 ClaI (50%) DNA fragment patterns detected by the RiboPrinter software, nine PstI and 14 ClaI ribogroups, respectively, were generated. Many of these ribopatterns were not well resolved with several faint fragments that could not visually be observed on the nylon membrane. Thus, the poor typeability obtained with PstI and ClaI, and the lack of discrimination (D.I. = 0.280) resulting from EcoRI digestion, made any cluster analysis of ribotyping data meaningless.

RAPD analysis. All of the 39 *C. botulinum* group II strains tested by RAPD, generated DNA fragment patterns with both primers used (OPJ-6 and OPJ-13). Both visual and computer analyses showed that the intensity of amplified products of the same strain varied greatly between amplifications. The lack of consistency in fragment intensity and the presence or absence of multiple fragments in one of the duplicate gels, caused difficulty in assessing the reproducibility of the procedure. Pairwise comparison of RAPD duplicates for primer OPJ-6 indicated that the procedure had poor reproducibility for all serotypes of *C. botulinum* group II with 57.4, 65.4 and 52.3% similarity coefficient for type B, E, and F, respectively. The RAPD fingerprints produced with primer OPJ-6 generally contained a large number of fragments, several of which were

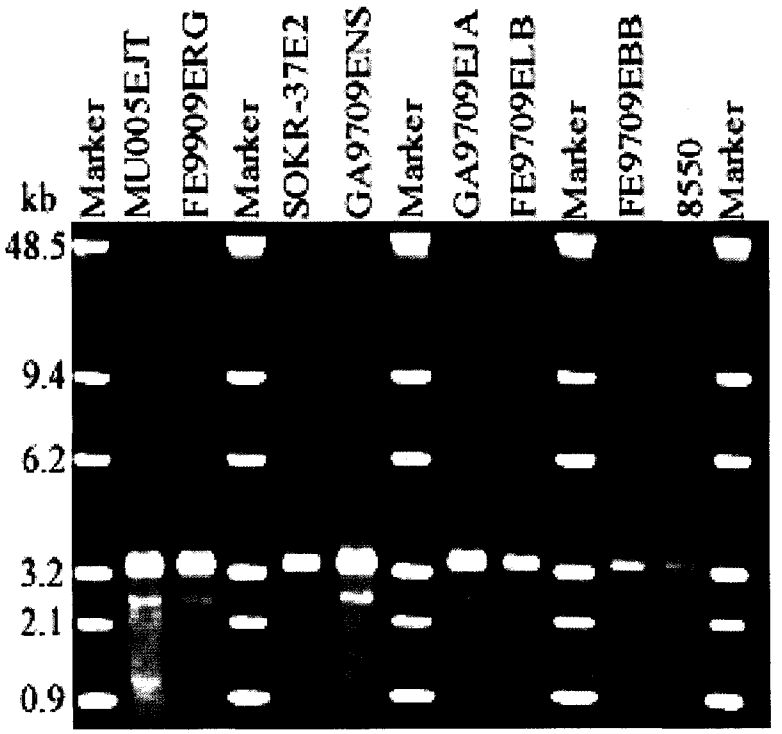


Figure 5.5. Typical EcoRI ribopatterns of *C. botulinum* type E strains using the automated RiboPrinter® system.

minor and very low in intensity (data not shown). The lack of reproducibility was mainly attributed to the presence of non-reproducible faint fragments. However, on several occasions, the fingerprints differed because one or two high-intensity fragments were present or absent in duplicate samples. Despite low reproducibility, many OPJ-6 duplicates shared a general common DNA fragment pattern.

The reproducibility of RAPD patterns performed with primer OPJ-13 was better than OPJ-6 for all group II strains with 85.3, 78.5 and 84.8% similarity coefficient for type B, E, and F, respectively. Up to 14 group II strains differed by ≤ 2 faint fragments and, regardless of serotype, most duplicate fingerprints shared a general common DNA fragment pattern. In contrast to the OPJ-6 fingerprints, those generated with primer OPJ-13 were characterized by a lower number of fragments, several of which were large and bright, facilitating the interpretation (Figure 5.6).

Cluster analysis was performed with RAPD fingerprints obtained with primer OPJ-13 only. The results of the dendrogram analysis should, however, be viewed with caution, due to the lack of reproducibility of the RAPD technique. Each dendrogram consisted of a main cluster of type E strains which diverged from a cluster of type B and F strains (Figure 5.7). The RAPD technique was discriminatory, producing up to 28 distinct RAPD types with a D.I. of 0.994 (Table 5.2). Among all the epidemiologically related food and clinical type E isolates, only two strains from outbreak 2 had identical RAPD patterns, and five strains from outbreak 3 grouped at >85% similarity level (Figure 5.7). However, the genetic relatedness observed among these strains was not reproduced when they were cultured and RAPD typed at different times. All the other food and clinical related isolates from outbreaks 1 and 4 showed distinct RAPD types.

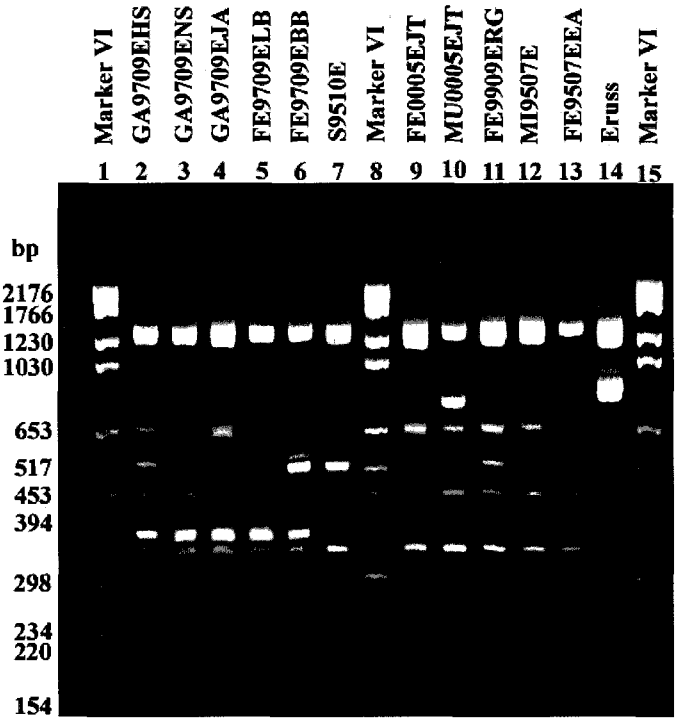
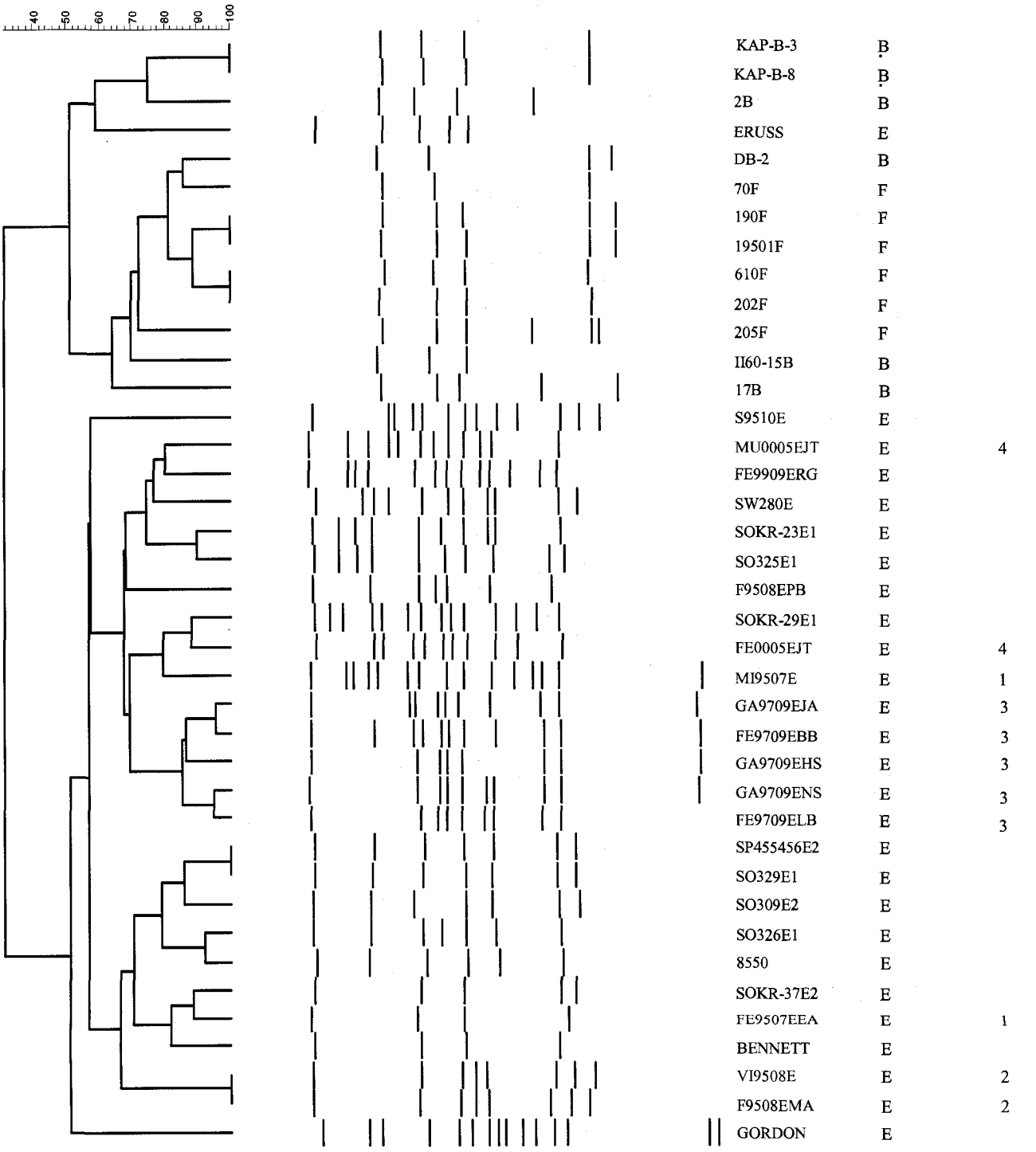


Figure 5.6. RAPD fragment patterns of nine epidemiologically related and three epidemiologically unrelated type E strains of *C. botulinum*. Lanes 1, 8, and 15, DNA molecular weight marker VI; lanes 2-6, outbreak 3 strains; lanes 9-10, outbreak 4 strains; lanes 12-13, outbreak 1 strains; lanes 7, 11 and 14, epidemiologically unrelated type E strains.

Figure 5.7. Dendrogram showing the genetic relatedness of 11 epidemiologically related type E strains originating from four different outbreaks analyzed among 39 *C. botulinum* group II and based on their OPJ-13 RAPD patterns. Only the epidemiologically-related strains (outbreak No. 1, 2, 3 and 4 in colour) from outbreak No. 2 yielded an identical PFGE pattern. The similarity (%) among strains was determined using the Dice Coefficient and the clustering was performed by UPGMA.



RAPD Pattern	Strain ID	Serotype	Out. No.
	KAP-B-3	B	
	KAP-B-8	B	
	2B	B	
	ERUSS	E	
	DB-2	B	
	70F	F	
	190F	F	
	19501F	F	
	610F	F	
	202F	F	
	205F	F	
	II60-15B	B	
	17B	B	
	S9510E	E	
	MU0005EJT	E	4
	FE9909ERG	E	
	SW280E	E	
	SOKR-23E1	E	
	SO325E1	E	
	F9508EPB	E	
	SOKR-29E1	E	
	FE0005EJT	E	4
	MI9507E	E	1
	GA9709EJA	E	3
	FE9709EBB	E	3
	GA9709EHS	E	3
	GA9709ENS	E	3
	FE9709ELB	E	3
	SP455456E2	E	
	SO329E1	E	
	SO309E2	E	
	SO326E1	E	
	8550	E	
	SOKR-37E2	E	
	FE9507EEA	E	1
	BENNETT	E	
	VI9508E	E	2
	F9508EMA	E	2
	GORDON	E	

No linkages could be established between the epidemiologically related type E isolates of any of the four outbreaks when only reproducible fragments were considered in the analysis. However, DNA fragment patterns were reproducibly identical (all fragments included) between KAP-B-3 and KAP-B-8, two *C. botulinum* type B strains isolated from kapchunka (uneviscerated, salted, air-dried whitefish) in 1981 (CDC, 1985), and two type E isolates (SP455456E2 and S0329E1) recovered from the same coastal environment in 2000.

5.4 Discussion

PFGE is the current gold-standard method for strain differentiation of bacterial pathogens of public health significance to assess the epidemic spread of infectious diseases in hospitals (Klaassen *et al.*, 2002; Strandén *et al.*, 2003) and trace foodborne outbreaks (Barrett *et al.*, 1994; Maslanka *et al.*, 1999; Ahmed *et al.*, 2000). Although PFGE is reproducible and discriminatory, some strains of *C. botulinum* type A and E have not been typeable using PFGE (Hielm *et al.*, 1998a,b; Franciosa *et al.*, 2004) due to DNA degradation, despite the use of formaldehyde prior to DNA isolation. In this study however, we have shown that addition of 50 μM thiourea to the running buffer overcomes the problems of DNA degradation, with all 21 type E isolates previously untypeable now producing high-quality fingerprints. At this thiourea concentration, typical smearing was consistently replaced by clear, interpretable DNA macrorestriction patterns that appeared sharp with reduced background. However, both inactivation of enzymes with formaldehyde prior to DNA isolation and the addition of thiourea during electrophoresis were necessary to achieve typeablity of all *C. botulinum* type E strains tested. Ray *et al.* (1992) first demonstrated that DNA degradation can occur during electrophoresis due to Tris buffer-dependent cleavage of double stranded DNA caused by the production of a peracid derivative of Tris formed at the anode (Ray *et al.*, 1995). The use of thiourea presumably prevents degradation of the DNA by scavenging the nucleolytic Tris derivative (Ray *et al.*, 1992).

While it is not known what makes some type E strains sensitive to DNA degradation during electrophoresis, while others are resistant, this trait was associated with specific sites of DNA modifications of *Streptomyces lividans*, which render the DNA susceptible to Tris-dependent cleavage (Evans *et al.*, 1994). None of the type B and type F *C. botulinum* group II strains in this study showed evidence of DNA

degradation without the use of thiourea, indicating that DNA degradation was limited to specific type E strains. Römling and Tümmler (2000) occasionally observed that only one isolate from a clonal group of variants of *Pseudomonas aeruginosa* was affected by DNA degradation and suggested that DNA degradation is not a stable trait. Some of the type E strains originally untypeable by PFGE were typed successfully at a different time without the addition of thiourea in the running buffer, but only when they were digested with SmaI, and not XhoI (data not shown). These results are difficult to explain since according to the manufacturer, the formulation of SmaI was not changed. Initial research on degradation of *S. lividans* DNA during electrophoresis revealed that DNA degradation was dependant upon batches of chemicals used in Tris-acetate-EDTA (TAE) buffer preparation (Zhou *et al.*, 1988). It was also demonstrated that the addition of ferrous ions to TAE buffer caused degradation of *S. lividans* DNA (Zhou *et al.*, 1988). Whether the electrophoretic conditions were different between some of the PFGE runs of *C. botulinum* type E DNA due to differences in ferrous iron concentrations in the running buffer or whether some other factors had an impact remains unknown, but nonetheless, thiourea was effective, enabling characterization of all type E strains previously not typeable without its inclusion.

PFGE was able to epidemiologically link related isolates from four type E botulism outbreaks using either SmaI or XhoI. Among the 31 epidemiologically unrelated group II strains, 18 and 23 different pulsotypes were produced with SmaI and XhoI, respectively. These results are in agreement with other researchers who found that XhoI was more discriminatory than SmaI for the subtyping of *C. botulinum* type E in fish and fishery products from Finland and other areas (Hielm *et al.*, 1998b; Hyytiä *et al.*, 1999b). The recognition sequence of SmaI (CCCGGG) has the same length as XhoI (CTCGAG), but has no AT bases. Since the genome of *C. botulinum* is low in GC

content with less than 30%, the SmaI restriction enzyme, being rich in GC recognition sites, may have generated larger fragments (>400 kb) not separated under the electrophoretic conditions which were used, but this remains to be verified.

All strains belonging to *C. botulinum* group II were typeable by automated ribotyping system using EcoRI, including the type E strains sensitive to DNA degradation. These results differed from a previous study which suggested that DNA degradation was the reason that only 2 of 13 type E strains were typeable with EcoRI (Skinner *et al.*, 2000). The different growth conditions and cell harvesting techniques between the two studies might have resulted in different DNA yields available for restriction digestion and hybridization. However, most EcoRI ribopatterns from *C. botulinum* group II strains were characterized by two fragments of approximately 3.2 and 2.1 kb. These results may indicate that most EcoRI restriction sites are conserved among *C. botulinum* group II and that these strains contained low copy numbers of rRNA operons and/or different rRNA operon copies located on fragments of similar molecular size.

The particular EcoRI ribopattern that we obtained was observed in only three of seven group II strains analyzed by automated ribotyping in another study (Skinner *et al.*, 2000) and several additional fragments were resolved with the typical intensively-stained fragment of approximately 3.2 kb from 19 group II strains analyzed by manual ribotyping (Hielm *et al.*, 1999). The lack of discrimination by automated ribotyping observed in the present study contrasts with the results of Hielm *et al.* (1999), who found that manual ribotyping was as good as PFGE in differentiating *C. botulinum* group II strains. However, because of difficulties in interpreting fragments in the region above 3 kb, the authors recognized that manual EcoRI ribotyping was not optimal for group II strains. The presence of some fragments larger than 3 kb was observed in some

ribopatterns generated in our study, but these were too weak in intensity to be selected as true fragments. Comparison of the two ribotyping techniques for the differentiation of clinical isolates of *Vibrio cholerae* O1 ribotypes also showed that small fragments weak in intensity that were detected in ribopatterns generated by manual ribotyping were not observed by the automated system (Dalsgaard *et al.*, 1999).

Automated ribotyping did not allow differentiation of *C. botulinum* group II strains below the group level when using EcoRI. A previous study (Skinner *et al.*, 2000) on the analysis of *C. botulinum* groups I and II by automated ribotyping using EcoRI showed that type B strains from group II can cluster under the same ribogroup as type B strains from group I, despite these groups belonging to two evolutionary distinct lineages based on 16S rRNA gene sequences (Collins and East, 1998). These unexpected findings have raised questions about the identity of some group II strains analyzed by automated ribotyping and were addressed in another report (Hielm *et al.*, 2001). In this study, all EcoRI ribopatterns of group II isolates were similar to each other, but very different from group I ribopatterns observed in our laboratory (data not shown). Our current database of ribotypes of *C. botulinum* group I and group II supports a clear taxonomic divergence between these two groups, in good agreement with the results obtained by manual ribotyping (Hielm *et al.*, 1999). Based on 16S rRNA sequence data, strains of non-proteolytic *C. botulinum* type B, E and F form a single phylogenetic group (Hutson *et al.*, 1993). The level of discrimination achieved by PFGE and RAPD was higher than automated ribotyping. Dendrograms generated from both RAPD and PFGE results indicated one large cluster of type E strains separated from another cluster composed of type B and F isolates. Cluster analysis of EcoRI ribopatterns of *C. botulinum* group II produced by manual ribotyping also resulted in the clustering of type B and F strains separate from type E strains (Hielm *et al.*, 1999).

Although all *C. botulinum* group II strains could be typed using RAPD, the technique was hampered by its low reproducibility, particularly with primer OPJ-6. Even with an average coefficient of similarity of 80.5%, primer OPJ-13 was not capable of adequately grouping most epidemiologically related isolates. Our results contrast with those of Hyytiä *et al.* (1999a), who obtained highly reproducible results with RAPD analysis of *C. botulinum* group I and II using the OPJ-6 and OPJ-13 primers. However, they did not specify if all faint fragments, including those that were not reproducible, were included in their analysis of reproducibility. In our study, all fragments were included in the analysis, which means that two replicate samples differing by only one faint fragment were considered two different RAPD patterns. Several factors affecting the reproducibility of arbitrary PCR amplified methods have been well documented (Meunier and Grimont, 1993; Tyler *et al.*, 1997), and include the selection of primers (Tang *et al.*, 1995). Bidet *et al.* (2000), working with *C. difficile,* obtained a reproducibility of 100% for PCR-ribotyping and PFGE analysis, while the reproducibility for arbitrarily primed PCR (AP-PCR) ranged from 33 to 88% using three different 10-mer primers.

Among the three typing methods, the highest D.I. was obtained with RAPD typing using primer OPJ-13. However, this result should be interpreted with caution as the reproducibility of the method was not 100%, which is usually required to calculate the D.I based on the Simpson's diversity index. The effect of reproducibility on the discriminatory indices of typing systems has been previously shown by Hunter (1990), who proposed the use of a standardized D.I based on a predetermined reproducibility for adequate comparison. However, the latter method has not been validated using typing methods generating DNA fragment patterns and such application would be

difficult when the compared typing methods yield remarkably different number of fragments such as RAPD and automated ribotyping.

Fingerprinting analysis of RAPD patterns of *C. botulinum* type E has been traditionally performed by visual examination, presumably because of the presence of a large number of low molecular weight fragments and the frequent occurrence of faint fragments, which makes computer analyses more difficult (Hyytiä *et al.*, 1999b). To control variation in DNA fragment patterns from run-to-run, non-reproducible faint fragments were excluded in the analysis of RAPD patterns (Hyytiä *et al.*, 1999b). Such fingerprint analysis is time consuming and would be very challenging when large datasets are being analysed. The epidemiological concordance of RAPD analysis performed with OPJ-13 did not improve between any of the epidemiologically related strains of the four outbreaks by selecting only reproducible faint fragments.

Comparison of typing methods showed that an optimized PFGE incorporating thiourea was the only fingerprinting method able to successfully identify epidemiologically related strains of *C. botulinum* type E belonging to four different outbreaks. RAPD was found to be unreliable for the molecular typing of group II strains and is therefore not recommended for epidemiological investigations. Despite being reproducible, EcoRI automated ribotyping system did not allow for the differentiation of *C. botulinum* group II below the group level. A new set of restriction enzymes may improve the discriminatory power of automated ribotyping. Our results have shown that PFGE is a reliable and discriminatory molecular typing method for outbreak investigations of type E botulism and molecular epidemiological studies in the Canadian Arctic.

CHAPTER 6

MICROBIAL SOURCE TRACKING AND GENETIC DIVERSITY OF *CLOSTRIDIUM BOTULINUM* TYPE E IN THE COASTAL ENVIRONMENT

6.1 Introduction

The link between botulism outbreaks and sources of *C. botulinum* contamination has not yet been demonstrated using molecular epidemiological tools. Genetic fingerprinting methods have been employed in molecular epidemiology to trace infectious diseases and determine common sources of infections in hospital (Kuijper *et al.*, 1987) and agricultural environments (Hosoglu *et al.*, 2003). Pulsed-field gel electrophoresis (PFGE) is considered the gold-standard method for bacterial typing (Struelens, 1998) and has recently been found to be a reproducible and discriminatory fingerprinting method for typing epidemiologically related isolates of *C. botulinum* type E (Leclair *et al.*, 2006). Several microbial source tracking (MST) methods including genotypic and phenotypic techniques are being developed to track sources of bacteria in water and food (Meays *et al.* 2004). Generally, these methods require optimization to reduce false-positive rates and the representation of known-source libraries has to be further developed to allow accurate classification (Griffith *et al.*, 2003; Myoda *et al.*, 2003; Stoeckel *et al.*, 2004).

PFGE has not been fully investigated for use in MST studies of bacterial pathogens (Scott *et al.*, 2002). However, the technique has been used for studying the genetic biodiversity of *C. botulinum* type E in aquatic environments (Hielm *et al.*, 1998a,b; Hyytiä *et al.*, 1999b) and was proposed to be used for tracing and identifying critical control points of contamination with *C. botulinum* type E in the fish and seafood industry (Hielm *et al.*, 1998b). Sources of coastal contamination of marine mammal meat with *C. botulinum* type E could be detected by field studies and traced by comparing isolates present in the aquatic environment in proximity to butchering sites, with those recovered from meat processed on the same site. Such data may provide insights on the origin and dispersion of *C. botulinum* spores in the coastal environment,

and help to identify transmission routes in marine mammal meat. The objectives of the study were to identify routes of transmission of *C. botulinum* type E in seal meat from endogenous and environmental sources by MST using PFGE and determine the genetic biodiversity of *C. botulinum* type E in the coastal environment.

6.2 Materials and Methods

Seal hunting and location of butchering sites. Two seal hunting trips were organized during the month of July 2001 at Diana Bay near Tasiujaq, and Big Island situated in the mouth of Whale River, east of Kuujjuaq. A detailed description of the harvest and the various butchering sites used for processing one ringed seal and three bearded seals in the coastal environment was provided in Chapter 4.

Sources and contamination routes. The coastal environment surrounding each butchering site was investigated to determine if the shoreline soil, rocks, and seawater contained spores of *C. botulinum* type E at the time of butchering. Both the skin and the intestines of each harvested seal were also investigated for the presence of endogenous spores. Collection of environmental and tissue samples was performed as previously described in Chapter 3. Prior to each butchering, the blades of the hunter's knife were cleaned with sterile double-distilled water and flamed with 95% ethanol. The animal was laid on its back and the hunter proceeded to the cutting, evisceration, and butchering of the carcass as normally done. During the butchering process, a series of meat and fat samples were collected to determine if any identified high-risk handling practices in the field could act as a mode of contamination of seal meat with *C. botulinum* type E spores. Following the observation of gross contamination resulting from a high-risk handling practice, a sample (average weight: 52.7 g) was cut from the butchered carcass using a surgical scalpel, transferred into a Whirl-pack™ bag (Fisher Scientific Ltd, Montreal, Quebec) and kept under cool conditions in the field until they were stored at –20°C in the Health Canada laboratories in Ottawa. The blade of the scalpel was changed or flamed between each set of samples to avoid cross-contamination. After butchering, the blade of the hunter's knife was swabbed with a

bacterial sponge (bio-spo™, Solar biologicals Inc., Ottawa, Ontario) to determine if the blade was contaminated with spores of *C. botulinum* type E.

Meat preparation and aging process. From each hunted seal, a preparation of meat and fat tissues was aged into *igunaq* and tested for viable *C. botulinum* and botulinum neurotoxin to determine if the end-products were contaminated during butchering. The carcass of the ringed seal (seal number 1) was cut into large pieces and placed on the surface of a coastal rock. The meat was further cut into long narrow pieces and immediately transferred into a large water bottle (11 L) through the small opening. In addition to meat, fat tissues from the large blubber layers attached to the skin were cut in the same way and mixed with the meat inside the container. The hunter included enough fat tissue to achieve a meat/fat ratio <1. The container was filled up to two-thirds (one-third headspace) and left opened overnight on the coastal rock. The preparation was then placed near the cabin for two days until it was transported to Kuujjuaq for aging into *igunaq*.

Similar to the ringed seal, two bearded seals (seals number 2 and 3) were butchered on the surface of large coastal rocks, and tissues were collected for use in preparations of *igunaq*. Tissues were cut into large pieces and put into plastic bins. The containers were filled to the top with no headspace, and sealed with plastic covers that were perforated by the hunter. A third bearded seal (seal number 4) was laid in the mud, tissues were carefully collected from the carcass and transferred into a similar plastic bin placed inside the boat. The container was filled with meat and fat tissues and sealed as previously described for seals number. 2 and 3. Upon arrival at Kuujjuaq, the four meat preparations were placed beside the *igunaq* producer's shed and covered with a old carpet and a sheet of plywood to protect the preparations from dogs. According to

the hunter, the perforated covers enabled the air to circulate inside the containers during the entire length of the aging process (July 19 to September 17). Preparations were monitored three to four times a week by the producer by cutting and examining a piece of aged meat during the morning for its appearance, smell and taste.

Detection of *Clostridium botulinum* type E. Cultivation of shoreline soil, bacterial sponges, skin and intestinal content samples of hunted seals was performed as described in Chapter 2. Initial samples of unprocessed raw meat collected from seals number 1 and 4 were cultured according to the official Health Canada method (Austin and Blanchfield, 1996), with minor modifications. Each sample was weighed and stomached with GPB in a 1:4 ratio for 5 and 10 min for meat and fat samples, respectively. Stomached samples were transferred in their entirety into a sterile 250-mL centrifuge bottle and centrifuged at 18,500 × *g* for 20 min (4°C). The supernatant was poured off and approximately half of the tissue pellet was transferred into a second bottle. Sixty millilitres of SPGY broth was added to each bottle, with one bottle being subjected to a heat treatment of 60°C for 20 min to reduce the background flora. All samples were then trypsinized (0.1% w/v), and incubated for 7-10 days in an anaerobic chamber (10% H_2, 10% CO_2, and 80% N_2) at room temperature then assayed for toxin production. Samples of seal number 2 were processed as described except that, due to the difficulty in adequately pelleting the samples during centrifugation, the GPB wash step was not performed for fat samples. The procedure was simplified for samples of seal number 3, for which no wash steps were performed for the meat samples. SPGY broth was therefore added directly to the pre-weighed fat and meat samples, and then processed accordingly.

Each of the four batches of aged meat, identified respectively as *igunaq* number 1 to *igunaq* number 4, were first tested for toxicity using the mouse bioassay. A sample of 32 mL of *misiraq* (oil part) was aspirated from the bottom of the container, mixed with 8 mL of GPB in a 100-mL Nalgene™ tube, and then centrifuged at 17,900 × *g* for 20 min (4°C). The extract or water phase was aspirated and kept refrigerated until tested by the mouse bioassay. The oil phase was decanted and the pellet suspended in 8 mL of GPB before being dispensed equally in four CMM culture tubes to determine the presence of viable spores or vegetative cells of *C. botulinum* type E. To reduce the background flora, two inoculated CMM tubes were heated at 60 and 75°C for 20 min, respectively, and one inoculum was treated with 50% ethanol for 1 h at room temperature. The alcohol-treated tube was centrifuged at 17,900 × *g* for 20 min (4°C) and then, after the alcohol fraction was decanted, the CMM broth was added to the sediment. The fourth inoculum was left untreated and transferred into a CMM culture tube. All cultures were incubated in an anaerobic chamber (10% H_2, 10% CO_2, and 80% N_2) at room temperature for 7 days for toxin production.

Following the finding that *igunaq* number 3 and 4 were toxic as determined by the mouse bioassay, it was decided to perform additional toxin assays and cultures of *misiraq* and *igunaq* samples to determine the extent of contamination and toxicity within each batch, starting with the non-toxic batches, i.e., *igunaq* number 1 and 2. Prior to sampling the *igunaq*, a 32 mL sample of top *misiraq* was aspirated from three locations, while another was collected after the *igunaq* preparation was stirred using a large sterile spatula (mixed *misiraq*). These *misiraq* samples were tested for toxicity and cultured as previously described for the bottom *misiraq* samples, but no pre-treatment at 75°C was performed. Ten large pieces of *igunaq* were then aseptically collected to avoid cross-contamination and approximately 50 g of surface meat from each piece was

cut off using sterile curved scissors. Small pieces of *igunaq* were pooled and stomached with 100 mL of GPB for 5 min and the mixture was then transferred in a 250-mL sterile culture tube and centrifuged at 18,500 × *g* for 20 min (4°C). Approximately 10 mL of the extract was aspirated and kept in a vial (20 mL) at 4°C until tested for toxicity, while the rest was decanted. Between 2 to 4 g of the sediment was used to inoculate each of the CMM tubes that were cultured using the same pre-treatments and medium as for the *misiraq* samples.

Field and laboratory quality control. Samples of raw meat and fat were collected from three harvested seals for quality control. Tissue samples were spiked in the field with a known number of *C. botulinum* type E spores to assess the effects of storage and transport on the quality of the samples and the performance of the laboratory procedures. A multiple-strain spore suspension composed of four laboratory type E strains at 10^4 spores per mL was prepared and brought to the field as described in Chapter 3. An inoculum of 1000 spores or 0.1 mL of the spore suspension was dispensed on the surface of each control tissue sample. Similarly to the non-spiked samples, the QCS were kept under cool conditions from field through transport, and then were stored at -20°C in the Health Canada's laboratory until analysis. In addition, a set of meat and fat samples were spiked in the laboratory with a low number of type E spores to assess the sensitivity of the laboratory procedures. A different spore suspension containing the same type E strains was prepared and used to spike six 50-g samples of meat or fat with approximately 10 spores. Samples were preheated, cultured, and tested for toxicity as described for the unprocessed raw meat samples. All spore suspensions used for spiking controls in this study were verified by plate counts according to the method described in Chapter 3.

Toxin assay and serotyping. All culture supernatants were assayed for botulinum neurotoxin using the mouse bioassay as previously described in Chapter 2. The *misiraq* and *igunaq* extracts were similarly tested except that they were not diluted prior to injection.

Isolation. Isolation of *C. botulinum* was performed by colony blot immunoassay as described in Chapter 2 and up to three confirmed colonies were purified and kept for PFGE analysis.

PFGE. Fingerprinting analysis of bacterial DNA was conducted to assess if the environmental or endogenous sources of *C. botulinum* type E were genetically related to those involved in the contamination of raw meat and finished products during butchering. PFGE analyses were performed according to the method described in Chapter 5. Isolates recovered from the ecological survey of *C. botulinum* type E were also typed by PFGE to determine the genetic diversity and the distribution of subtypes in the coastal environment of Nunavik. The PFGE patterns of each environmental isolate was classified into subtypes designated by a Roman numeral based on the combination of SmaI and XhoI pulsotypes they produced.

6.3 Results

Tracing contamination of seal meat with *C. botulinum* type E at Diana Bay. The number of fat and meat samples collected from seal No. 1 for tracing contamination during butchering was limited (n=4; includes 2 QCS). Due to the size of the animal, most pieces of meat and fat that were butchered were primarily kept to produce the first batch of *igunaq* (*igunaq* No. 1). A sample of meat and another one of fat were collected from tissues that were directly in contact with the surface of the coastal rock. One culture of the single raw fat sample was found toxic by the mouse bioassay, indicating that contamination of tissues occurred during the butchering process (Table 6.1). However, *C. botulinum* type E was not isolated from the broth culture by the colony blot procedure.

The blade surfaces of the knife used to butcher the animals was not found contaminated with spores of *C. botulinum* type E at the end of butchering. Analysis of the ready-to-eat *igunaq* No. 1 initially showed that two samples of *misiraq* (fat dissolved in oil during aging) and three of *igunaq* (aged meat) were contaminated with *C. botulinum* type E, while all extracts of *igunaq* and *misiraq* samples (n=13) from *igunaq* No. 1 were negative for botulinum toxin. Figure 6.1 shows preparations of seal *igunaq* before and after the aging process. The PFGE patterns of six isolates recovered from these positive samples were all identical and indistinguishable from the PFGE pattern of a type E strain isolated from another *igunaq* preparation (*igunaq* No. 3). These results suggest possible cross-contamination of the type E isolate from *igunaq* No. 3 to *igunaq* No. 1 at a late stage of the aging process, since none of the samples from *igunaq* No. 1 contained type E botulinum neurotoxin. All positive cultures of *misiraq* and *igunaq* samples from *igunaq* No. 1 were therefore considered negative.

Table 6.1. Sources and contamination of seal meat with *C. botulinum* type E at different butchering sites

Seal ID	Environmental sources		Animal sources		Contaminated surfaces[c]		Contaminated tissues and *igunaq*				Toxicity	
	Shoreline soil	Seawater	Intestinal content	Skin	Coastal rock	Knife	Raw meat	Raw Fat	Aged meat[a]	Oil[b]	Aged meat	Oil
No. 1	Positive	Negative	Negative	Negative	Negative	Negative	0/1[d]	1/1	0/10	0/3	0/10	0/3
No. 2	Positive	Negative	Positive	Negative	Negative	Negative	0/4	0/5	0/10	0/3	0/10	0/3
No. 3	Positive	Negative	Negative	Negative	Negative	Negative	0/7	0/6	6/10	3/3	1/10	2/3
No. 4	Positive	Positive	Negative	ND[e]	Negative	Negative	0/4	¼	8/10	3/3	10/10	3/3

[a]Aged meat means *igunaq* in Inutittut.
[b]Oil part of the *igunaq* preparation is called *misiraq*. Note that there is a traditional condiment also called *misiraq*, which is produced by aging fat without meat.
[c]Surfaces of the knife and the coastal rocks were swabbed after butchering.
[d]Number of positive samples/total number of samples.
[e]ND: not determined.

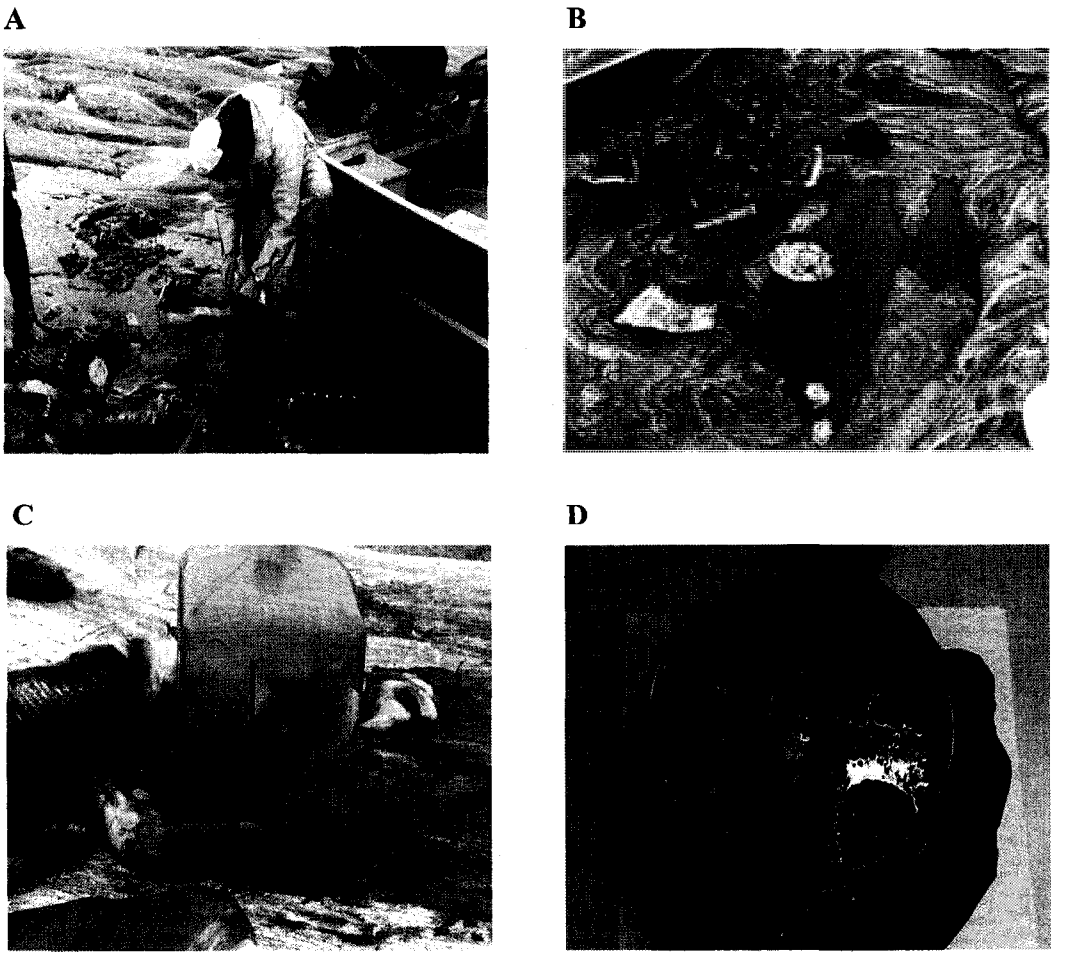


Figure 6.1. Seal *igunaq* preparations before and after the aging process. Prior to *igunaq* preparation, panel A shows the sampling of meat tissues after being rinsed with seawater. Panel B shows *igunaq* No. 2 preparation made with tissues from seal No. 2 at Big Island. Panel C shows the preparation of seal *igunaq* No. 1 contained in a water bottle brought at Diana Bay. The arrow shows a temperature datalogger inserted in the preparation for the monitoring of temperature during the aging process. Panel D shows one of the four *igunaq* preparations after two months of storage outside from mid-July to mid-September in Kuujjuaq. Note the presence of a large amount of oil released from the fat tissues during the aging process and the changes in the colour of the tissues due to oxidation.

Spores of *C. botulinum* type E were not detected from the abdominal skin sample or the intestines of seal No. 1. At the butchering site, spores of *C. botulinum* type E were detected in the shoreline soil, but not in the seawater sample or on the surface of the coastal rocks used for butchering seal No. 1. The origin of the coastal spores was investigated by sampling two aquatic systems surrounding the campsite of Diana Bay, the Dancelou River and a peat bog network behind the campsite (Figure 6.2). Two of eight cultures of sediments collected from a peat bog network and three cultures of sediments from the Dancelou River were positive by the mouse bioassay, indicating the presence of viable *C. botulinum* type E cells. Of nine type E isolates recovered from these aquatic environments, eight distinguishable PFGE subtypes were identified with both SmaI and XhoI restriction enzymes, indicating a high degree of genetic diversity. None of the sediment strains collected from upstream waters were found identical to shoreline soil isolate SOKR3602E1. However, two sediment isolates (SOKR-34E5 and SOKR-49E1) isolated from two locations in the Dancelou River had identical PFGE patterns, as well as two isolates (PBKR-41E1 and SOKR-33E1) recovered from two closely located areas of the peat bog network (Figure 6.3). The peat bog isolates originated from a plateau situated above the river channel and differed from SOKR-49E2, a strain isolated from the river bed, by only two bands with SmaI (>88% similarity level), and were identical with XhoI.

Tracing contamination of seal meat with *C. botulinum* type E at Whale River. The first two bearded seals (seals No. 2 and No. 3) harvested in Whale River were butchered on large flat rocks lining Big Island at high tide. Of nine samples of meat or fat collected from seal No. 2 that were visually contaminated with dirt from the rock surface, none was found to be contaminated with type E spores of *C. botulinum*.

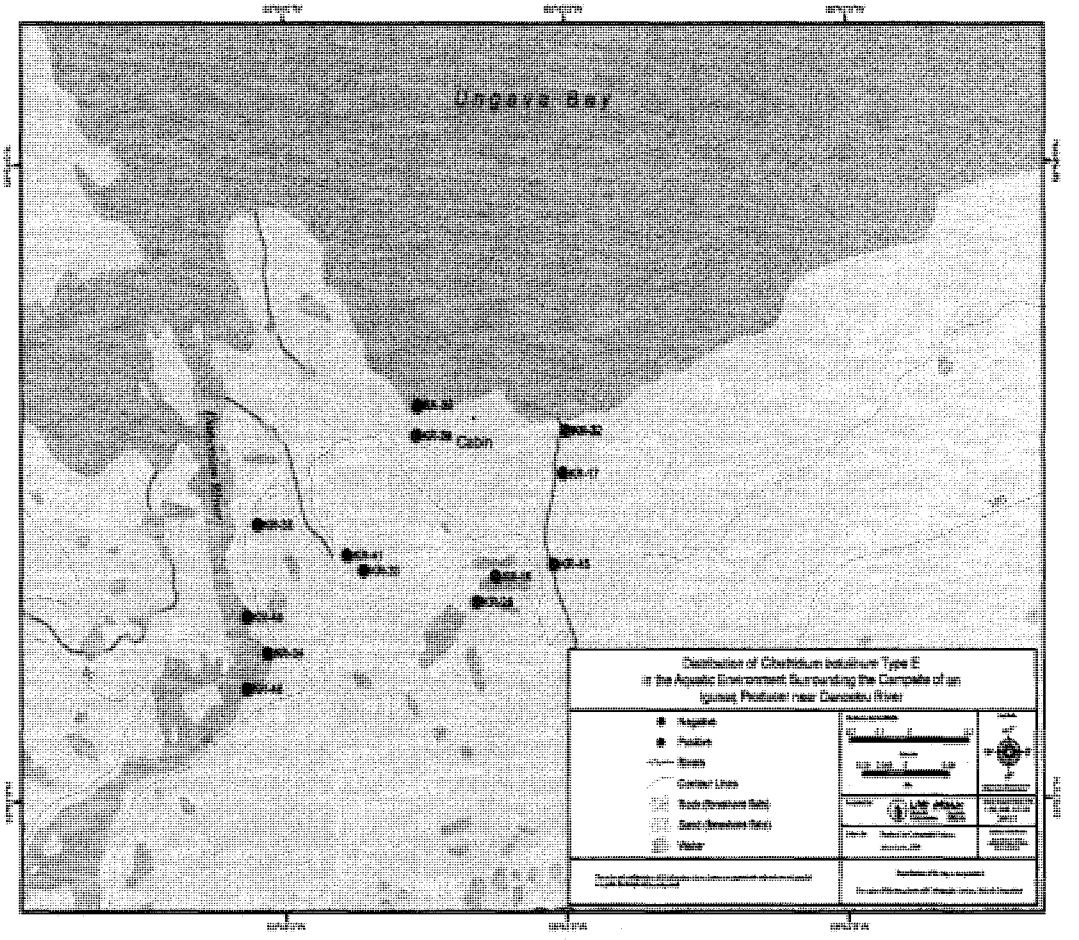
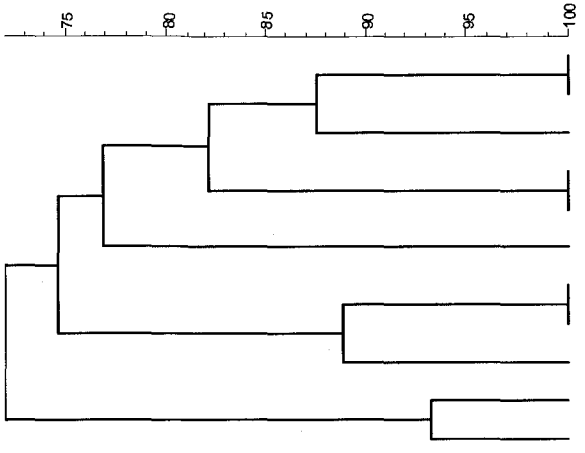
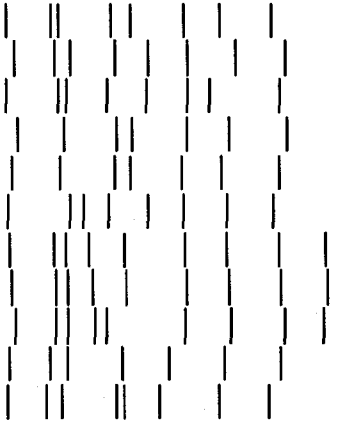


Figure 6.2. Distribution of *C. botulinum* type E in the aquatic environments surrounding the campsite of Diana Bay located near the mouth of the Dancelou River.

Figure 6.3. Dendrograms showing the relationship between *C. botulinum* type E isolates recovered from different aquatic environments surrounding butchering site No. 1 based on their SmaI and XhoI macrorestriction fragment patterns. The similarity (%) among strains was determined using the Dice Coefficient and the clustering was performed by UPGMA.

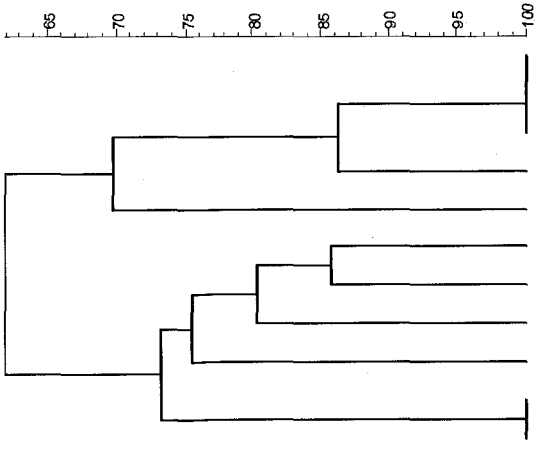
SmaI

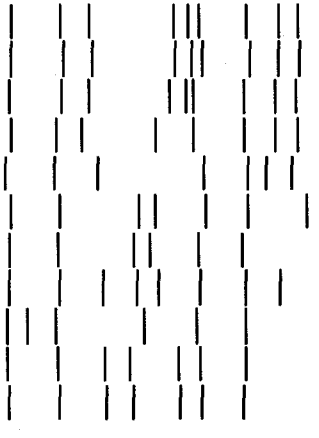




Strain ID	Sample type	Serotype
RMRS-1M2E1	Seal meat (QCS)	E
SOKR-34E2	Freshwater sediment	E
FWSKR4802E1	Freshwater sediment	E
SOKR-34E5	Freshwater sediment	E
SOKR-49E1	Freshwater sediment	E
SOKR3602E1	Shoreline soil	E
PBKR-41E1	Peat bog	E
SOKR-33E1	Peat bog	E
SOKR-49E2	Freshwater sediment	E
SOKR-34E1	Freshwater sediment	E
SOKR-42E1	Shoreline soil	E

XhoI





PBKR-41E1	Peat bog	E
SOKR-33E1	Peat bog	E
SOKR-49E2	Freshwater sediment	E
SOKR3602E1	Shoreline soil	E
RMRS-1M2E1	Seal meat (QCS)	E
SOKR-34E1	Freshwater sediment	E
SOKR-42E1	Shoreline soil	E
SOKR-34E2	Freshwater sediment	E
FWSKR4802E1	Freshwater sediment	E
SOKR-34E5	Freshwater sediment	E
SOKR-49E1	Freshwater sediment	E

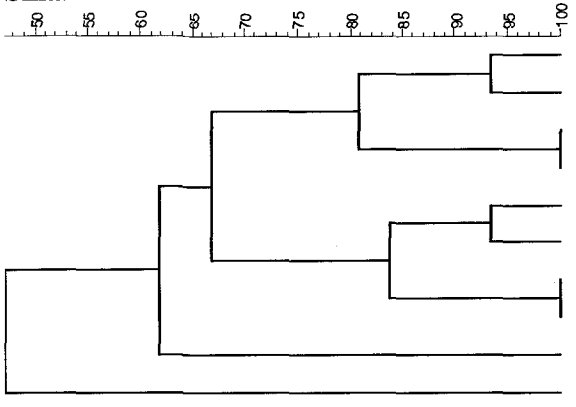
None of the *misiraq* and *igunaq* samples made with seal No. 2 meat (*igunaq* No. 2) were found positive for *C. botulinum* spores or botulinum toxin (Table 6.1), despite the presence of spores in the intestines of seal No. 2 and the surrounding aquatic environment. *C. botulinum* type E was found in shoreline soil, but not in seawater or on the surfaces of coastal rocks. The sample of seawater was collected at high tide whereas shoreline soil composed of mud, was collected on the next day at low tide, approximately 20 meters from the butchering site. The intestine of the seal No. 2 contained spores of *C. botulinum* type A and type E, but its skin sample was negative.

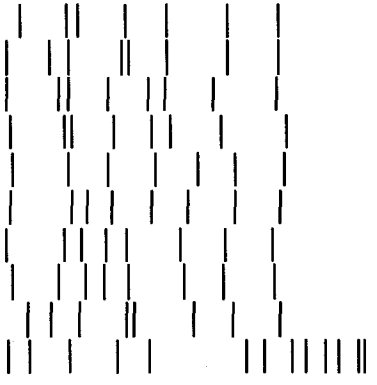
No spores of *C. botulinum* type E were detected on the blade of the hunter's knife at the end of butchering of seal No. 2, as well as after the butchering of two other bearded seals (Table 6.1). PFGE patterns of the intestinal strains of seal No. 2 was compared to the PFGE patterns of environmental isolates isolated from the shoreline soil collected near the butchering site and from samples of seawater, coastal rocks, and shoreline soil collected at another butchering site (KR-35), located within the same aquatic environment (along the mouth of the Whale River). The PFGE patterns of the intestinal isolates were different from all environmental isolates and a close relationship was only observed between an isolate from a coastal rock (RSKR-68E3) and a shoreline soil isolate (SOKR-19E1) that were not isolated from the same butchering site (Figure 6.4).

Similarly to the samples from seal No. 2, none of the eight samples of meat or fat from seal No. 3 were found to be contaminated with spores of *C. botulinum*, although they were collected from large pieces of butchered meat rinsed with seawater (to clear off the blood), or dragged across rocks for a distance of approximately 20 meters (in order to be protected from the tide). No spores were detected on the surface of the coastal rock or from the seawater sample taken from the rock cavity that was used to

Figure 6.4. Dendrograms showing the relationship between *C. botulinum* type E isolates recovered from the intestines of seal No. 2 butchered on the shoreline of Big Island and those from the environment of Whale River based on their SmaI and XhoI macrorestriction fragment patterns. The similarity (%) among strains was determined using the Dice Coefficient and the clustering was performed by UPGMA.

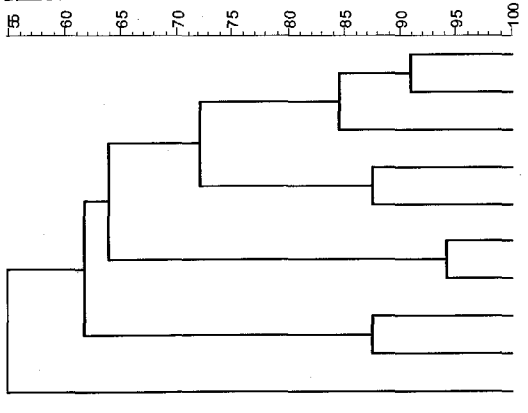
SmaI

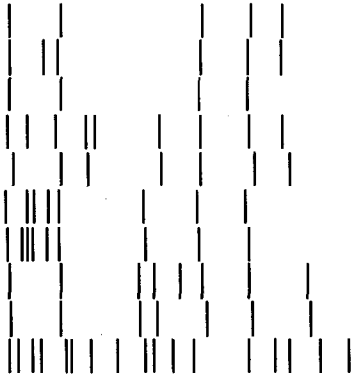




Strain ID	Sample type	Serotype
RSKR-68E2	Coastal rock	E
SOKR-35E3	Shoreline soil	E
RSKR-68E3	Coastal rock	E
SOKR-19E1	Shoreline soil	E
IN01SE63E1	Seal intestine	E
SWKR07E1	Seawater	E
SOKR-35E1	Shoreline soil	E
SOKR-35E2	Shoreline soil	E
RSKR-68E1	Coastal rock	E
IN01SE63A3	Seal intestine	A

XhoI



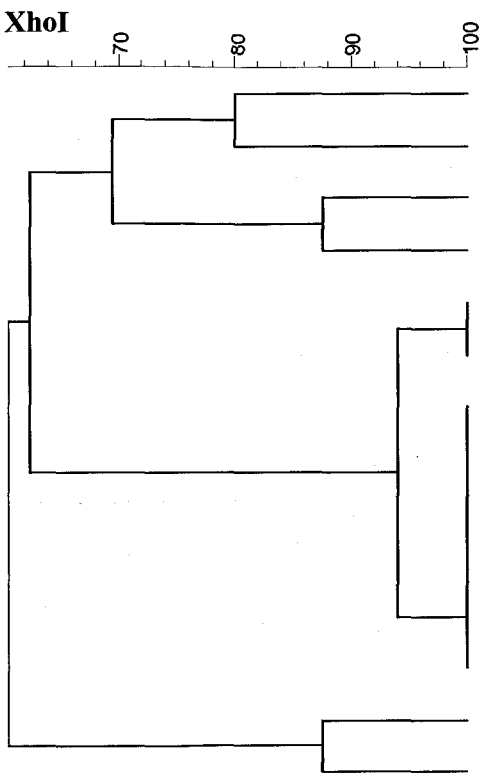


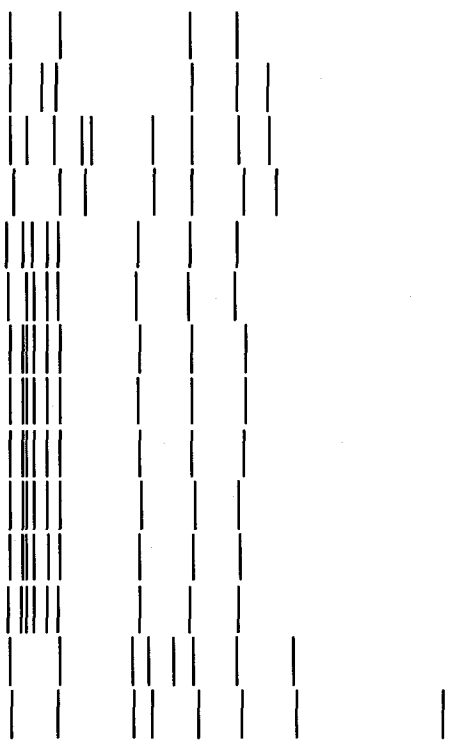
IN01SE63E1	Seal intestine	E
SOKR-35E2	Shoreline soil	E
RSKR-68E1	Coastal rock	E
SOKR-35E1	Shoreline soil	E
SWKR07E1	Seawater	E
RSKR-68E3	Coastal rock	E
SOKR-19E1	Shoreline soil	E
RSKR-68E2	Coastal rock	E
SOKR-35E3	Shoreline soil	E
IN01SE63A3	Seal intestine	A

rinse meat. Cultures of skin and intestinal content samples from seal No. 3 were also negative. However, *igunaq* No. 3 prepared with meat and fat tissues from seal No. 3 was found toxic and several samples of *igunaq* (6/10) and *misiraq* (3/3) contained viable *C. botulinum* type E (Table 6.1). Type E neurotoxin was detected in one and two extracts of the *igunaq* and *misiraq* samples, respectively. PFGE analysis of multiple isolates (n=19) recovered from three *misiraq* samples (collected at the bottom of the container) and four *igunaq* pieces, showed that the preparation was predominantly contaminated with a single type E strain or subtype (Figure 6.5). Cluster analysis of PFGE patterns generated from *igunaq* No. 3 isolates and those from the Whale River environment showed that the predominant subtype from *igunaq* No. 3 was also present in the shoreline soil (SOKR-19E1) collected near the butchering site of seal No. 3. These data suggested that the source of contamination of *igunaq* No. 3 was most likely of environmental origin. Furthermore, strain number IG3MB02E6 isolated from the oil part (*misiraq*) of *igunaq* No. 3 and closely-related to SOKR-19E1 (one band difference), yielded a PFGE pattern identical to that of the coastal isolate RSKR-68E3 isolated from another butchering site situated along the mouth of Whale River (KR-35).

The third bearded seal (seal No. 4), however, was butchered in mud at low tide. Due to the strong prevailing winds, the boat had to be anchored in a small bay where the field team could be sheltered from the waves. Once the tide receded, the *igunaq* producer cut and butchered the animal where it had lain in the mud bed. Eight samples of meat or fat were arbitrarily collected from the upper part of the pail to assess potential contamination from the mud during butchering, while the rest of the meat preparation was aged into *igunaq* (*igunaq* No. 4). All cultures of fat and meat samples collected from seal No. 4 were negative for type E spores, but one culture of raw fat sample contained a type B strain of *C. botulinum* group I. However, 80% (8/10) of the

Figure 6.5. Dendrogram showing the relationship between *C. botulinum* type E isolates recovered from the *igunaq* No. 3 preparation made with seal No. 3 meat and those from the environment of Whale River based on their XhoI macrorestriction fragment patterns. The similarity (%) among strains was determined using the Dice Coefficient and the clustering was performed by UPGMA.





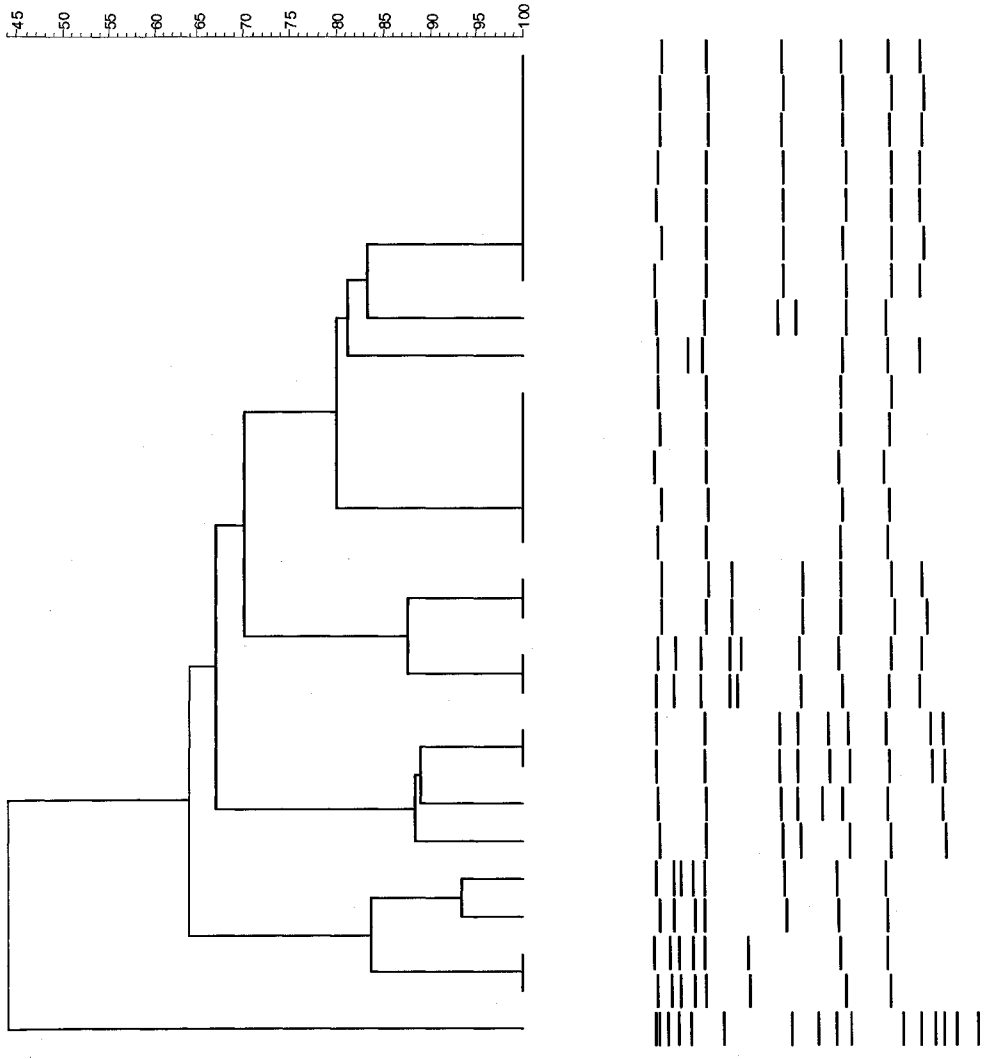
Strain ID	Sample type	Serotype
RSKR-68E1	Coastal rock	E
SOKR-35E2	Shoreline soil	E
SOKR-35E1	Shoreline soil	E
SWKR07E1	Seawater	E
IG3MB02E6	Misiraq	E
RSKR-68E3	Coastal rock	E
IG3MB02E1	Misiraq	E
IG3ME1402E1	Igunaq	E
IG3ME1802E1	Igunaq	E
IG3ME1902E1	Igunaq	E
IG3ME2202E1	Igunaq	E
SOKR-19E1	Shoreline soil	E
RSKR-68E2	Coastal rock	E
SOKR-35E3	Shoreline soil	E

igunaq and all three *misiraq* samples contained viable *C. botulinum* type E and all extracts of these samples contained BoNT/E (Table 6.1). Both the shoreline soil and seawater samples collected at the beginning of the rising tide were positive for *C. botulinum* type E, while no spores were detected in the intestinal tract of seal No. 4. PFGE analysis of food and environmental isolates from the Whale River showed that both *igunaq* preparation No. 4 and the mud sample (SOKR-29) collected by the seal, contained multiple and unrelated type E strains (Figure 6.6). However, some strains isolated from the environment of KR-35 butchering site along the mouth of the Whale River shared PFGE patterns identical to strains from the environment surrounding seal No. 4. For instance, the PFGE pattern of SWKR0402E2 isolated from seawater collected near Big Island (KR-29 butchering site) was identical to that of isolate SWKR07E1 present in seawater near KR-35 site. Similarly, isolates SOKR-29E1 and SOKR-35E1 yielded indistinguishable pulsotypes with SmaI and XhoI, although they originated from shoreline soil samples collected from two different butchering sites separated by approximately 4 km.

Genetic diversity of *C. botulinum* type E in the coastal environment. Between one and three strains of *C. botulinum* were isolated from each positive field sample (n=33) collected from the coastal environment of Nunavik, for a total of 75 isolates; three type A, three type B, and 69 type E. Cluster analysis of PFGE patterns was performed using only isolates yielding different SmaI or XhoI pulsotypes within each sample. Figure 6.7 shows a dendrogram based on XhoI PFGE patterns, of 53 isolates of *C. botulinum* from two peat bog, seven coastal rock, five seawater, six sediment, and 33 shoreline samples. As previously shown (Chapter 5), XhoI was again to be found more discriminatory than the SmaI restriction enzyme, producing up to 39 pulsotypes as compared to 23 for SmaI

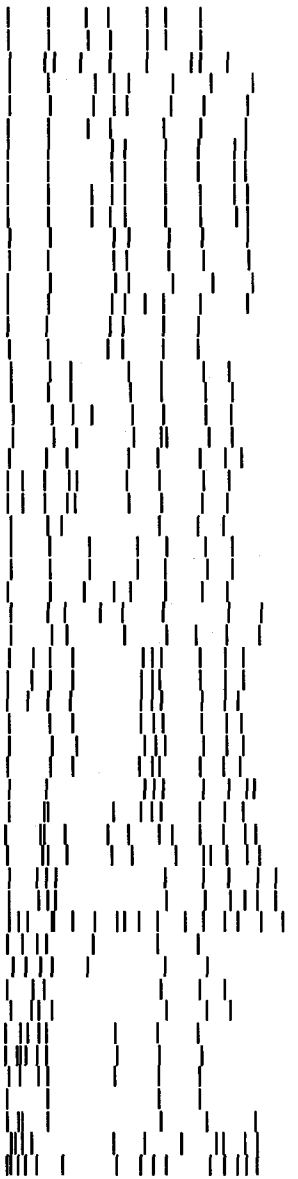
Figure 6.6. Dendrogram showing the relationship between *C. botulinum* type E isolates recovered from the *igunaq* No. 4 preparation made with seal No. 4 meat and those from the environment of Whale River based on their XhoI macrorestriction fragment patterns. The similarity (%) among strains was determined using the Dice Coefficient and the clustering was performed by UPGMA.

XhoI



Strain ID	Sample type	Serotype
IG4MB02E4	Misiraq	E
IG4ME2402E1	Igunaq	E
IG4ME2602E2	Igunaq	E
IG4ME2702E1	Igunaq	E
IG4ME2802E1	Igunaq	E
IG4ME3002E2	Igunaq	E
IG4MT02E1	Misiraq	E
IG4ME3102E1	Igunaq	E
SOKR-35E2	Shoreline soil	E
IG4MB02E2	Misiraq	E
IG4ME2602E1	Igunaq	E
IG4ME3102E2	Igunaq	E
IG4MM02E2	Igunaq	E
RSKR-68E1	Coastal rock	E
SWKR0402E2	Seawater	E
SWKR07E1	Seawater	E
SOKR-29E1	Shoreline soil	E
SOKR-35E1	Shoreline soil	E
IG4ME2802E2	Igunaq	E
IG4ME3002E1	Igunaq	E
RSKR-68E2	Coastal rock	E
SOKR-35E3	Shoreline soil	E
RSKR-68E3	Coastal rock	E
SWKR0402E1	Seawater	E
IG4ME2502E1	Igunaq	E
IG4MM02E1	Misiraq	E
BLBS-3F4B1	Raw fat	B

Figure 6.7. Dendrogram showing the relationship between 53 *C. botulinum* type E isolates isolated from the coastal environment of Nunavik based on their XhoI macrorestriction fragment patterns. Note the existence of extensive genetic biodiversity of *C. botulinum* type E strains in the coastal environment. The similarity (%) among strains was determined using the Dice Coefficient and the clustering was performed by UPGMA.



Strain ID	Sample type	Serotype	Village	Region	Location
SOKR-34E5	Freshwater sediment	E	Kuujjuaq	Ungava Bay	Dancelou River
SOKR-49E1	Freshwater sediment	E	Kuujjuaq	Ungava Bay	Dancelou River
SO309E2	Shoreline soil	E	Puvimituq	Hudson Bay	Bigfinger pointe
SO303E1	Shoreline soil	E	Umiujaq	Hudson Bay	Le Goulet
SO325E2	Shoreline soil	E	Tasiujaq	Ungava Bay	Leaf Bay
SP417E (NT)	Coastal rock	E	Puvimituq	Hudson Bay	Bigfinger pointe
SO303E5	Shoreline soil	E	Umiujaq	Hudson Bay	Le Goulet
SO304E1	Shoreline soil	E	Inukjuak	Hudson Bay	Qikiqtaruqtatuq
SO303E4	Shoreline soil	E	Umiujaq	Hudson Bay	Le Goulet
SO305E1	Shoreline soil	E	Inukjuak	Hudson Bay	Harrison Island
SOKR-34E1	Freshwater sediment	E	Kuujjuaq	Ungava Bay	Dancelou River
SOKR-35E3	Shoreline soil	E	Kuujjuaq	Ungava Bay	Mouth of Whale River
SO322E1	Shoreline soil	E	Kangirsuk	Ungava Bay	Payne River
RSKR-68E2	Coastal rock	E	Kuujjuaq	Ungava Bay	Mouth of Whale River
SOKR-42E1	Shoreline soil	E	Tasiujaq	Ungava Bay	Tasiujaq Islands
SP417E (Alc)	Coastal rock	E	Puvimituq	Hudson Bay	Bigfinger pointe
SWKR0402E2	Seawater	E	Kuujjuaq	Ungava Bay	Big Island, Whale River
SWKR07E1	Seawater	E	Kuujjuaq	Ungava Bay	Mouth of Whale River
SO325E1	Shoreline soil	E	Tasiujaq	Ungava Bay	Leaf Bay
SO326E1	Shoreline soil	E	Tasiujaq	Ungava Bay	Leaf Bay
SOKR3602E1	Shoreline soil	E	Kuujjuaq	Ungava Bay	Diana Bay
SOKR-29E1	Shoreline soil	E	Kuujjuaq	Ungava Bay	Big Island, Whale River
SOKR-35E1	Shoreline soil	E	Kuujjuaq	Ungava Bay	Mouth of Whale River
SP457/458E	Coastal rock	E	Kuujjuaq	Ungava Bay	Mouth of Koksoak River
SO329E1	Shoreline soil	E	Kuujjuaq	Ungava Bay	Mouth of Koksoak River
SP455456E2	Rock surface	E	Kuujjuaq	Ungava Bay	Mouth of Koksoak River
SOKR-34E2	Freshwater sediment	E	Kuujjuaq	Ungava Bay	Dancelou River
FWSKR40E1	Freshwater sediment	E	Kuujjuaq	Ungava Bay	*Tributary*
SO330E1	Shoreline soil	E	Kuujjuaq	Ungava Bay	Mouth of Koksoak River
SO304E2	Shoreline soil	E	Inukjuak	Hudson Bay	Qikiqtaruqtatuq
SO305E2	Shoreline soil	E	Inukjuak	Hudson Bay	Harrison Island
SO321E1	Shoreline soil	E	Kangirsuk	Ungava Bay	Payne River
PBKR-41E1	Peat bog	E	Kuujjuaq	Ungava Bay	Diana Bay
SOKR-33E1	Peat bog	E	Kuujjuaq	Ungava Bay	Diana Bay
SOKR-49E2	Freshwater sediment	E	Kuujjuaq	Ungava Bay	Dancelou River
SO301E	Shoreline soil	E	Kuujjuaraapik	Hudson Bay	Karjurtuit Point
SO309E1	Shoreline soil	E	Puvimituq	Hudson Bay	Bigfinger pointe
SO307E1	Shoreline soil	E	Puvimituq	Hudson Bay	Hotel beach
SO325E	Shoreline soil	E	Tasiujaq	Ungava Bay	Leaf Bay
SOKR-44E1	Shoreline soil	E	Kuujjuaq	Ungava Bay	Mouth of Koksoak River
SOKR-44E2	Shoreline soil	E	Kuujjuaq	Ungava Bay	Mouth of Koksoak River
SO300A1	Shoreline soil	A	Kuujjuaraapik	Hudson Bay	Boat opening
SOKR-44E3	Shoreline soil	E	Kuujjuaq	Ungava Bay	Mouth of Koksoak River
SW279E	Seawater	E	Kuujjuaq	Ungava Bay	Mouth of Koksoak River
SOKR-35E2	Shoreline soil	E	Kuujjuaq	Ungava Bay	Mouth of Whale River
SW280E	Seawater	E	Kuujjuaq	Ungava Bay	Mouth of Koksoak River
RSKR-68E3	Coastal rock	E	Kuujjuaq	Ungava Bay	Mouth of Whale River
SOKR-19E1	Shoreline soil	E	Kuujjuaq	Ungava Bay	Big Island, Whale River
SWKR0402E1	Seawater	E	Kuujjuaq	Ungava Bay	Big Island, Whale River
RSKR-68E1	Coastal rock	E	Kuujjuaq	Ungava Bay	Mouth of Whale River
SOKR-29E2	Shoreline soil	E	Kuujjuaq	Ungava Bay	Big Island, Whale River
SO303E3	Shoreline soil	E	Umiujaq	Hudson Bay	Le Goulet
SO327B1	Shoreline soil	B	Kangiqsualuj.	Ungava Bay	*North of peninsula*

using the same strains. A total of 44 different PFGE subtypes were generated from 53 isolates using SmaI and XhoI pulsotypes (Table 6.2), indicating the existence of a genetically diverse population of *C. botulinum* type E within the coastal environment of Nunavik. Although very distant geographically, some strains originating from Ungava Bay and Hudson Bay coasts, two regions separated by the Hudson Strait (Figure 3.4), yielded the same PFGE subtype (XXX, XXXII). The PFGE patterns of isolate SO305E2 from Inukjuak were indistinguishable from isolate SO321E1 from Kangirsuk, as well as isolates SO303E1 and SO325E2 from Umiujaq and Tasiujaq, respectively. Some subtypes of *C. botulinum* type E were isolated from two different butchering sites located within the same aquatic environment. Subtypes II and III were isolated from seawater and shoreline soil, respectively, taken at two butchering sites (KR-29 and KR-35) along the mouth of the Whale River. Comparison of PFGE patterns of isolates recovered from the same butchering site showed that a clonal subtype was isolated from different substrates in only one of three butchering sites (Figure 6.7). Isolate SO329E1 recovered from the shoreline soil collected near the BL-329 butchering site located along the mouth of the Koksoak River, was found indistinguishable from the coastal rock isolate SP455456E2 by PFGE analysis, suggesting that the spores of *C. botulinum* type E present on the surface of coastal rocks may originate from the shoreline soil or sediment surrounding the butchering site.

Table 6.2. Distribution of *C. botulinum* type E subtypes among environmental isolates originating from butchering sites along the Nunavik coastline

Sources of samples	No. of isolates	PFGE subtypes[a]
Ungava Bay coast		
Kangiqsualujjuaq (north of peninsula)		
Shoreline soil	1	I
Mouth of the Whale River		
Shoreline soil	6	I, III (2)-V, X-XI
Coastal rock	3	VI-VIII
Seawater	3	II (2), IX
Kuujjuaq (mouth of the Koksoak River)		
Shoreline soil	5	XIII-XVII
Coastal rock	2	XII-XIII
Seawater	2	XVIII-XIX
Diana Bay and Dancelou River		
Shoreline soil	2	XXI, XXVII
Sediment	5	XXIII-XXV (2), XXVI
Peat bog	2	XXII (2)
Tasiujaq (Leaf Bay)		
Shoreline soil	4	XXVIII-XXXI
Kangirsuk (Payne River)		
Shoreline soil	2	XXXII-XXXIII

Table 6.2 (Continued). Distribution of *C. botulinum* type E subtypes among environmental isolates originating from butchering sites along the Nunavik coastline

Sources of samples	No. of isolates	PFGE subtypes[a]
Hudson Bay coast		
Puvirnituq		
Shoreline soil	3	XXXIV-XXXV, XXXVIII
Coastal rock	2	XXXVI-XXXVII
Inukjuak		
Shoreline soil	4	XXXII, XXXIX-XLI
Umiujaq		
Shoreline soil	4	XXX, XXXIX, XLI-XLII
Kuujjuaraapik		
Shoreline soil	2	XLIII-XLIV

[a]Subtypes were generated with SmaI and XhoI pulsotypes. Each unique subtype was designated by a Roman numeral. The number in brackets corresponds to the number of isolates yielding the same subtype.

6.4 Discussion

Placing meat or fat tissue from harvested seals into direct contact with the surface of coastal rocks during butchering may lead to the contamination of tissues as demonstrated for the seal No. 1. The spores present in shoreline soil are likely carried to the rocky shore by the sea waves that break and splash on the littoral during the rising tide. Although none of the coastal rocks used by the hunter at Diana Bay were positive at the time of sampling, the presence of spores of *C. botulinum* type E was detected on the surface of coastal rocks of four butchering sites located at the mouth of large rivers during the ecological survey. The analysis of PFGE patterns from strains of *C. botulinum* type E isolated from shoreline soil, rocks or seawater from the same butchering site showed existence of local biodiversity, with up to six different subtypes within a single site. The presence of the same PFGE subtype of *C. botulinum* type E in shoreline soil and on coastal rocks of the same butchering site was also demonstrated at BL-329 site at the mouth of the Koksoak River. Thus, tracing contamination of seal meat with environmental sources of *C. botulinum* type E to a specific handling meat practice becomes challenging with the presence of multiple subtypes, and when several handling meat practices leading to contamination are used by the hunter during butchering.

Rinsing seal meat or immersing the entire carcass in seawater could lead to contamination of meat with spores of *C. botulinum* type E present in seawater. This practice was observed once in the field at butchering site No. 3 in Whale River, but this did not result in the contamination of the sampled meat and fat tissues with type E spores. The residual seawater collected from the rock depressions from which the rinsing water originated also tested negative for *C. botulinum*.

The presence of the organism in seawater was only detected near butchering site No. 4 at the mouth of Whale River during the microbial tracking study, but the hunter did not rinse any meat with seawater. Based on cluster analysis of PFGE patterns, none of the seawater isolates (SWKR0402E1 and SWKR0402E2) recovered from butchering site No. 4 could be closely linked with any of the multiple type E isolates recovered from *igunaq* preparation No. 4 or those isolated from the mud sample collected near the harvested seal, indicating the existence of a high level of genetic diversity of *C. botulinum* type E strains in the environment of the mouth of Whale River. Similarly, surveys in Finland showed the existence of local biodiversity of *C. botulinum* type E strains in sediments and fish from lakes and farms (Hyytiä *et al.*, 1999b). However, based on PFGE analysis, water isolate SWKR0402E2 from butchering site No. 4 was found to be identical to water isolate SWKR07E1 obtained from the KR-35 site also located at the mouth of Whale River, but sampled during the ecological survey. In addition, two shoreline soil isolates originating from these two butchering sites (SOKR-29E1 and SOKR-35E1) were found to be identical based on PFGE analysis. Similarly, an environmental link was established between SW279E and SOKR-44E3, two type E isolates recovered from seawater and shoreline soil, respectively from two different butchering sites in the mouth of the Koksoak River. Thus, several subtypes of *C. botulinum* type E strains were encountered in more than one butchering site located in the same estuary environment, indicating that these areas contain multiple clonal strains, possibly originating from common headwaters or terrestrial sources (Chapter 3).

With the exception of seal No. 4, which was butchered in mud, the other two bearded seals were cut and butchered on a flat coastal rock at high tide, thus preventing contact with shoreline soil exposed during low tide. The risk of contamination of seal meat from environmental sources is less likely when the animal is butchered on a flat

coastal rock than when on a pebble beach or a shoreline area covered by a mud substrate. Butchering seal No. 4 in mud was theoretically considered the most high-risk butchering practice observed in the field. This was confirmed in the laboratory by the isolation of up to seven different subtypes of *C. botulinum* type E in *igunaq* No. 4. None of the food isolates were be closely linked with those obtained from seawater and shoreline soil samples collected near seal No. 4, supporting the existence of a high genetic biodiversity of *C. botulinum* type E in the environment of Whale River. As such, the presence of several subtypes of *C. botulinum* type E in a suspect food sample could complicate an epidemiological investigation and would require the isolation of multiple strains from related clinical and food specimens to allow identification of the outbreak strain. The simultaneous contamination of foods by more than one subtype of *C. botulinum* type E was previously demonstrated by Hyytiä *et al.* (1999b) in fresh fish and fishery products from Finland.

Despite the existence of a high biodiversity of *C. botulinum* type E in the environment of Whale River, strain number SOKR-19E1 (shoreline soil isolate) recovered near butchering site No. 3, was found to be indistinguishable from multiple type E isolates recovered from *igunaq* No. 3 when using cluster analysis of PFGE patterns. In contrast to *igunaq* preparation No. 4, *igunaq* No. 3 was contaminated with a single strain of *C. botulinum* type E; the shoreline soil was thought to be the origin of this contamination. However, seal No. 3 was butchered on a flat coastal rock at high tide, which prevented any contact of the butchered meat with shoreline soil (collected at low tide). Therefore, contamination of meat could either have occurred from contact with residual spores present on the rock left by the previous tide, or from spores in water that were carried onto the rock while pulling the animal out of the water. Strain SOKR-19E1 was not detected on the surface of coastal rocks or in seawater collected

near butchering site No. 3, but strain RSKR-68E3, a closely-related isolate to SOKR-19E1 was found on the surface of a coastal rock of another butchering site (KR-35), located across Big Island.

Two strains of *C. botulinum* were isolated from the intestinal contents of seal No. 2 and included a type A (IN01SE63A3) and a type E (INSE63E1) *C. botulinum*. No linkage could be established between isolate IN01SE63E1 and any of the type E environmental isolates from butchering site No. 2. Although the prevalence of type E spores in the intestines of seals harvested in Nunavik is low (Chapter 3), good hygienic practices should be followed in the field to minimize the risk of contamination of meat with intestinal materials that could occasionally contain botulinum spores and zoonotic protozoan parasite (Santin *et al.*, 2005). A different subtype of *C. botulinum* group I type A was also isolated from the shoreline soil collected near the Northern village of Kuujjuaraapik during the ecological survey. This was the first report of *C. botulinum* type A detected in the Canadian sea coasts. No outbreaks of botulism have been caused by *C. botulinum* types A or B in Nunavik. In Alaska, types A and B are considered indigenous and are increasingly being reported as etiologic agents of botulism (Barrett *et al.*, 1977; Wainwright *et al.*, 1988). An incident of botulism has even been related to the consumption of seal *misiraq* containing both BoNT/ A and E (Wainwright *et al.*, 1988).

The genetic biodiversity of *C. botulinum* type E isolates observed in the coastal environment of Nunavik was high, with 83% of isolates yielding distinct PFGE subtypes. High genetic biodiversity was also reported among *C. botulinum* type E isolates in fish and fishery products from Finland, where 62 subtypes from 92 type E isolates (67%) were observed when using PFGE and randomly amplified polymorphic DNA (RAPD) methods (Hyytia *et al.*, 1999b). Despite this level of biodiversity,

identical pulsotypes were seen among type E isolates originating from different regions of Finland (Hyytia *et al.*, 1999b) and among North American isolates from the Pacific coast of United States, Alaska, and Canada (Hielm *et al.*, 1998a). Identical PFGE patterns were also observed among Arctic isolates of *C. botulinum* type E originating from dry mutton in the Faeroe Islands and two samples of seal meat from Greenland (Hielm *et al.*, 1998a). In Nunavik, no distinct clustering of type E strains by region was observed, although some coastal isolates recovered from three Northern villages of the Hudson Bay region yielded identical or closely-related pulsotypes. Two subtypes of *C. botulinum* type E isolated from the coastal environment of Ungava Bay were found along the eastern Hudson Bay coast. Water currents and migration of marine species are potential vehicles that could carry spores over large distances and contribute to the large-scale geographic distribution of subtypes of *C. botulinum* type E.

In summary, the risk for contamination of seal meat from placing butchered tissues in direct contact with the surfaces of coastal rocks was demonstrated by bacteriological analysis, but not during the practices of rinsing meat with seawater or dragging meat across rocks over long distances. However, the risk for contamination of seal meat during the butchering process was high, as the organism was detected in the environments of these butchering sites and two of four seal *igunaq* preparations (end products for human consumption) contained BoNT/E. Genomic analysis of coastal isolates of *C. botulinum* type E by PFGE revealed the existence of a high level of genetic biodiversity in the environments of the butchering sites which could make the tracing of food contamination sources difficult.

CHAPTER 7

MOLECULAR EPIDEMIOLOGY OF FOODBORNE BOTULISM IN THE CANADIAN ARCTIC

7.1 Introduction

In Canada, foodborne botulism occurs sporadically in Arctic regions where type E outbreaks often occur as a result of the consumption of aged marine mammal products by the Inuit population (Austin and Dodds, 2000). Marine mammal meat, mostly seals, were the main food sources implicated in type E botulism outbreaks recorded from the Canadian Arctic between 1960 and 1984 (Dolman, 1974; Hauschild and Gauvreau, 1985). Food sources of botulism outbreaks have been traditionally identified through epidemiological investigations and confirmed by detection of botulinum neurotoxin and viable cells of *C. botulinum* of the same serotype present in clinical and food specimens.

The epidemiological link between the occurrence of an outbreak and a common food source can be established at the strain level by comparing DNA fingerprints of strains isolated from food and clinical specimens. Pulsed-field gel electrophoresis (PFGE) has recently been found to be a more reliable and discriminatory DNA fingerprinting method than randomly amplified polymorphic DNA (RAPD) or automated ribotyping for use in epidemiological investigations of type E botulism outbreaks in the Canadian Arctic (Leclair *et al.*, 2006). Currently, only a limited number of strains of *C. botulinum* type E causing outbreaks in Arctic regions have been characterized by molecular typing methods (Hielm *et al.*, 1998a; Hyytiä *et al.*, 1999b; Leclair *et al.*, 2006). The genetic diversity and distribution of outbreak strains of *C. botulinum* type E affecting the Inuit population remain unknown. The objectives of this study were to i) study the molecular epidemiology of type E botulism in the Canadian Arctic; ii) determine the prevalence and geographical distribution of various PFGE subtypes of outbreak strains, and iii) assess their occurrence in aquatic environments where the disease is endemic.

7.2 Materials and Methods

Bacterial strains and growth media. Eighty-seven isolates of *C. botulinum* isolated from 49 clinical and food specimens submitted to the BRS for the investigation of 24 botulism incidents that occurred in the Canadian Arctic between 1995 and 2004 (Table 7.1), were selected for this molecular epidemiological study. Between one to three isolates (up to six) of *C. botulinum* was generally isolated from each specimen. In addition, 100 environmental isolates of *C. botulinum* type E recovered from 41 samples of water, sediment, shoreline and terrestrial soil collected in the area of the Koksoak River in Nunavik, were included in the study (Table 7.2). These environmental samples were collected downstream and upstream from Kuujjuaq, where 60% of recorded botulism incidents from Nunavik occurred between 1964 and 2004. The isolates were archived on Microbank™ beads at -86°C and were grown on McClung Toabe medium for 2-3 days anaerobically, and then transferred into 8 ml of SPGY broth for overnight growth as previously described in Chapter 5.

Epidemiological data. A review of the botulism incidents (outbreaks and single cases) in the Canadian Arctic was conducted during the period 1995-2004 using publications of the Canada Communicable Disease Report, the EpiNorth newsletter, and the laboratory reference books of the BRS. Data collected include: number of clinical cases, implicated food, time period (month/year) of the outbreak, and the name of the Northern village.

PFGE. DNA isolation, restriction digestion, and separation of DNA fragments by PFGE were performed according to the method of Leclair *et al.* (2006), described in detail in Chapter 5.

Fingerprint analysis. Comparison of DNA fragment patterns and cluster analysis were performed using BioNumerics version 3.5 software (Applied Maths, Kortrijk, Belgium). The level of similarity between DNA fragment patterns was estimated using the Dice coefficient correlation and cluster analysis of fingerprints was performed using the UPGMA method. Low fragment sizes (<23.1 kb) generated by SmaI digestions were ignored in the analysis (Chapter 5). Differences of one or more bands between PFGE fragment patterns were considered significant. DNA fragment patterns showing similarity levels greater than 97% with less than one band difference were considered identical. Pairwise comparison of PFGE patterns was performed to determine the number of distinguishable subtypes of *C. botulinum* type E for each specimen and only those subtypes were included in the cluster analysis. The PFGE patterns of each isolate were classified into subtypes designated by a Roman numeral based on the combination of SmaI and XhoI pulsotypes they produced.

7.3 Results

Genomic relationship among epidemiologically related isolates of *C. botulinum* type E. By selecting distinguishable PFGE subtypes generated from each specimen, 57 out of 87 isolates (65.5%) of *C. botulinum* composed of 56 type E and one type A were compared by cluster analysis. Nineteen SmaI and 29 XhoI (Figure 7.1) different PFGE fragment patterns were produced generating 28 (50%) different subtypes of *C. botulinum* type E. The isolation of *C. botulinum* type E from both clinical and food specimens during a botulism investigation was possible for 50% or 12 incidents. The analysis of dendrograms showed that the *C. botulinum* type E isolates recovered from related food and clinical specimens were identical in 10 (83.3.%) of these incidents using either SmaI or XhoI macrorestriction fragment patterns (Figure 7.1). The PFGE patterns of clinical and food isolates did not match for botulism incidents No. 9 and No. 23 and some clinical cases of incident No.12. Three distinct PFGE subtypes of *C. botulinum* type E (XXV-XXVII) were isolated from the submitted beluga fat sample in incident No. 23 and none of them matched the subtype of the related fecal isolate FE0211E1DK (XXVIII) recovered from the patient (Table 7.1). Similarly, isolate MI9706E recovered from the suspect seal *igunaq* involved in incident No. 9 and its related clinical isolate GA9706EMA yielded different PFGE subtypes, IX and X, respectively. In both of these incidents, the food specimens were positive for BoNT, but not the corresponding clinical specimens (data not shown). Based on the number of fragments that differed between related XhoI PFGE patterns, using criteria developed by Tenover *et al.* (1995), the food and clinical isolates from incidents No.9 and No.23 were considered possibly related (4 to 6 fragments) or unrelated (≥7 fragments).

Figure 7.1. PFGE dendrogram showing the relationship among 57 clinical and food isolates of *C. botulinum* recovered during the investigation of 24 botulism incidents in the Canadian Arctic between 1995 and 2004, based on their XhoI macrorestriction fragment patterns. The similarity (%) among strains was determined using the Dice Coefficient and the clustering was performed by UPGMA.

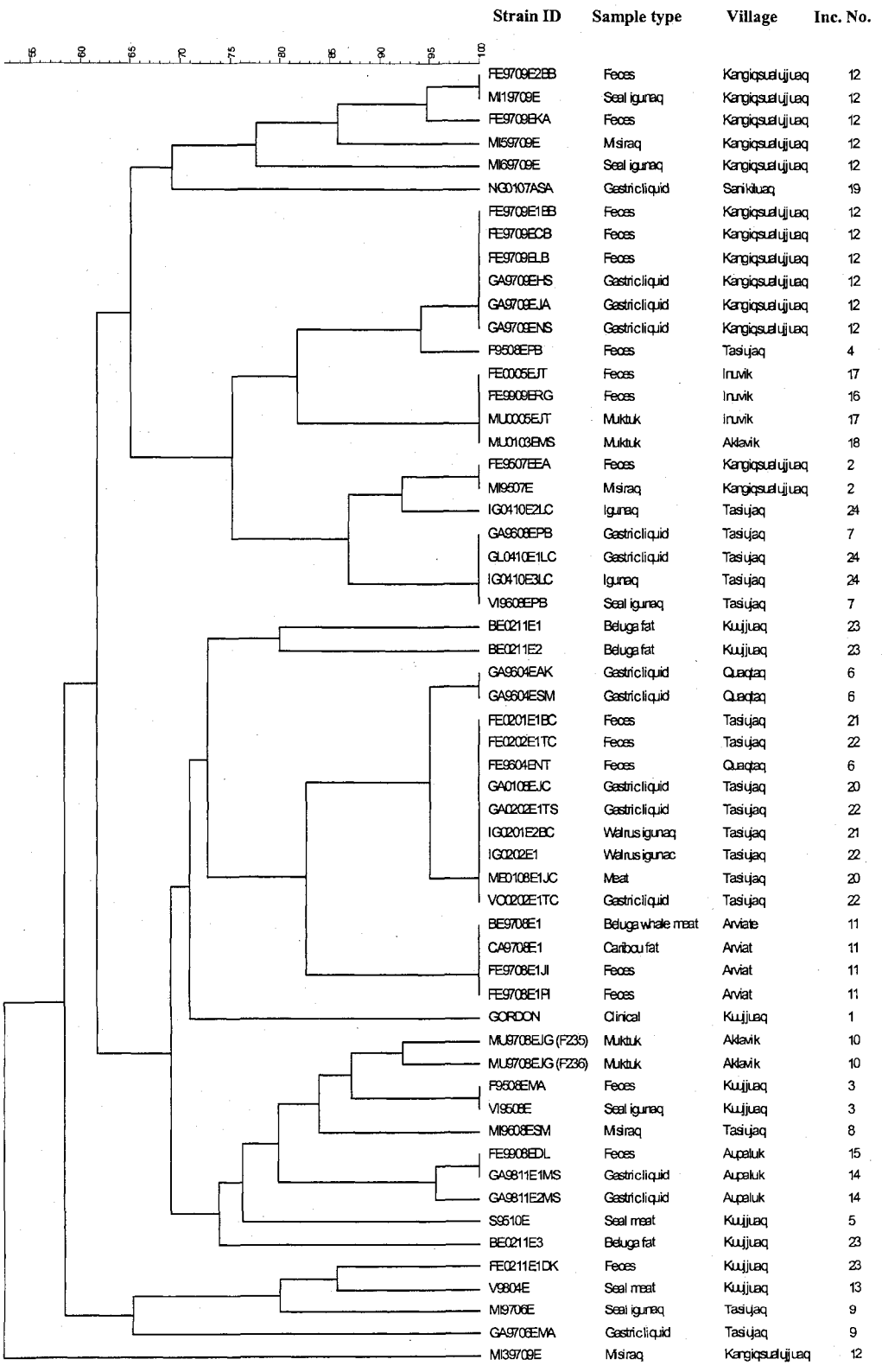

Strain ID
Sample type
Village
Inc. No.
55
60
65
70
75
80
85
90
95
100
FE9709E2BB Feces Kangiqsualujjuaq 12
MI19709E Seal igunaq Kangiqsualujjuaq 12
FE9709EKA Feces Kangiqsualujjuaq 12
MI59709E Misiraq Kangiqsualujjuaq 12
MI69709E Seal igunaq Kangiqsualujjuaq 12
NG0107ASA Gastric liquid Sanikiluaq 19
FE9709E1BB Feces Kangiqsualujjuaq 12
FE9709ECB Feces Kangiqsualujjuaq 12
FE9709ELB Feces Kangiqsualujjuaq 12
GA9709EHS Gastric liquid Kangiqsualujjuaq 12
GA9709EJA Gastric liquid Kangiqsualujjuaq 12
GA9709ENS Gastric liquid Kangiqsualujjuaq 12
P9508EPB Feces Tasiujaq 4
FE0005EJT Feces Inuvik 17
FE9909ERG Feces Inuvik 16
MU0005EJT Muktuk Inuvik 17
MU0103EMS Muktuk Aklavik 18
FE9507EEA Feces Kangiqsualujjuaq 2
MI9507E Misiraq Kangiqsualujjuaq 2
IG0410E2LC Igunaq Tasiujaq 24
GA9608EPB Gastric liquid Tasiujaq 7
GL0410E1LC Gastric liquid Tasiujaq 24
IG0410E3LC Igunaq Tasiujaq 24
VI9608EPB Seal igunaq Tasiujaq 7
BE0211E1 Beluga fat Kuujjuaq 23
BE0211E2 Beluga fat Kuujjuaq 23
GA9604EAK Gastric liquid Quaqtaq 6
GA9604ESM Gastric liquid Quaqtaq 6
FE0201E1BC Feces Tasiujaq 21
FE0202E1TC Feces Tasiujaq 22
FE9604ENT Feces Quaqtaq 6
GA0108EJC Gastric liquid Tasiujaq 20
GA0202E1TS Gastric liquid Tasiujaq 22
IG0201E2BC Walrus igunaq Tasiujaq 21
IG0202E1 Walrus igunac Tasiujaq 22
ME0108E1JC Meat Tasiujaq 20
VO0202E1TC Gastric liquid Tasiujaq 22
BE9708E1 Beluga whale meat Arviate 11
CA9708E1 Caribou fat Arviat 11
FE9708E1JI Feces Arviat 11
FE9708E1PI Feces Arviat 11
GORDON Clinical Kuujjuaq 1
MU9708EJG (FZ35) Muktuk Aklavik 10
MU9708EJG (FZ36) Muktuk Aklavik 10
P9508EMA Feces Kuujjuaq 3
VI9508E Seal igunaq Kuujjuaq 3
MI9608ESM Misiraq Tasiujaq 8
FE9908EDL Feces Aupaluk 15
GA9811E1MS Gastric liquid Aupaluk 14
GA9811E2MS Gastric liquid Aupaluk 14
S9510E Seal meat Kuujjuaq 5
BE0211E3 Beluga fat Kuujjuaq 23
FE0211E1DK Feces Kuujjuaq 23
V9804E Seal meat Kuujjuaq 13
MI9706E Seal igunaq Tasiujaq 9
GA9706EMA Gastric liquid Tasiujaq 9
MI39709E Misiraq Kangiqsualujjuaq 12

Table 7.1. Distribution of *C. botulinum* type E subtypes among clinical and food specimens isolated during the investigation of 24 botulism incidents in the Canadian Arctic from 1999 to 2004

Location	Incident No.	Date of incident	No. of cases	Origin of isolates	No. of isolates	PFGE subtypes[a]
Nunavik						
Kangiqsualujjuaq	2	07/1995	1	Seal *misiraq*, feces	2	II
	12	09/1997	9	Seal *igunaq*, feces (4), gastric liquid (3)	12	XIV-XIX
Kuujjuaq[b]	1	07/1975	1	Clinical	1	I
	3	08/1995	2	Seal *igunaq*, feces	2	III
	5	09/1995	1	Seal meat	1	V
	13	04/1998	1	Seal meat	1	XX
	23	11/2002	1	Beluga fat, feces	4	XXV-XXVIII
Tasiujaq	4	08/1995	5	Feces	1	IV
	7	07/1996	1	Seal *igunaq*, gastric liquid	2	VII
	8	08/1996	1	Seal *misiraq*	1	VIII
	9	06/1997	1	Seal *igunaq*, gastric liquid	2	IX, X
	20	07/2001	1	Meat, gastric liquid	2	VI

Table 7.1 (Continued). Distribution of *C. botulinum* type E subtypes among clinical and food specimens isolated during the investigation of 24 botulism incidents in the Canadian Arctic from 1999 to 2004.

Location	Incident No.	Date of incident	No. of cases	Origin of isolates	No. of isolates	PFGE subtypes[a]
Tasiujaq	21	01/2002	1	Walrus *igunaq*, feces	2	VI
	22	02/2002	3	Walrus *igunaq*, feces, gastric liquid (2)	4	VI
	24	10/2004	1	*Igunaq*, gastric liquid	3	VII, XXIX
Aupaluk	14	11/1998	1	Gastric liquid	2	XXI-XXII
	15	08/1999	1	Feces	1	XXI
Quaqtaq	6	04/1996	3	Feces, gastric liquid (2)	3	VI
Northwest Territories						
Aklavik	10	08/1997	1	*Muktuk* (2)	2	XI, XII
Inuvik	16	09/1999	1	Feces	1	XXIII
Inuvik	17	05/2000	1	*Muktuk*, feces	2	XXIII
Aklavik	18	03/2001	1	*Muktuk* oil	1	XXIII

Table 7.1 (Continued). Distribution of *C. botulinum* type E subtypes among clinical and food specimens isolated during the investigation of 24 botulism incidents in the Canadian Arctic from 1999 to 2004.

Location	Incident No.	Date of incident	No. of cases	Origin of isolates	No. of isolates	PFGE subtypes[a]
Nunavut						
Arviat	11	08/97	4	Beluga fat, caribou meat, feces (2)	4	XIII
Sanikiluaq	19	07/2001	1	Gastric liquid	1	XXIV

[a]Subtypes were generated with SmaI and XhoI pulsotypes. Each unique subtype was designated by a Roman numeral. The number in brackets corresponds to the number of isolates yielding the same subtype.
[b]Includes one botulism incident in 1975.

In incident No. 12, three subtypes of *C. botulinum* type E were isolated from seven clinical specimens belonging to seven different patients, while four subtypes of *C. botulinum* type E were isolated from the incriminated seal *igunaq*. Two subtypes of *C. botulinum* type E were isolated from a fecal specimen of which, one strain (FE9709E2BB) yielded the same PFGE patterns (XVI) as that of seal *igunaq* isolate MI19709E. However, the other fecal strain (FE9709E1BB) from this patient had the PFGE subtype XIV also recovered from five other patients. As the XhoI PFGE patterns of these two strains differed by ≥ 7 fragments, it appears that at least two outbreak strains of *C. botulinum* type E were involved in incident No.12. A total of six different subtypes of *C. botulinum* type E were isolated in this incident, and only one perfect match was observed between a food and a clinical isolate despite up to six food isolates were characterized by PFGE. According to Tenover's criteria, the four food subtypes of *C. botulinum* type E were either closely related (2 to 3 fragments) or unrelated based on XhoI macrorestriction patterns.

The finding of multiple strains in submitted food or clinical specimens were also observed in incident No. 14 and No. 24. In incident No. 24, two different subtypes of *C. botulinum* type E were isolated from the suspect *igunaq* of unknown animal origin, of which isolate IG0410E3LC was found to be identical to the related gastric liquid isolate GL0410E1LC based on PFGE analysis, yielding the same PFGE subtype (VII). Similarly, two PFGE subtypes of *C. botulinum* type E (XXI, XXII) were isolated from the same gastric liquid specimen from a patient (incident No. 14). In each of these incidents, the XhoI PFGE patterns of these isolates were either identical or closely related, indicating that they all were likely derived from the outbreak strain.

Genomic relationship among epidemiologically unrelated incident strains of *C. botulinum*. The classification of strains of *C. botulinum* type E in subtypes based on SmaI and Xho PFGE patterns showed that 11 of the 23 (47.8%) type E incidents were caused by subtypes that were isolated during two or more botulism incidents (Table 7.1). Thus, the same subtype of *C. botulinum* type E that was isolated from three patients in Quaqtaq in April 1996 (incident No. 6), was recovered from five clinical and three food specimens associated with incidents No. 20, 21, and 22 that occurred in Tasiujaq on August 2001, January 2002, and February 2002, respectively. Similarly, the subtype of *C. botulinum* type E that caused botulism in a patient from Tasiujaq on August 1996 (incident No. 7) was the same PFGE subtype that affected the patient from incident No. 24 that occurred in the same community on October 2004, more than seven years later. The same subtype of *C. botulinum* type E was also isolated from two patients in Aupaluk that were affected with botulism in November 1998 and August 1999, respectively. All of these eight incidents from Nunavik were associated with the consumption of aged marine mammal products originating from either seal or walrus. These results indicate that some subtypes of *C. botulinum* type E may be more abundant in some regions or areas where marine mammals are commonly butchered. Figure 7.2 shows the geographic distribution of *C. botulinum* type E subtypes isolated during the investigation of botulism incidents in Nunavik between 1995 and 2004. In contrast, all outbreak strains from the five unrelated botulism incidents from Kuujjuaq yielded distinct PFGE subtypes of *C. botulinum* type E and none of them were found to be identical to the subtypes isolated from any of the food or clinical specimens recovered during the investigation of two and seven botulism incidents from Kangiqsualujjuaq and Tasiujaq, respectively, which are the two closest villages to Kuujjuaq.

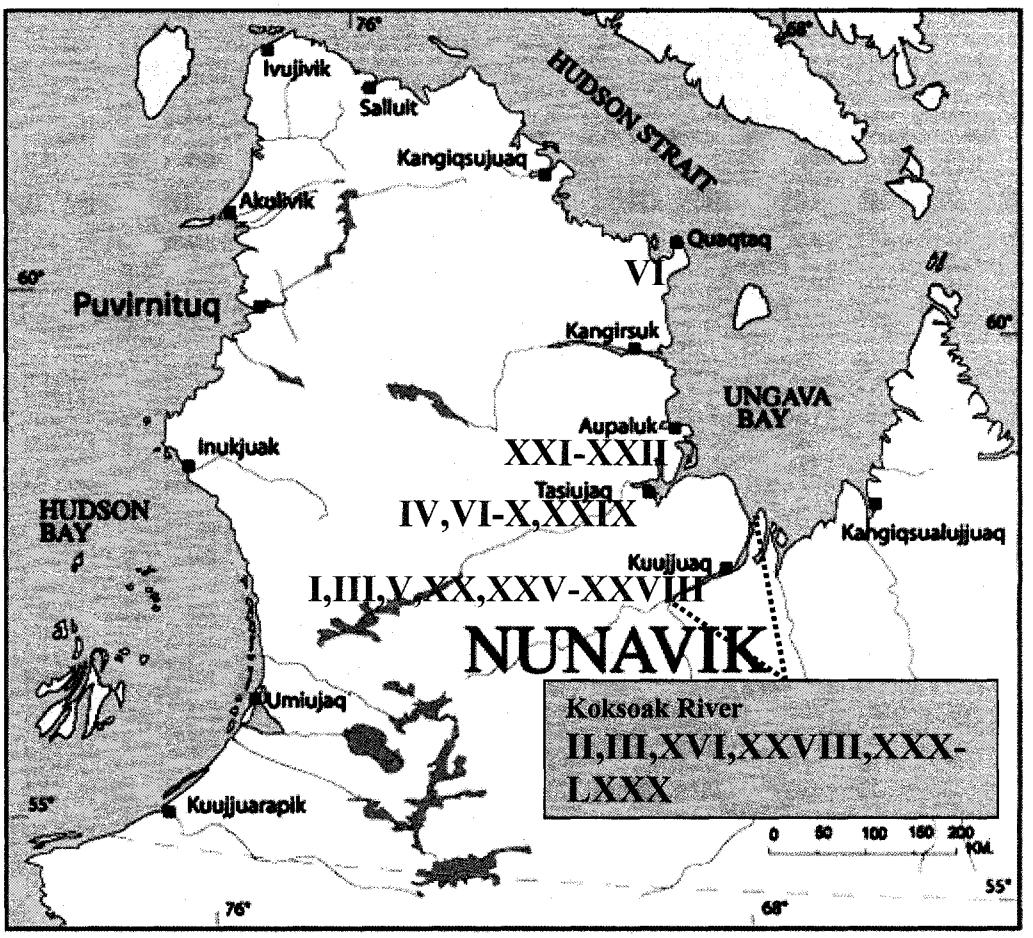


Figure 7.2. Geographic distribution of *C. botulinum* type E subtypes isolated from food and clinical specimens during the investigation of botulism incidents in Nunavik between 1995 and 2004. The environmental subtypes of *C. botulinum* type E isolated from the Koksoak River are included for comparison. Note that the environmental subtypes II, III and XXIX were also isolated from previous botulism incidents in Kangiqsualujjuaq, Kuujjuaq and Tasiujaq, respectively. Source of map: http://www.makivik.org.

Figure 7.3. PFGE dendrogram showing the relationship among 73 environmental isolates of *C. botulinum* type E recovered from the Koksoak River area based on their XhoI macrorestriction fragment patterns. The similarity (%) among strains was determined using the Dice Coefficient and the clustering was performed by UPGMA.

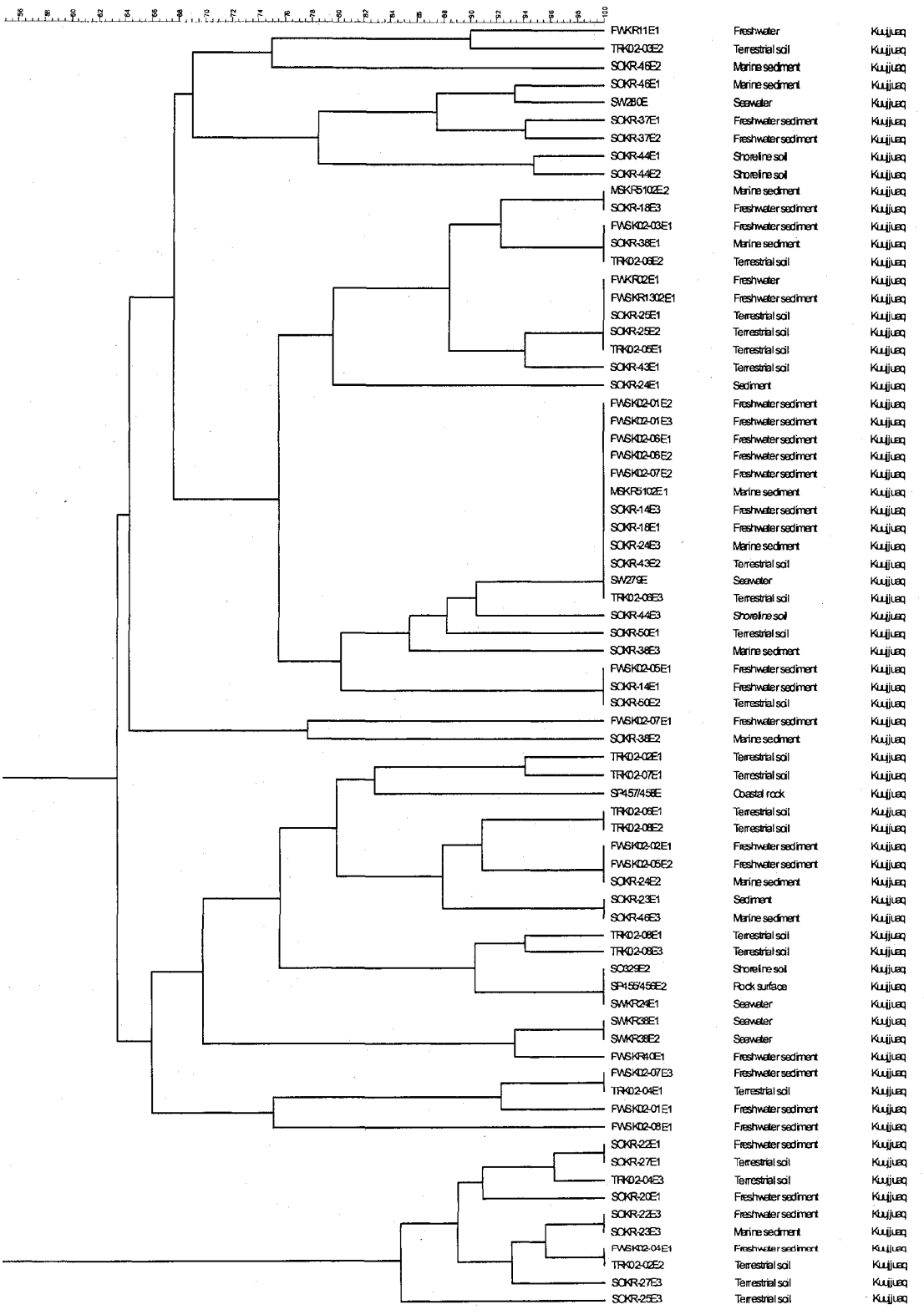

Strain ID
Sample type
Village
56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100
FWKR11E1 Freshwater Kujjuaq
TRK02-03E2 Terrestrial soil Kujjuaq
SOKR-46E2 Marine sediment Kujjuaq
SOKR-46E1 Marine sediment Kujjuaq
SW280E Seawater Kujjuaq
SOKR-37E1 Freshwater sediment Kujjuaq
SOKR-37E2 Freshwater sediment Kujjuaq
SOKR-44E1 Shoreline soil Kujjuaq
SOKR-44E2 Shoreline soil Kujjuaq
MSKR5102E2 Marine sediment Kujjuaq
SOKR-18E3 Freshwater sediment Kujjuaq
FWSK02-03E1 Freshwater sediment Kujjuaq
SOKR-38E1 Marine sediment Kujjuaq
TRK02-09E2 Terrestrial soil Kujjuaq
FWKR02E1 Freshwater Kujjuaq
FWSKR1302E1 Freshwater sediment Kujjuaq
SOKR-25E1 Terrestrial soil Kujjuaq
SOKR-25E2 Terrestrial soil Kujjuaq
TRK02-05E1 Terrestrial soil Kujjuaq
SOKR-43E1 Terrestrial soil Kujjuaq
SOKR-24E1 Sediment Kujjuaq
FWSK02-01E2 Freshwater sediment Kujjuaq
FWSK02-01E3 Freshwater sediment Kujjuaq
FWSK02-06E1 Freshwater sediment Kujjuaq
FWSK02-06E2 Freshwater sediment Kujjuaq
FWSK02-07E2 Freshwater sediment Kujjuaq
MSKR5102E1 Marine sediment Kujjuaq
SOKR-14E3 Freshwater sediment Kujjuaq
SOKR-18E1 Freshwater sediment Kujjuaq
SOKR-24E3 Marine sediment Kujjuaq
SOKR-43E2 Terrestrial soil Kujjuaq
SW279E Seawater Kujjuaq
TRK02-08E3 Terrestrial soil Kujjuaq
SOKR-44E3 Shoreline soil Kujjuaq
SOKR-50E1 Terrestrial soil Kujjuaq
SOKR-38E3 Marine sediment Kujjuaq
FWSK02-05E1 Freshwater sediment Kujjuaq
SOKR-14E1 Freshwater sediment Kujjuaq
SOKR-50E2 Terrestrial soil Kujjuaq
FWSK02-07E1 Freshwater sediment Kujjuaq
SOKR-38E2 Marine sediment Kujjuaq
TRK02-02E1 Terrestrial soil Kujjuaq
TRK02-07E1 Terrestrial soil Kujjuaq
SP457/458E Coastal rock Kujjuaq
TRK02-08E1 Terrestrial soil Kujjuaq
TRK02-09E2 Terrestrial soil Kujjuaq
FWSK02-02E1 Freshwater sediment Kujjuaq
FWSK02-05E2 Freshwater sediment Kujjuaq
SOKR-24E2 Marine sediment Kujjuaq
SOKR-23E1 Sediment Kujjuaq
SOKR-46E3 Marine sediment Kujjuaq
TRK02-08E1 Terrestrial soil Kujjuaq
TRK02-09E3 Terrestrial soil Kujjuaq
SO329E2 Shoreline soil Kujjuaq
SP455/456E2 Rock surface Kujjuaq
SWKR24E1 Seawater Kujjuaq
SWKR38E1 Seawater Kujjuaq
SWKR38E2 Seawater Kujjuaq
FWSKR40E1 Freshwater sediment Kujjuaq
FWSK02-07E3 Freshwater sediment Kujjuaq
TRK02-04E1 Terrestrial soil Kujjuaq
FWSK02-01E1 Freshwater sediment Kujjuaq
FWSK02-08E1 Freshwater sediment Kujjuaq
SOKR-22E1 Freshwater sediment Kujjuaq
SOKR-27E1 Terrestrial soil Kujjuaq
TRK02-04E3 Terrestrial soil Kujjuaq
SOKR-20E1 Freshwater sediment Kujjuaq
SOKR-22E3 Freshwater sediment Kujjuaq
SOKR-23E3 Marine sediment Kujjuaq
FWSK02-04E1 Freshwater sediment Kujjuaq
TRK02-02E2 Terrestrial soil Kujjuaq
SOKR-27E3 Terrestrial soil Kujjuaq
SOKR-25E3 Terrestrial soil Kujjuaq

Table 7.2. Distribution of *C. botulinum* type E subtypes among sediment, water, shoreline and terrestrial soil samples collected in the Koksoak River area

Source of sample	No. of isolates	PFGE subtypes[a]
DOWNSTREAM FROM KUUJJUAQ		
Mouth of the Koksoak River		
Shoreline soil	4	XXX, XXXIV-XXXVI
Coastal rock	2	XXX, XXXII
Seawater	2	XXXI, XXXIII
Marine portion of the Koksoak River		
Seawater	3	XLI, XLIV-XLV
Sediment	13	II, XVI, XXVIII, XXXVII-XL, XLII-XLIII, XLVI-XLIX
Tributaries		
Sediment	5	XLIX-LII
UPTREAM FROM KUUJJUAQ		
Freshwater portion of the Koksoak River		
Sediment	11	XL, XLVIII, LII-LVII
Tributaries		
Freshwater	2	LXII-LXIII
Sediment	9	XXXIX, LVIII-LXI, LXIV-LXVII
Inland area (25 to 100 m from the tributaries)		
Terrestrial soil	22	III, XXVIII, XL, L, LII, LX, LXIV, LXVII-LXXX

[a]Subtypes were generated with SmaI and XhoI pulsotypes. Each unique subtype was designated by a Roman numeral.

There were three unrelated botulism incidents (Nos. 16-18) from the Northwest Territories and all were associated with the consumption of aged beluga products and caused by the same subtype (XXIII) of *C. botulinum* type E (Figure 7.1). Two of these incidents occurred in Inuvik in September 1999 and May 2000, respectively, and the other in Aklavik in March 2001; two Northern villages located near the mouth of the Mackenzie River.

Genomic relationship among environmental strains of *C. botulinum* type E from the Koksoak River area. The sampling of the Koksoak River area was performed throughout the river and its tributaries upstream and downstream from Kuujjuaq (Figure 3.4) and was previously described in detail in Chapter 3. Of 100 isolates recovered from the 41 environmental samples, 73 were compared by cluster analysis following the removal of identical subtypes originating from a common sample. Twenty-nine SmaI and 42 XhoI (Figure 7.3) different PFGE fragment patterns were produced generating 55 (75.3%) different subtypes of *C. botulinum* type E, indicating the presence of a heterogeneous population of *C. botulinum* type E in the Koksoak River area.

For comparison purposes, the different subtypes of *C. botulinum* type E were grouped according to the source of samples and the sampling area along the Koksoak River (Table 7.2). A fairly high proportion of subtypes was specific to each sampling area, i.e., marine, freshwater and inland areas. Thus, 69.6% of subtypes of *C. botulinum* type E from the inland area were not found in the Koksoak River, as well as 59.1% and 78.3% of subtypes from the freshwater and marine environments, respectively were not encountered elsewhere. However, ten subtypes of *C. botulinum* type E were found in more than one sampling area of which seven were found in terrestrial soil, upstream from Kuujjuaq. These telluric subtypes were found in various locations along the

Koksoak River, either in freshwater (XXVIII, XL, LII) or marine (XXVIII, XL) sediments of the river, or in freshwater sediments of tributaries such as in small lakes (L), creeks (L, LII, LXIV) and merging rivers (LXVII). One subtype of *C. botulinum* type E was found at the same location, but in different substrates. Terrestrial isolate SOKR-27E1 and isolate SOKR-22E1 found in freshwater sediment of a creek, were collected approximately 25 meters from each other, and both belonged to PFGE subtype LX. In another case, a PFGE subtype was found in two locations at a considerable distance from each other. The PFGE subtype L was found in terrestrial soil upstream from Kuujjuaq, about 30 km from the beginning of the Koksoak River and also in the sediment of a small lake near the mouth of the river, about 100 km from the other site. In contrast, none of the *C. botulinum* type E subtypes isolated from samples of seawater, coastal rocks, or shoreline soil collected at the mouth of the river were found upstream, even in the marine portion of the river. However, two subtypes of *C. botulinum* type E (XLVIII and XLIX) isolated from marine sediments near the mouth of the river, were found in freshwater sediments upstream from Kuujjuaq, and in the small lake near the mouth of the river, respectively, suggesting that the freshwater environment could contribute to the pool of coastal type E isolates.

Genomic relationship among strains of *C. botulinum* type E from the Koksoak River area and strains recovered from botulism incidents of southern Ungava Bay. Based on the comparison of SmaI and XhoI PFGE patterns, four environmental strains of *C. botulinum* type E from the Koksoak River area were found to be identical to outbreak strains from two Northern villages of Ungava Bay, Kuujjuaq and Kangiqsualujjuaq (Table 7.3). Thus, terrestrial isolate SOKR-27E3 originating from an inland location upstream from Kuujjuaq, had the same PFGE subtype (III) as the

Table 7.3. Identical subtypes of *C. botulinum* type E observed among environmental isolates from the Koksoak River area and isolates from botulism incidents in southern Ungava Bay 1995-2004

Subtype[a]	Locations in the Koksoak River area	Incident No.	Northern Village	Date of incident
II	Marine portion of the river	2	Kangiqsualujjuaq	07/1995
III	Inland area upstream from Kuujjuaq	3	Kuujjuaq	08/1995
XVI	Marine portion of the river	12	Kangiqsualujjuaq	09/1997
XXVIII	Inland area upstream from Kuujjuaq, marine and freshwater portions of the river	23	Kuujjuaq	11/2002

[a]Subtypes were generated with SmaI and XhoI pulsotypes.

outbreak strain from incident No. 3 that affected at least two people in Kujjuuaq on August 1995. The PFGE subtype XXVIII found in the sediments of the freshwater (FWSK02-03E1) and marine (SOKR-38E1) portions of the river as well as the inland soil (TRK02-06E2) upstream from Kuujjuaq, was observed in a fecal specimen of a botulism case that occurred in Kuujjuaq on November 2002. Geographically distant, two environmental subtypes of *C. botulinum* type E recovered from the Koksoak River area were also isolated during two botulism incidents that occurred in Kangiqsualujjuaq, approximately 175 km east from Kuujjuaq. SOKR-23E1, a type E strain isolated from marine sediment of the Koksoak River had the same PFGE patterns as the food and clinical isolates of incident No. 2 that occurred in Kangiqsualujjuaq on July 1995.

Similarly, SOKR-46E2 recovered from the marine sediments of the river had the same PFGE patterns as those produced by one of the two identified outbreak strains from incident No. 12 that affected nine people in Kangiqsualujjuaq on September 1997. These results indicate that the Koksoak River is a reservoir of *C. botulinum* type E strains that have the ability to grow and produce toxin in aged marine mammal products and cause botulism in humans.

Some environmental strains of *C. botulinum* type E were found closely related to outbreak strains and differed by only one fragment when using the XhoI or SmaI restriction enzyme. Isolate SOKR-46E3 from marine sediments of the Koksoak River yielded a PFGE subtype closely related to that of *misiraq* isolate MI9507E from incident No. 2 that occurred in Kangiqsualujjuaq in 1995. Similarly, freshwater sediment isolate FWK02-01E1 recovered at the dock of Kuujjuaq and isolate GA9706EMA isolated from a Tasiujaq patient, had closely related PFGE patterns. These results suggest that the distribution of some subtypes of *C. botulinum* type E

observed in the Koksoak River area extend further along the coastline of the southern Ungava Bay, east and west, up to the neighbouring villages.

7.4 Discussion

The rapid identification of common food sources causing human botulism is critical during an outbreak investigation, particularly when the suspect food is a commercial product that has been widely distributed. Laboratory confirmation of the implicated food is currently performed by demonstrating the presence of the same type of botulinum toxin and/or viable *C. botulinum* organisms in both food and clinical specimens using the mouse bioassay. PFGE has been recently applied for the investigation of foodborne (Lindström *et al.*, 2004) and infant botulism incidents (Johnson *et al.*, 2005; Nevas *et al.*, 2005b) and has been found useful to confirm suspect sources based on molecular evidence.

The results of this study showed that 83.3% of type E outbreaks, for which both clinical and food isolates were obtained, have yielded isolates with identical SmaI and XhoI PFGE patterns. This indicates that each cluster of epidemiologically related isolates are derived from a common outbreak strain. The finding of identical PFGE subtypes of *C. botulinum* type E in both clinical and food specimens enables (the clinical microbiologist or epidemiologist) to conclude with a higher level of confidence that the suspect food was the source of botulinum intoxication. The interpretation becomes challenging when different subtypes of *C. botulinum* are isolated from related food and clinical specimens, and this requires further investigation to determine the presence of multiple isolates in the diagnostic specimens or in other potential food sources. The detection of multiple type E isolates was demonstrated in both clinical and food specimens from incident No. 12 from which six different subtypes of *C. botulinum* were isolated. One common subtype (XIV) was isolated from six clinical specimens belonging to six different patients of the same incident, but not from the suspect seal *igunaq*. The molecular link between the food source and the outbreak cases was

established by isolating a second isolate (FE9709E2BB) from one of these clinical specimens which produced indistinguishable PFGE fragment patterns from isolate MI19709E recovered from the suspect seal *igunaq*. These results highlight that in order to be able to establish an epidemiological linkage based on molecular evidence, it is necessary to routinely obtain several isolates (at least three) from both food and clinical specimens during an investigation of a botulism outbreak.

In some cases, the molecular link could not be established between a food and a clinical specimen because the submitted food sample was not the food consumed that had caused botulism. In incident No. 23, three isolates of *C. botulinum* type E were recovered from a beluga fat sample involved in a botulism case in Kuujjuaq in November 2002, but none of their PFGE patterns matched those of the related clinical isolate FE0211E1DK. The epidemiological investigation revealed that the submitted beluga fat sample was not consumed, but rather used to produce *misiraq* (marine mammal oil resulting from the aging of fat) about two years before the incident. The investigation also traced the food source to a traditional Inuit food called "tingnak", which is the solid fat part not dissolved in oil when making *misiraq*. The suspect tingnak was kept frozen for two years and was entirely consumed by some inhabitants of Kuujjuaq and affected one person. Nonetheless, the presence of multiple subtypes of *C. botulinum* type E found in the submitted fat sample indicated that the fat was not safe for aging into *misiraq*.

The contamination of seal *igunaq* by multiple type E isolates likely reflects the diversity of *C. botulinum* in the Arctic environment where marine mammals are butchered, as was previously shown in Chapter 6. Analysis of 73 isolates of *C. botulinum* type E from the Koksoak River area resulted in 55 different PFGE subtypes, indicating the existence of a high biodiversity of *C. botulinum* type E in the area. The

spores were widely distributed in the Koksoak River and some subtypes encountered in inland areas, south of Kuujjuaq, were also found in sediments of the river and its tributaries, sometimes several kilometres apart. The spores present in these inland and freshwater areas would naturally reach the coastline environment, north of Kuujjuaq, through runoff waters and river flow, and then either be carried out by water currents along the coastline or settle locally near potential butchering sites. Two botulism incidents from Kuujjuaq (Nos. 3 and 23) and two from Kangiqsualujjuaq (Nos. 2 and 12) were caused by subtypes of *C. botulinum* type E that were isolated from the Koksoak River environment. Subtype XXVIII which was involved in incident No. 23, was isolated from an inland location upstream from Kuujjuaq and also from the freshwater and marine sediments of the river. The epidemiological investigation of incident No. 23 revealed that the suspect aged beluga product (tingnak) originated from a beluga whale harvested at Dry Bay, just west of the mouth of the Koksoak River. These results suggest that the Koksoak River was a possible environmental source of subtype XXVIII involved in this incident.

Contamination of marine mammal meat with *C. botulinum* type E may occur at the butchering site through contact with shoreline soil, seawater, or surface of coastal rock from the local environment (Chapter 6). The location of the butchering sites of the seals involved in the incidents Nos. 2, 3 and 12 was only documented for incident No. 12 for which the seal was butchered on Kaugaq Island, approximately 50 km northwest from Kangiqsualujjuaq, or 80 km northeast from the mouth of the Koksoak River (Figure 3.4, site BL-328). A butchering site from this island was investigated during the survey of the coastline environment in 2000 (Chapter 3), but none of the environmental samples collected at the butchering site contained spores of *C. botulinum*. However, one of four subtypes of *C. botulinum* type E detected in the seal *igunaq* preparation which

was identified as an outbreak strain (subtype XVI) involved in the incident No. 12, was found in the sediments of the marine portion of the Koksoak River. This river and possibly other large rivers such as the George River near Kangiqsualujjuaq, are abundant sources of *C. botulinum* type E spores which may be carried by water current along the coast and contaminate the shoreline environment of inshore islands. The involvement of large river systems as a potential source of outbreak strains was further supported by the finding of a clinical subtype of *C. botulinum* type E from Tasiujaq detected at a common butchering site along the Leaf Bay, a catchment basin for the Leaf River. The PFGE patterns of SO325E1, a type E strain isolated from shoreline soil also collected during the coastline survey were found to be identical to the PFGE patterns of strain FE9508EPB, which was isolated in 1995 from a Tasiujaq patient affected with botulism after eating walrus *igunaq*.

Three incidents from Tasiujaq (incidents No. 20-22) and one from Quaqtaq (incident No.6), unrelated in time, were caused by the same subtype of *C. botulinum* type E (VI). The three incidents from Tasiujaq affecting five people occurred consecutively within a 7-month period from July 2001 to February 2002, and were all associated with the consumption of walrus *igunaq*. Following these three incidents, interviews were conducted in Tasiujaq with five *igunaq* producers and revealed that the suspect *igunaq* preparations were made by two producers using meat, fat and skin tissues from walruses that were all harvested in July 2001 (Suppa and Proulx, 2002) at Imilik, a popular area for hunting walruses during their summer migration and commonly used for butchering marine mammals. Imilik is located near Quaqtaq where botulism incident No.6 associated with the consumption of seal *igunaq* occurred in 1996, and which affected three people. The location of the butchering site used for the seal involved in the incident No.6 was not reported. However, it appeared that the

walrus meat used in the production of three different *igunaq* preparations by Tasiujaq producers, was contaminated by the same environmental strain of *C. botulinum* subtype VI, most likely during the butchering process in Imilik. Thus, the distribution of outbreak subtypes of *C. botulinum* type E based on the location of the incidents may not reflect the environmental origin or distribution of these subtypes.

Similarly, two unrelated botulism incidents (Nos. 14 and 15) from Aupaluk that occurred on November 1998 and August 1999 were caused by the same genotype of *C. botulinum* type E (XXII). The food source implicated in incident No. 15 was not identified, while incident No. 14 was associated with the consumption of seal *igunaq*. Three unrelated incidents (Nos. 16-18) from Aklavik and Inuvik in the Northwest Territories (NWT) that occurred between September 1999 and March 2001 were all associated with PFGE subtype XXIII. These incidents occurred in two Northern villages located near the mouth of the McKenzie River, and were all associated with the consumption of aged beluga whale products. There was no information on the origin of the implicated products involved in the Aupaluk and NWT incidents, whether they were collected from the same harvested animal, from different animals or whether they were butchered at the same site. Collecting this information would be useful to determine if some butchering sites are more often linked to outbreak cases and therefore should be avoided by hunters who wish to age meat and fat of marine mammals into *igunaq* or *misiraq*. Repeated use of butchering sites could promote accumulation of tissue materials and nutrients for the local bacterial flora. If presented with adequate environmental conditions, *C. botulinum* will germinate, decompose the biological materials and increase its population level in the local environment. Sporulation will allow survival of these "blooms" of growth for decades. The sporadic growth of *C. botulinum* type E resulting from the decomposition of marine mammal carcass residues

left on the shoreline (Huss, 1980) and the natural flow of spores originating from the inland and freshwater environments, are potential sources contributing to the spore load present in the coastal area.

No type A or type B botulism outbreaks have been recorded in the Canadian Arctic, except for a single case of type B botulism which occurred in Baker Lake, Nunavut associated with the consumption of raw lake trout. This association was based on epidemiological evidence. A type B strain of *C. botulinum* group II was isolated from the liver of the patient, but no viable microorganism or toxin was detected in the submitted gastric liquid (Hauschild, 1985). A type A strain of *C. botulinum* group I was isolated for the first time from gastric liquid from a botulism case in the Canadian Arctic in July 2001. The case occurred in Sanikiluaq, Nunavut (Incident No.19) and was epidemiologically linked to the consumption of aged beluga whale meat that had been buried in the ground for some time before consumption. Fecal and a gastric liquid specimens were received for testing, but no food samples were submitted for analysis. The type A strain was isolated from the gastric liquid of the patient, but no BoNT or viable *C. botulinum* type E was isolated from the feces or gastric liquid of the patient. Thus, it was not possible to determine whether the detection of the type A strain in the gastric liquid had any clinical significance. A similar situation occurred in Finland where a *C. botulinum* group I type B strain was isolated from the gastric liquid of a patient, and yet it was demonstrated that the patient was in fact affected with type E botulism (Lindström *et al.*, 2004).

A number of type A and type B botulism cases have been increasingly reported from Alaska since 1971, and all of them have been related to the consumption of fish or land mammals (Barrett *et al.*, 1977; Wainwright *et al.*, 1988). Type A or types B and F *C. botulinum* group I strains have not been isolated from the coastal area of Alaska,

suggesting that these strains may originate from inland areas. A type A strain was recently isolated from shoreline soil near a butchering site at Big Finger Point near Kuujjuaraapik in the eastern part of Hudson Bay. Sanikiluaq is also situated in the eastern Hudson Bay, in the Belcher Inslands. Comparison of PFGE fragment patterns of the two type A isolates showed that they were of different subtypes (data not shown). The geographical distribution and genetic diversity of *C. botulinum* group I strains in Arctic regions is unknown. However, a recent study examined the genetic diversity of *C. botulinum* strains of European and American origin and showed that group I was a heterogeneous species based on PFGE, although the presence of some clusters was observed (Nevas *et al.*, 2005a). *C. botulinum* group I strains were not isolated from the extensive survey conducted in the Koksoak River. However, the isolation procedure used involved the use of affinity-purified rabbit antibodies against type E botulinum neurotoxin only, and not against type A, B, and F toxin. Therefore, it is theoretically possible that some of the positive samples could also have contained other serotypes of *C. botulinum*, besides type E.

The demonstration of BoNT in diagnostic specimens remains the optimal confirmatory procedure for use in investigation of botulism outbreaks. The use of coproexamination for *C. botulinum* is particularly of diagnostic aid when the submitted clinical and food specimens are negative for BoNT (Dowell *et al.*, 1977). However, the detection of fecal isolates of *C. botulinum* type E in healthy individuals may be higher in the Inuit population than in the southern populations. The frequent consumption of marine mammals, particularly by adults and elders, and the abundance of spores of *C. botulinum* type E in the coastal areas, may lead to a greater contamination of the gastrointestinal tract of this population. Finding the same PFGE subtype in two different clinical specimens or a clinical and a food specimen, would however confirm the

diagnosis. PFGE was found useful in tracing and confirming food sources involved in several type E botulism incidents in the Canadian Arctic and identifying a cross-contamination event. The same subtype of *C. botulinum* type E (XIII) was isolated from the clinical specimens of two patients and two different suspect food samples submitted for the investigation of the botulism outbreak that occurred in Arviat, Nunavut in August 1997, which affected four people. The two suspect foods involved caribou fat and beluga whale meat, and both were collected from the same garbage bin. As the beluga meat was the only food sample containing type E neurotoxin, it is reasonable to assume that the isolation of subtype XIII from caribou fat resulted from a cross-contamination event.

In summary, several distinct outbreak subtypes of *C. botulinum* type E were characterized by PFGE in Inuit villages of southern Ungava Bay, indicating the existence of a high genetic diversity of *C. botulinum* type E in the environment. Some of these subtypes were detected in inland soil and freshwater and marine sediments of the Koksoak River that flow into southern Ungava Bay, the latter of which is considered a potential source of *C. botulinum* type E spores present in the coastal environment where marine mammals are butchered on the shoreline. Some PFGE subtypes of *C. botulinum* type E were found commonly among unrelated botulism incidents from the NWT and Nunavik regions, and were attributed to the use of common butchering sites and/or geographical regions where these cases had occurred. Systematic isolation and examination of multiple isolates of *C. botulinum* from clinical specimens and suspect foods is recommended during any outbreak investigation of botulism in the Canadian Arctic, due to possible contamination of traditional foods with multiple type E strains.

CHAPTER 8

FACTORS AFFECTING THE GROWTH AND TOXICITY OF *CLOSTRIDIUM BOTULINUM* TYPE E IN AGED SEAL MEAT (*IGUNAQ*)

8.1 Introduction

In Canada, most botulism outbreaks have been associated with the consumption of traditional northern native foods, such as aged fish and marine mammal products, and more rarely, with commercial products (Austin and Dodds, 2000). A popular and traditional Inuit food delicacy called "*igunaq*", made from the natural aging or putrefaction of marine mammal meat, was the most frequent food type associated with type E botulism outbreaks in Arctic regions of Canada. The traditional process consists of aging a large amount of marine mammal meat and fat in sewn skin pouches made from the animal's hide and maintained outside in a cache for several weeks or months. Preparation methods are now highly variable and involve the use of different containers (plastic pouches, plastic and galvanized containers) and storage areas (sheds, wooden boxes).

Under certain conditions, these preparations support the growth of *C. botulinum* type E and the production of its neurotoxin. The use of plastic bags in lieu of skin pouches, a high incubation temperature, lack of fat in the preparation and low air flow inside a cache, are considered factors that can lead to the production of poisonous *igunaq* (Weetaluktuk, 1997). The potential of each factor or their combined effects to induce growth of *C. botulinum* type E and toxin formation has not been determined under laboratory conditions. To reduce the risk of botulism from eating aged foods, it is recommended to age foods below 2°C and to use traditional methods that allow air to circulate, and not to use airtight plastic and glass jars, buckets or plastic bags (Castrodale, 2005).

Various methods are used to control the growth of *C. botulinum* and toxin development in meat and meat products. Methods include heat treatment, lowering of pH, and increasing salt content, combined with a low storage temperature (Lücke and

Roberts, 1993). These control measures were established to ensure that high-risk products could not support the growth of *C. botulinum* and toxin production when challenged with high spore loads at abuse temperatures. Salt, fermentable carbohydrates or bacterial cultures are not added to seal meat prior to aging by *igunaq* producers to prevent growth of *C. botulinum* type E, nor is a heat treatment used to destroy botulinum neurotoxin that could have been produced during the aging process. Except for low storage temperature, use of the other control procedures would inevitably alter the taste of the *igunaq*. The objectives of this study were to evaluate the time to toxicity of traditional and various modern seal *igunaq* preparations inoculated with a range of spore loads of *C. botulinum* type E, incubated at recommended and abuse temperatures, under different atmosphere conditions.

8.2 Materials and Methods

Source of seal meat, fat and skin tissues. The choice of the bearded seal as a source of meat and fat for challenge studies was based on interviews conducted with 20 *igunaq* producers from Nunavik, between April 2001 and June 2002 (Chapter 4). These interviews revealed that meat of bearded seal was preferred over that of ringed seals. Up to three bearded seals were harvested by the NRC staff in hunting areas near the Nothern Village of Kuujjuaq to provide sufficient amounts of meat, fat and skin tissues for five challenge studies carried out in the laboratories of Health Canada. The first bearded seal was hunted at the end of September 2002 in the Koksoak River at Savarkuluk, about 58 km upstream of Kuujjuaq. The animal was butchered on a gravel beach and large pieces of meat and fat were cut and placed into separate pails. The entire skin, with superficial fat tissues, was put into large plastic bags and transported to the NRC where tissues were kept refrigerated until shipping. The second bearded seal was hunted on July 7, 2003 at the mouth of the Koksoak River at high tide and butchered on a large flat rock at a butchering site located on the east side of the mouth of the river (Figure 3.4, KR-44 site). The third seal was hunted and butchered at Dry Bay at low tide, about 17 km west of KR-44 site on a large flat rock covered with slime, on September 20, 2003. The superficial fat attached to the skin of these two last seals was removed on-site and kept for the challenge studies. All samples were sent in large coolers with ice-packs to Health Canada where they were either pre-cut and/or immediately frozen until inoculation.

Experimental design. Two experiments were conducted to evaluate the time-to-toxicity of seal *igunaq* preparations using a single high inoculation dose (10^3 spores of *C. botulinum* type E per gram of meat), three temperatures (4, 10 and 20°C), two types

of containers (polystyrene jars [Exp. No. 1] and seal skin pouches [Exp. No. 2]) and a maximum storage time of 42 or 49 days. The selection of abusive temperatures was based on the results of three temperature data loggers (Optic Shuttle™, Intermountain Environmental, Inc., Logan, Utah, USA) kept in different *igunaq* preparations aged in Kuujjuaq between mid-July and mid September, 2001 (Chapter 6). The average temperature of the four batches was 10°C and ranged from 2 to 23°C during the aging period. The storage time (6 weeks) corresponds to one and one-half (1.5) times the maximum incubation time reported in the survey with the *igunaq* producers from Nunavik. Sample containers were opened to air bi-weekly to simulate periodic monitoring of preparations and assess the effect of air exposure on *C. botulinum* growth and toxin production. Sampling times were set at 2, 4, and 6 weeks, at which time duplicate and triplicate samples of *igunaq* aged in seal skin pouches and polystyrene jars, respectively, were removed and tested for toxicity and growth parameters.

A large-scale challenge study (Exp. No. 3), with weekly sampling intervals was conducted to assess the effect of three levels of inocula (10^1, 10^2, and 10^3 spores per g of meat) and three temperatures (4, 10 and 20°C) over a long storage time (84 days) on toxin production of *C. botulinum* type E in seal *igunaq*. The *igunaq* was also prepared in triplicate in polystyrene jars covered with cheese cloth. The replacement of the impermeable plastic cover by the cheese cloth was done to simulate the use of thin fabric that is currently used by some *igunaq* producers from Quaqtaq (Chapter 4), where only one botulism incident has ever been reported. The use of cheese cloth allowed for a continuous air exchange inside the preparations. Similarly, two experiments were conducted to evaluate the effect of the same inoculation levels and temperatures on botulinum toxin formation in aged *igunaq*, but using different containers and atmosphere conditions. Inoculated seal meat and fat tissues were also aged in seal skin

pouches (Exp. No. 4) and in high-barrier bags (Exp. No. 5) kept closed and stored for 56 days, with sampling being done every two weeks for toxin. The small sutured seal skin pouches and high-barrier bags of low oxygen transmission rate were used in these experiments to simulate, respectively, the traditional dish called "*puurtaq*" made with seal skin, and the high-barrier domestic plastic bags, where containers were not opened periodically in order to simulate a "worst case scenario".

Selection of *Clostridium botulinum* type E strains. All food challenge studies were conducted with *C. botulinum* type E strains, as all recorded foodborne outbreaks from Nunavik were associated with strains of this serotype. MI9507E, VI9508E, MI9706E, GA9604EAK, and GA9709ENS are type E strains that were either isolated from incriminated aged seal products or clinical specimens of patients that had consumed such foods. Table 8.1 describes their origin, location and year of isolation. Prior to inoculum preparation, each strain was first grown in SPGY broth to assess their level of toxin production by the quantitative slot blot procedure, as described by Cadieux *et al.* (2005). The levels of toxicity of the five strains ranged from 1258 to 7151 MLD, while the minimum toxic level recommended for challenges studies is 1000 MLD (Doyle, 1991).

Production, preparation and enumeration of spore cocktail. Spore crops were produced on McClung Toabe agar (Bacto™, Becton, Dickinson and Company, Sparks, Maryland, USA) at room temperature stored under anaerobic conditions for 5 to 7 days for each of the five type E strains. After verification of the presence of spores by phase contrast microscopy, spores were harvested in 5 mL of sterile distilled water using a hockey stick spreader and transferred into a 40-mL Nalgene tube with a sterile Pasteur

Table 8.1. List of food and clinical strains of *C. botulinum* type E selected for the inoculation studies with seal *igunaq*

Strain ID	Origin	Northen Village	Isolation date	Source
MI9507E	*Misiraq*	Kangiqsualujjuaq	95-11-17	BRS[a]
VI9508E	Seal meat	Kuujjuaq	95-11-28	BRS
MI9706E	Seal *igunaq*	Tasiujaq	98-02-13	BRS
GA9604EAK	Gastric liquid	Quaqtaq	96-06-12	BRS
GA9709ENS	Gastric liquid	Kangiqsualujjuaq	98-11-17	BRS

[a]BRS: Botulism Reference Service for Canada, Health Canada, Ottawa, Ontario, Canada.

pipette. Following harvesting, spores were washed four times with sterile distilled water using the following centrifugation speeds and times to remove cell fragments and media: one centrifugation at 14,470 × *g* for 10 min at 4°C and three at 7,093 × *g* for 10 min at 4°C. An aliquot of each spore suspension was heat-shocked at 60°C for 20 min to inactivate vegetative cells and then enumerated by direct plate counting on McClung Toabe agar. Based on spore counts, an appropriate volume of each strain was mixed to make a cocktail with equal numbers of spores of each strain. The stock suspension was diluted and divided in aliquots at the desired volume and concentration and stored at –86°C until use. The spore concentration of the composite was verified by plate counting. Dilutions of the stock suspension were plated (100 µL) in duplicate onto McClung Toabe agar medium and lipase-positive colonies were counted under fluorescent light after three days of incubation at room temperature in an anaerobic chamber with an atmosphere of 10% H_2, 10% CO_2, and 80% N_2.

Preparation and storage of various types of seal *igunaq*. The amounts of meat and fat tissues in all challenge studies were kept constant at 60 and 90 g respectively, for a meat/blubber ratio of 1:1.5. This ratio was based on the survey conducted with the *igunaq* producers which recommended including 50 to 60% fat to avoid the production of bad *igunaq* preparations (Chapter 4). Meat and fat tissues were cut into large pieces and pre-weighed in separate 120-mL plastic containers. Two types of containers were employed for experiment numbers (Exp. No.) 1 and 2. Clear wide-mouth polystyrene containers (150 mL) allowing examination of the decomposition process were used to simulate the large plastic containers in Exp. No. 1 (Figure 8.1). The plastic covers were kept loose to allow air exchange. Seal skin pouches were made in Exp. No. 2 to simulate the traditional seal *igunaq* (*puurtaq*), which consists of aging meat and fat

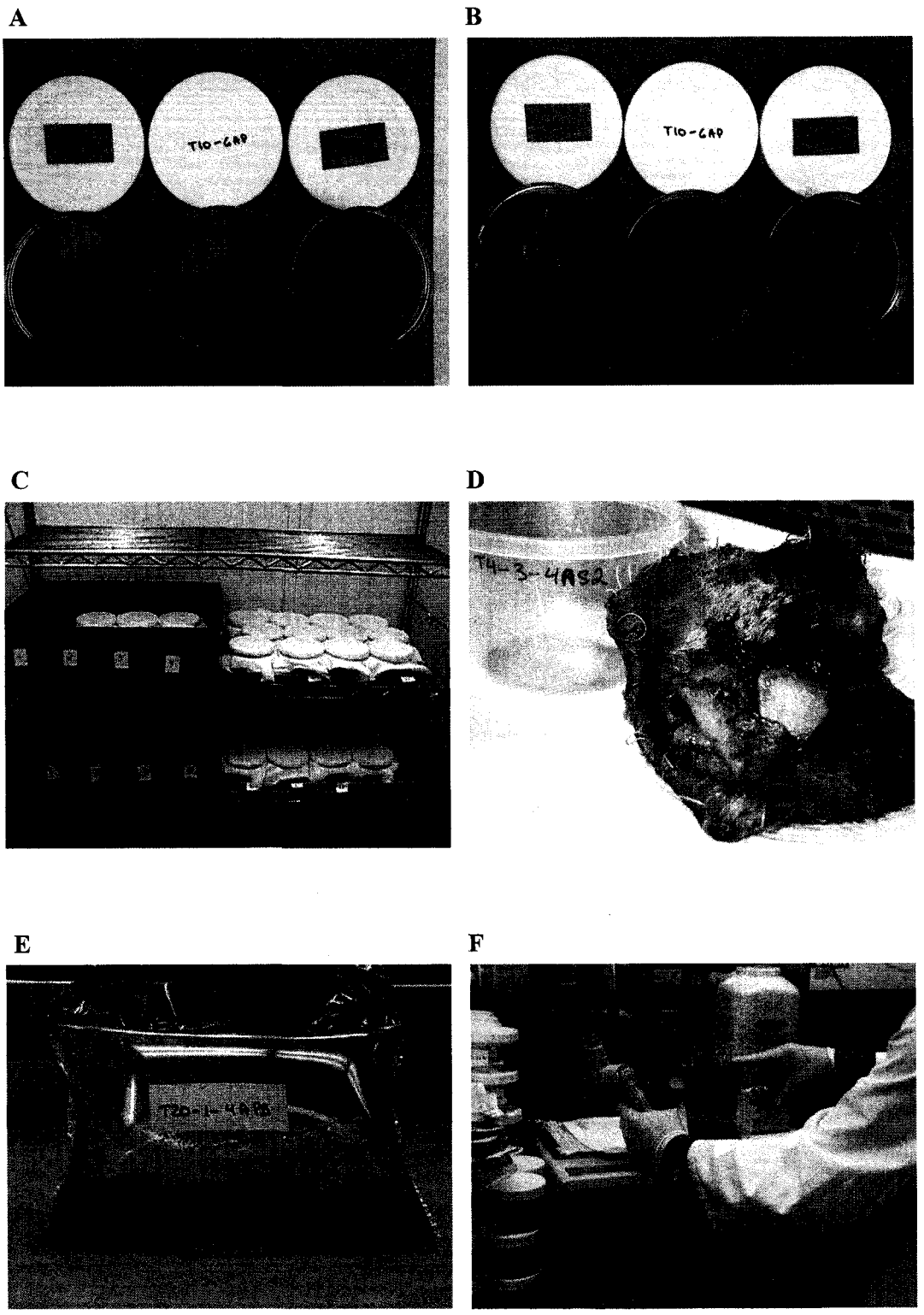


Figure 8.1. Different types of seal *igunaq* preparations tested in this study. Panels A and B: seal *igunaq* in plastic containers after 7 and 42 days of incubation, respectively; panel C: seal *igunaq* in plastic containers covered with cheese cloth and maintained in a walk-in incubator; panel D: seal *igunaq* aged in seal skin pouch; panel E: seal *igunaq* in a high-barrier oxygen bag; panel F: measurement of headspace gas in a seal skin pouch using the Servomex gas analyser.

tissues within a large inverted skin pouch in which the skin and hairs are in direct contact with meat and fat tissues. Large pieces of skin (approximately 9.4 cm × 16 cm), were cut from the animal's pelt, inverted, and sewn tight with a simple continuous appositional suture using nonabsorbable polyamide pseudomonofilament (Supramid® 3+4). The opening of the pouch was shut using large paper clips placed on two wooden sticks set on the edges of the pouch to allow periodic opening.

The same type of polystyrene containers was used for Exp. No. 3, except that the opening of the container was covered with a piece of cheese cloth (Absorbant doublefold gauze 90M / 3A, Kandall Canada Inc., Peterborough, Ontario, Canada) of 225 cm^2 in size and folded over three times. Similar to Exp. No. 2, Exp. No. 4 was conducted using seal skin pouches of 8.8 cm (width) × 15.7 cm (height) which were sewn using the same suture type and material. In Exp. No. 5, high gas barrier bags (Cryovac Sealed Air Corp., Mississauga, Ontario, Canada) of 210 mm (width) × 205 mm (height) in size with an oxygen transmission rate of 12 $cc/m^2/day$ at 24°C were used. Inoculated seal meat and fat tissues were packaged in air and sealed with an Impulse heat sealer (Model SP-300H, Zenda Industrial Co.,). The Cryovac bags and *puurtaq* preparations were placed in individual containers to avoid any contact or cross-contamination events among these preparations due to possible leakage of oil during the aging process. All *igunaq* preparations were stored in large upright or walk-in incubators each equipped with an external temperature display and internal thermometers and hygrometers. The temperature and relative humidity of each of the incubators was monitored and recorded at least three times per week.

Inoculation doses and method. The first two experiments were conducted using a high inoculation dose of 10^3 spores/g of meat to determine if toxin could be detected in seal

igunaq. A fresh working spore suspension was heat-shocked at 60°C for 20 min immediately prior to inoculation. Three pieces of meat from each *igunaq* sample were surface-inoculated with a small volume (Exp. No. 1: 41 μL; Exp. No. 2: 35 μL) of inoculum and then randomly placed with the remaining meat and fat tissues in the container. The inoculum level should reflect the contamination level of raw material with *C. botulinum* type E. The background levels of spores in raw seal meat was therefore estimated for two bearded seals using a five-tube MPN procedure whereby three decimal amounts (0.5 g, 5 g, and 50 g) of seal meat were each cultured in five tubes and assayed by the mouse bioassay for type E botulinum neurotoxin, as described in Chapter 2. None of the MPN tubes led to toxicity in mice, which resulted in a MPN count of < 4 spores per kg of meat, indicating that the contamination level was very low. Thus, the effect of inoculation dose was tested using three 10-fold levels of spore inoculum, i.e., 10^1, 10^2 and 10^3 spores/g of meat or 600, 6000, and 60000 spores per 60 g of meat, respectively, for Exp. Nos. 3-5.

Detection of botulinum toxin and serotyping. Following the removal of two 5-g samples kept for the measurement of growth parameters, the entire *igunaq* sample, including the oil, was stomached in 300 mL of GPB for 5 min and a portion (135 g) of the homogenate was transferred into a sterile 250-mL bottle. The extract was collected following centrifugation at 18,500 × *g* for 20 min at 4°C and filter-sterilized through a 0.45 μm filter. The undiluted extract was trypsinized and injected into mice as described in Chapter 2. *Igunaq* extracts showing toxicity in mice at the earliest sampling time, were seroneutralized with monovalent antitoxin E and tested in mice to confirm that the cause of toxicity was type E botulinum neurotoxin.

Estimation of *C. botulinum* type E level. The quantification of *C. botulinum* type E in seal *igunaq* was performed for Exp. Nos. 1 and 2 only. Serial dilutions of the *igunaq* homogenate (150 g of *igunaq* in 300 mL of GPB) were prepared with GPB dilutions and each dilution (10^{-2} to 10^{-8}) was streaked onto two CBI agar plates. The inoculated CBI plates were incubated in an anaerobic chamber with an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 for three days at room temperature. Dilutions with 30 to 100 lipase-positive colonies on plates were counted using a Darkfield Quebec® colony counter model 3327 (American Optical Corporation, Buffalo, New York, USA) and their numbers confirmed by colony blot immunoassay and expressed as CFU/g of *igunaq* (Chapter 2).

Water activity and surface pH measurements. A 5-g meat sample was collected from each *igunaq* sample at each sampling time (including at day 0) of Exp. Nos. 1 and 2 for water activity (a_w) and pH measurements. The sample was placed in a small plastic dish and inserted in the AquaLab water activity meter Series 3 (Decagon, Pullman, Washington, USA) for a_w measurements. Using the same sample, the pH was measured using an Orion Perphect Ross flat surface electrode connected to a Hanna Instruments pH meter model HI 931400 (Hanna Instruments, Bedfordshire, United Kingdom). The pH was recorded at three different locations on the sample. Each a_w and pH value reported represented the average of two and three measurements, respectively.

Estimation of aerobic colony counts. A 5-g meat sample was collected from each *igunaq* sample (Exp. Nos. 1 and 2) at each sampling time (including at day 0) for the aerobic plate count (APC). The sample was stomached in 50 mL of 0.1% peptone-water and the homogenate (0.1 mL) was serially diluted in peptone-water. Each dilution (10^{-6}

to 10^{-8}) was spread plated (0.1 mL) onto two tryptic soy agar (Difco™, Becton, Dickinson and Company) plates using a sterile microbiological spreader and incubated at 20°C for 4 days (Lindroth *et al.*, 1986). Colonies were counted on a Darkfield Quebec® colony counter and expressed as CFU/g of meat.

Redox measurements. The redox potential (E_h) in seal *igunaq* was performed using a Thermo Orion platinum electrode model 96-78 connected to a portable Orion meter model 250Aplus (Thermo Fisher Scientific Inc., Waltham, Massachusetts). Preliminary E_h measurements performed on a seal meat surface and in a slurry made of seal meat and deoxygenated water at a 1:3 ratio required more than 2 to 3 h to reach a stable E_h reading. Instead of measuring each sample at the sampling time, the E_h electrode was installed in a representative *igunaq* sample and daily E_h values were recorded at least five days a week over the entire length of the experiment. The E_h was recorded from three *igunaq* samples inoculated with different levels of *C. botulinum* type spores and stored under various temperatures. The E_h electrode was set inside a *puurtaq* preparation inoculated with 10^3 spores/g and stored at 10°C in Exp. No. 2, while in Exp. No. 3 using plastic container covered with cheese cloth, the E_h electrode was set in an *igunaq* preparation that was inoculated with the same dose, but stored at 4°C. In Exp. No. 4, the E_h electrode was set in a *puurtaq* preparation that was inoculated with 10^2 spores/g and stored at 20°C. The E_h electrode was decontaminated with 10% sodium hydroxide for botulinum toxin and disinfected with an Orion cleaning solution (900022) between each experiment. Calibration was performed against an Orion redox standard (967901, E_h = +420 ±3 mV) and the E_h measurements performed in *igunaq* samples were correlated to a normal hydrogen electrode.

Oxygen and carbon dioxide gas measurements. Carbon dioxide and oxygen gas were measured in the headspaces of each *puurtaq* preparation and high gas barrier bag of Exp. Nos. 2 and 4, and Exp. No. 5, respectively. The measurements of both O_2 and CO_2 gas in *puurtaq* preparations made for Exp. No. 2 was performed using the 1450B3 Servomex Food Pack Analyser (Novatech, Montreal, Quebec) equipped with an external vacuum pump. Headspace gas was withdrawn from the *puurtaq* using the manual pump connected to a 20 G 1 1/2 inch needle, which was carefully inserted between suture points and above the meat and fat tissues. Calibration of the gas analyser was done by aspirating and analyzing ambient air at each sampling time.

The measurements of oxygen and carbon dioxide gas for Exp. Nos. 4 and 5 were performed using two different gas analyzers, the Mocon PG-100 oxygen headspace analyzer and the Mocon HS750 carbon dioxide gas analyzer (Modern Controls, Inc., Minneapolis, Minnesota, USA). The headspace gases of the high gas barrier bags were aspirated through a septum affixed to the outside of the bag using a side pore stainless steel needle of 4 cm long and connected to a 10-mL syringe. A modified 10-mL syringe punctured with a small hole and sealed with a septum was used to aspirate the headspace gas from the *puurtaq* preparations using a standard 20 G 1 1/2 inch needle, as the fine stainless steel needle would have been obstructed following its insertion through the suture points. Subsequently, the other syringe connected to the stainless steel needle was used to aspirate the headspace gas contained in the modified syringe through the septum. Calibration of the Mocon gas analyzers was verified against two sets of internal gas standards prepared with a Multivac gas mixer (model A300, Multivac Inc., Kansas City, Missouri, USA) at the following concentrations: 30% O_2, 70% CO_2; 10% O_2, 90% CO_2. Two high gas barrier bags were filled with these gas mixtures prior to each sampling time.

8.3 Results

Preliminary field studies. Waterproof data loggers for temperature measurements were introduced into four separate seal *igunaq* preparations produced by an *igunaq* producer from Kuujjuaq during the field trip of July 2001. The preparations were aged in pails and kept outdoors in the Northern village of Kuujjuaq from mid-July to mid-September. Analysis of the three recovered data loggers revealed that the average temperature inside all batches was 10°C and ranged from 2 to 23°C during the two-month period. Once the natural incubation was complete, the four batches were tested for E_h, pH, a_w and botulinum toxins. Analysis of the basic growth factors in ready-to-eat *igunaq* showed that the E_h (<-300 mV), pH (7.3), and a_w (0.97) were at adequate levels to support the growth of *C. botulinum* type E. Botulinum toxins were detected in two batches which were originally considered edible by the producer, while the two non-toxic batches were perceived as unfit for food consumption (Chapter 6). Results of this preliminary study were used to design the following inoculation studies conducted under controlled laboratory conditions.

Variation in pH and a_w. Table 8.2 shows the pH and a_w measurements performed in seal *igunaq* preparations of Exp. Nos. 1 and 2. The a_w of seal *igunaq* aged in unsealed plastic containers opened bi-weekly remained above an a_w of 0.97 during the entire length of the storage period, regardless of the incubation temperature. A small decrease in a_w was observed over time in *igunaq* preparations aged in seal skin pouches opened bi-weekly and incubated at 10 and 20°C, with the lowest mean a_w value of 0.95 being measured in *puurtaq* preparations stored at 20°C for 49 days. All the other *puurtaq* preparations had a mean a_w value of $\geq$0.97, the minimal value required for the growth of *C. botulinum* type E.

Table 8.2. Monitoring of growth parameters in seal *igunaq* preparations inoculated with *C. botulinum* type E strains (10^3spores/g) and aged in unsealed plastic containers and seal skin pouches

Growth parameters[b]	Day 0	Sampling time (days)[a]					
		Plastic containers			Seal skin pouches		
		14	28	42	14	28	49
4°C							
a_w	0.986	0.982	0.984	0.984	0.987	0.980	0.985
PH	5.5	6.9	7.8	7.7	6.1	7.2	7.5
APC	6.15	10.41	10.21	10.18	9.84	9.90	9.93
CBC	2.59	ND	ND	ND	ND	ND	ND
10°C							
a_w	0.986	0.982	0.987	0.983	0.987	0.980	0.969
PH	5.5	7.2	7.8	7.7	5.4	7.3	7.8
APC	6.15	10.25	9.81	9.46	9.69	9.93	9.66
CBC	2.59	<6.0	5.76	5.28	<6.0	6.40	4.65
20°C							
a_w	0.986	0.978	0.982	0.975	0.979	0.967	0.950
PH	5.5	7.0	7.5	6.6	6.7	6.4	5.8
APC	6.15	9.82	9.24	8.94	9.44	9.51	8.26
CBC	2.59	7.91	7.77	6.28	7.57	8.16	8.05

[a]Three samples of seal *igunaq* aged in plastic containers and two aged in seal skin pouches were tested at each sampling time.
[b]All values are reported as arithmetic means. a_w: water activity; APC: aerobic plate count (log CFU/g); CBC: *Clostridium botulinum* count (log CFU/g); ND: not done.

The surface pH of seal *igunaq* preparations aged in plastic containers increased from 5.5 (pH value in raw seal meat) to a neutral pH at day 14 and remained near a neutral pH for the rest of Exp. No. 1, regardless of the incubation temperature (Table 8.2). In Exp. No. 2, the surface pH of *puurtaq* preparations incubated at 4 and 10°C reached neutral pH values at day 28 and remained near neutral pH after 49 days of storage. However, the pH of the *puurtaq* preparations incubated at 20°C reached pH 6.7 after two weeks of storage and then decreased to 5.8 by day 49, which was the closest value to the minimum growth pH value for *C. botulinum* group II strains (pH 5.0) observed in this study. Thus, regardless of the incubation temperature and preparation methods, all *igunaq* samples from Exp. Nos. 1 and 2 remained above the minimum pH and a_w for growth of *C. botulinum* type E within 42 and 49 days of storage, respectively, except for the *puurtaq* preparations aged at 20°C, where the a_w was below the minimum growth value on day 49 of storage.

Variation in levels of aerobic bacteria and *C. botulinum* type E. The APC was initially high (~10^6 CFU/g) in raw seal meat at day 0 and increased to ~10^9 to ~ 10^{10} CFU/g by day 14, regardless of incubation temperatures and types of *igunaq* preparation (Table 8.2). The APC counts remained within the range of ~10^9 to ~10^{10} CFU/g until the end of the storage periods except for *igunaq* samples aged in plastic containers and seal skin pouches incubated at 20°C and sampled at days 42 and 49, respectively, for which the APC slightly decreased to ~10^8 CFU/g. The APC counts were generally higher in *igunaq* samples aged at 4°C, followed by those aged at 10 and 20°C at each sampling time, regardless of the types of *igunaq* preparations.

The inoculation dose used for Exp. Nos. 1 and 2 was 10^3 spores of *C. botulinum* type E per g of meat.

However, the initial estimation of spore level in raw seal meat at day 0 was 2.6 log CFU/g, indicating that the *C. botulinum* type E counts (CBC) were underestimated, and that this could also have occurred for *igunaq* samples tested at later sampling times. CBC counts were only done on *igunaq* samples containing botulinum neurotoxin. CBC counts were not done for *igunaq* samples aged at 10°C at day 14, due to a lack of lower dilutions prepared for levels of *C. botulinum* type E within the range of ~10^5 and ~10^6 CFU/g. However, it was possible to estimate that the levels of *C. botulinum* type E from these toxic *igunaq* samples were below ~10^6 CFU/g. Regardless of the types of *igunaq* preparations, samples incubated at 10°C had ~10^6 CFU/g by day 28, which then slightly decreased to ~10^5 CFU/g at the end of the storage period. *Igunaq* samples aged at 20°C contained higher CBC counts at the corresponding sampling times. The CBC counts of *igunaq* samples aged in seal skin pouches and unsealed plastic containers and incubated at 20°C increased from ~10^3 to ~10^8 CFU/g by day 14 and then remained at a similar level or decreased to ~10^6 CFU/g on days 49 and 42, respectively.

Variation in headspace oxygen and carbon dioxide gas. Concentrations of headspace O_2 gas in seal skin *puurtaq* preparations inoculated with 10^3 spores/g and exposed to air on a bi-weekly basis (Exp. No. 2) remained constant, i.e., ~20% throughout the storage period, regardless of the incubation temperature (Figure 8.4). Concentrations of headspace CO_2 also did not vary significantly in these preparations, and remained around 1 to 2%, whether they were incubated at 4, 10, or 20°C, except for a set of *puurtaq* samples incubated at 20°C, which had an average CO_2 level of 5.4% on day 29. These results indicated that there was a continuous exchange of gasses with the atmosphere, and that spores of *C. botulinum* type E in *puurtaq* preparations were subjected to an atmosphere similar to air for the entire storage period.

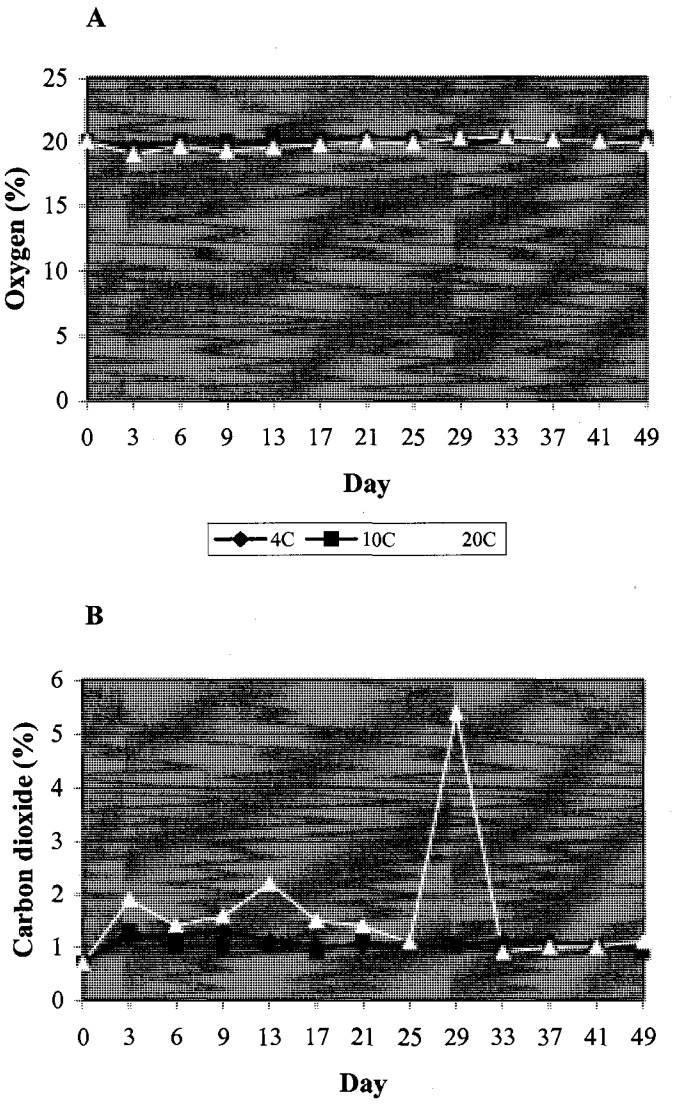


Figure 8.2. Variations in headspace oxygen (panel A) and carbon dioxide (panel B) gas in *puurtaq* preparations inoculated with 10^3 spores of *C. botulinum* type E per gram and incubated at 4, 10, and 20°C.

Similar variations in headspace O_2 and CO_2 gas were observed in *puurtaq* preparations inoculated with different doses (10^1, 10^2, 10^3 spores/g) and incubated at 4, 10, or 20°C, respectively, without being opened during the storage period (Exp. No. 4). The O_2 concentrations in *puurtaq* preparations ranged from 16 to 21%, but most had O_2 levels close to 20%, regardless of the storage temperature or inoculation dose. Most of these *puurtaq* preparations contained less than 2% CO_2 over the entire storage period, except for one set of *puurtaq* samples incubated at 10°C, which contained on average, 8.4% CO_2 and 15.7% O_2 on day 42. Apart from this set of samples, these results indicated that air was able to diffuse through the sutures and provided an aerobic atmosphere similar to air, even though the skin pouches were not opened periodically during the storage period. Table 8.3 shows the final headspace O_2 and CO_2 concentrations in *puurtaq* preparations inoculated with various spore loads and incubated at 4, 10, and 20°C.

As opposed to seal skin pouches, inoculated seal meat aged in high-barrier bags (Exp. No. 5) contained high levels of headspace CO_2 gas over time and depleted all O_2 within two weeks of storage (Table 8.4). The level of CO_2 in bags containing seal meat inoculated with 10 spores/g increased as a function of storage temperature (Figure 8.3), which was visually observed by the degree of expansion of the bags. The CO_2 level in bags stored at 20°C increased from < 1 to 61.2% after 14 days and reached 69.7% by day 28. Due to the high degree of expansion at day 28, all bags incubated at 20°C were processed to avoid potential explosion of the bags. The CO_2 level in bags incubated at 10°C increased to 56.3% by day 42, while in bags incubated at 4°C the level increased to 41.3% by day 56. The variation in CO_2 level in high-barrier bags incubated at 4°C was similar over time whether the meat was inoculated with 10, 100 or 1000 spores of *C. botulinum* type E per gram of meat.

Table 8.3. Headspace oxygen and carbon dioxide gas in seal *igunaq* inoculated with different levels of *C. botulinum* type E spores and aged into seal skin pouches (*puurtaq*)

Igunaq type	Air exposure (pouch opening)	Inoculation (spores/g)	Storage temp. (°C)	Storage time (days)	Headspace gas (%v/v)			
					Initial		Final	
					O_2	CO_2	O_2	CO_2
Exp. No. 2								
Puurtaq	Bi-weekly	10^3	4	49	20.1	0.7	20.2	1.0
Puurtaq	Bi-weekly	Control	4	49	20.1	0.7	20.2	0.9
Puurtaq	Bi-weekly	10^3	10	49	20.1	0.7	20.3	0.9
Puurtaq	Bi-weekly	Control	10	49	20.1	0.7	20.1	1.1
Puurtaq	Bi-weekly	10^3	20	49	20.1	0.7	19.9	1.1
Puurtaq	Bi-weekly	Control	20	49	20.1	0.7	20.1	0.9
Exp. No. 4								
Puurtaq	None	10^1	4	56	20.7	<1	20.0	0.1
Puurtaq	None	10^2	4	56	20.7	<1	19.6	0.1
Puurtaq	None	10^3	4	56	20.7	<1	20.3	0.2
Puurtaq	None	Control	4	56	20.7	<1	20.2	0.1
Puurtaq	None	10^1	10	42	20.7	<1	15.7	8.4
Puurtaq	None	10^2	10	42	20.7	<1	19.8	0.2
Puurtaq	None	Control	10	42	20.7	<1	20.7	<1
Puurtaq	None	10^1	20	42	20.7	<1	19.3	0.9
Puurtaq	None	10^2	20	42	20.7	<1	19.7	0.2
Puurtaq	None	Control	20	42	20.7	<1	15.8	10.4

Table 8.4. Headspace oxygen and carbon dioxide gas in seal *igunaq* inoculated with different levels of *C. botulinum* type E spores and aged into high-barrier bags

Igunaq type	Packaging treatment	Air exposure (bag opening)	Inoculation (spores/g)	Storage temp. (°C)	Storage time (days)	Headspace gas (%v/v)			
						Initial		Final	
						O_2	CO_2	O_2	CO_2
Exp. No. 5									
High-barrier bag	Air	None	10^1	4	56	20.7	<1	<1	41.3
High-barrier bag	Air	None	10^2	4	56	20.7	<1	<1	36.1
High-barrier bag	Air	None	10^3	4	56	20.7	<1	<1	36.9
High-barrier bag	Air	None	Control	4	56	20.7	<1	0.2	35.1
High-barrier bag	Air	None	10^1	10	42	20.7	<1	<1	56.3
High-barrier bag	Air	None	Control	10	42	20.7	<1	<1	52.4
High-barrier bag	Air	None	10^1	20	28[a]	20.7	<1	<1	69.7
High-barrier bag	Air	None	Control	20	28[a]	20.7	<1	<1	52.0

[a]Due to the high degree of expansion on day 28, all bags incubated at 20°C were processed to avoid potential explosion of bags later during the storage period.

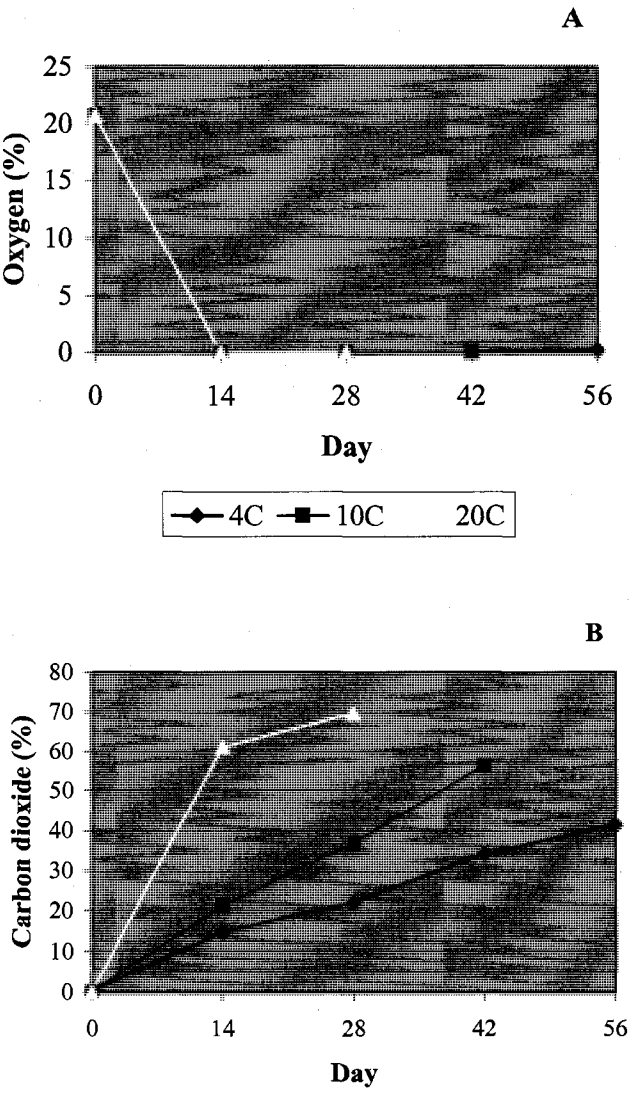


Figure 8.3. Variations in headspace oxygen (panel A) and carbon dioxide (panel B) gas in *igunaq* preparations inoculated with 10 spores of *C. botulinum* type E per gram and aged in high-barrier bags incubated at 4, 10, and 20°C.

Monitoring of redox potentials. The E_h was monitored several times a week (up to seven) in three seal *igunaq* preparations aged in different containers and incubated at 4, 10, and 20°C, respectively. The E_h of the three preparations decreased rapidly into the negative range within four days, and remained between -275 to -374 mV for up to two thirds of the storage periods, before slowly increasing. At no time did the E_h reach positive values by the end of the three experiments (Figure 8.4). The *puurtaq* preparation incubated at 20°C and kept closed during the entire storage period rapidly achieved a negative E_h, i.e.,–178.6 mV by day 1 and –321.7 mV by day 10, before increasing to –92.9 mV after 40 days of storage. The *puurtaq* preparation incubated at 10°C reached a negative E_h later on by day 4 (–133.3 mV) and -373.7 mV by day 23, despite the skin pouch being opened twice a week. The E_h of this *puurtaq* remained generally low for the remaining period increasing to –237.2 mV by day 49. In contrast, the E_h of the seal *igunaq* aged in a plastic container covered with cheese cloth and incubated at 4°C reached a negative E_h range at day 2 (–34.9 mV) further decreased to –286.4 mV by day 6, and then plateaued until day 31 before increasing to –189.9 mV on day 42. Despite the fact that they were not sealed and were exposed to various degrees of air, all three *igunaq* preparations remained in the lower negative E_h range during the storage period, and provided adequate E_h conditions for the growth of *C. botulinum* type E. The maximum E_h values that are permissive for the growth of *C. botulinum* type E range from +100 to +250 mV, depending on the substrate (Sperber, 1982).

Time to toxicity. Time to toxicity for seal *igunaq* preparations inoculated with 10^3 spores/g and aged at 10 and 20°C in seal skin pouches or unsealed plastic containers occurred within two weeks, regardless of the preparation method (Table 8.5). Opening both types of containers to air on a bi-weekly basis failed to prevent the growth and

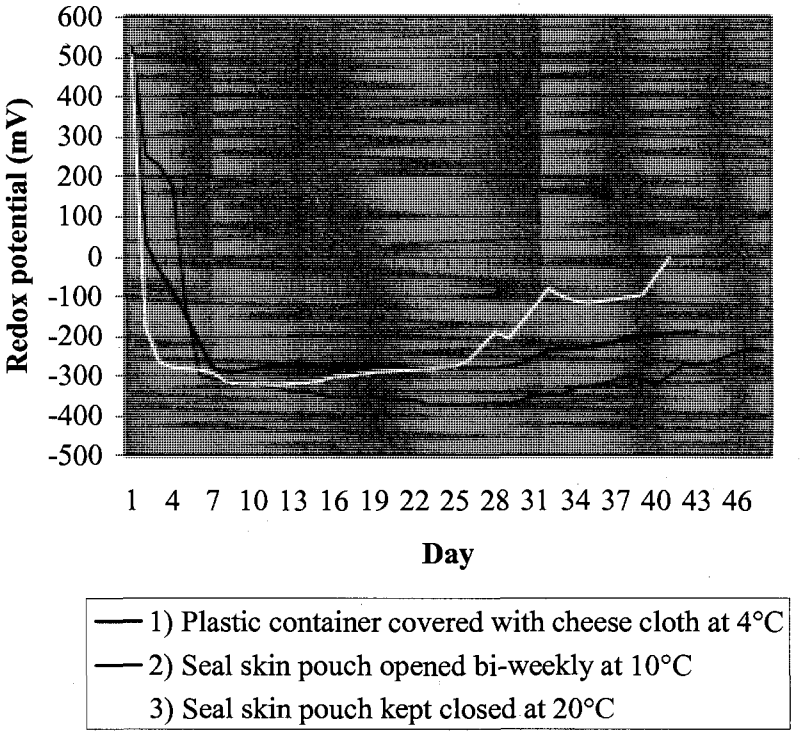


Figure 8.4. Monitoring of the E_h in seal *igunaq* aged in different containers and at various temperatures for a six-week period. 1) seal skin pouches incubated at 10°C and opened bi-weekly; 2) plastic containers covered with cheese cloth and incubated at 4°C; 3) seal skin pouches incubated at 20°C and kept closed.

Table 8.5. Challenge studies with seal meat preparations inoculated (10^3 spores/g) with *C. botulinum* type E and aged in unsealed plastic containers and seal skin pouches

Temperature	Time to toxicity (days)					
	Plastic containers			Seal skin pouches		
	14	**28**	**42**	**14**	**28**	**49**
4°C	0/3[a]	0/3	0/3	0/2	0/2	0/2
10°C	3/3	2/3	2/3	1/2	2/2	2/2
20°C	3/3	3/3	3/3	2/2	2/2	2/2

[a]Number of positive samples/total number of samples.

toxin production of *C. botulinum* type E, indicating that such a practice cannot be used as a control measure. However, all samples incubated at 4°C remained non-toxic for the entire length of both Exp. Nos. 1 and 2.

The large scale challenge study designed with a one-week sampling interval, and using plastic containers covered with cheese cloth to allow continuous gas exchange (Exp. No. 3), showed a similar time to toxicity results (Table 8.6). *Igunaq* samples aged at 10 and 20°C became toxic within two and one week, respectively, regardless of the inoculation dose, which ranged from 10^1 to 10^3 spores per gram. These results confirm the ability of *C. botulinum* type E to grow and produce toxin in *igunaq* preparations constantly exposed to air, using a low inoculation dose and two different abuse temperatures. All *igunaq* samples aged at 4°C did not support the production of BoNT/E for the entire length of the three-month storage period (84 days). However, one *igunaq* sample inoculated with 10^3 spores/g and another inoculated with 10^2 spores of *C. botulinum* type E per gram were unexpectedly found positive for type B neurotoxin at 4°C by day 42 and 84, respectively. A type B strain of *C. botulinum* group I was isolated from the *igunaq* sample collected on day 84, but the minimum growth temperature for this group is 10°C and therefore it is very unlikely that the isolated strain could have been responsible for the production of BoNT/B in the *igunaq* sample. Toxicity of the *igunaq* extract was only achieved only when the sample was trypsinized, suggesting that in addition to the indigenous proteolytic type B strain, a psychrotrophic type B strain from *C. botulinum* group II was also present. Further work was carried out in an attempt to recover a psychrotrophic type B strain from the *igunaq* sample, but this was not successful.

Similar time to toxicity results were also observed in *igunaq* samples aged in high gas barrier bags and seal skin pouches, regardless of the selected inoculation doses.

Table 8.6. Challenge study with seal meat preparations inoculated with *C. botulinum* type E and aged in plastic containers covered with cheese cloth

Incubation temperature	Inoculum (spores/g)	Time to toxicity (days)							
		7	**14**	**21**	**28**	**35**	**42**	**56**	**84**
4°C	10^1	0/3[a]	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	10^2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1[b]/3
	10^3	0/3	0/3	0/3	0/3	0/3	1[b]/3	0/3	0/3
10°C	10^1	0/3	2/3	2/3	3/3	2/3	3/3	C	
	10^2	0/3	3/3	3/3	C				
	10^3	0/3	2/3	3/3	3/3	C			
20°C	10^1	1/3	3/3	2/3	2/3	3/3	3/3		
	10^2	3/3	3/3	C					
	10^3	3/3	3/3	C					

[a]Number of positive samples/total number of samples. C means completed; we stopped when two sets of samples were all positive (3/3).
[b]Results from neutralizations revealed that mice were intoxicated with botulinum neurotoxin type B and not type E. This indicates that the *igunaq* was naturally-contaminated with a *C. botulinum* type B strain that is able to grow at 4°C and produce toxin.

Table 8.7. Challenge study with seal meat preparations inoculated with *C. botulinum* type E and aged in seal skin pouches and high-barrier oxygen bags

Incubation temperature	Inoculum (spores/g)	Time to toxicity (days)							
		Seal skin pouches				High-barrier oxygen bags			
		14	28	42	56	14	28	42	56
4°C	10^1	0/2[a]	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	10^2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
	10^3	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
10°C	10^1	0/2	2/2	2/2	ND	1/2	2/2	2/2	ND
	10^2	0/2	1/2	2/2	ND	ND	ND	ND	ND
20°C	10^1	2/2	2/2	C		2/2	4[b]/4	C	
	10^2	2/2	2/2	C					

[a]Number of positive samples/total number of samples. C means completed; we stopped when two sets of samples were all positive (2/2). ND means not done.
[b]Bags were highly inflated with gas produced by bacteria and samples were therefore processed earlier to obviate potential breakage of bags.

Both types of seal *igunaq* preparations incubated at 10 and 20°C showed toxicity within two or four weeks, whether they were inoculated with 10^1 or 10^2 spores per gram (Table 8.7). Again, all samples incubated at 4°C, including those incubated in high-barrier bags, remained non-toxic for the entire length of the study (56 days).

Serotyping. From 33 selected seal *igunaq* extracts kept frozen at -20°C for 19 to 26 months, six originating from *igunaq* samples aged at 10°C lost their toxicity over the storage period when assayed for botulinum neurotoxin by the mouse bioassay. The remaining 27 *igunaq* extracts which induced toxicity in mice, were all neutralized by monvalent antitoxin E, confirming the cause of toxicity in *igunaq* samples as being botulinum neurotoxin E.

8.4 Discussion

Igunaq has been traditionally prepared by the Inuit to preserve surplus meat and fish harvested and to ensure access to food when there is a scarcity of fish, terrestrial and marine mammals to hunt over the winter period. The traditional seal *igunaq* is usually aged in a pouch made of the skin of the ringed seal and stored in a cache, while today, new types of containers and various storage areas are employed to age seal meat by *igunaq* producers (Weetalutuk, 1997). The objectives of this study were to simulate and challenge the most common types of seal *igunaq* preparations made in Nunavik with *C. botulinum* type E, to determine the main factors enabling its growth and production of botulinum neurotoxin.

The results of the challenge studies showed that the type of containers employed in this study did not influence the time to toxicity of seal *igunaq* preparations aged at abuse temperatures (10 and 20°C), regardless of inoculation dose. The use of inverted seal skin as a container did not inhibit the growth and toxin production of *C. botulinum* type E in *puurtaq* preparations and cannot be seen as a safe aging method to prevent botulism. However, all *igunaq* preparations incubated at 4°C were negative for type E botulinum toxin, whether they were aged in seal skin pouches, plastic containers, or even in high-barrier bags after 56 days of storage.

The use of plastic bags was first discouraged by the public health authority of Alaska in 1976, following an increase in botulism incidents associated with beaver tails and fish aged or stored in plastic bags (Eisenberg and Bender, 1976a). It was thought that the plastic bags acted as an oxygen barrier providing anaerobic conditions for the growth of *C. botulinum*. The authors also reported that in two of five botulism incidents, bags were placed under a stove to allow rapid aging of beaver tails within two weeks. Traditionally, Inuit from Yupik, Alaska, used to age fish heads in clay pits dug in the

ground where the temperature was cold, but over the years they changed their practices significantly by using wooden barrels, followed by plastic containers, and plastic bags set above the ground or even placed indoors (Shaffer *et al.*, 1990). Similarly, in some communities of Nunavik, particularly in the Ungava Bay region, the traditional *igunaq* aged in seal skin and buried under gravel beach, or placed in a cache, was replaced by aging methods involving the use of these modern-type containers placed above the ground (Weetaluktuk, 1997). All these changes in food aging practices inevitably resulted in warmer and faster production of aged fish and marine mammal meat products, but also enabled any spores of *C. botulinum* type E present in the preparations to grow and produce toxin at a faster rate.

Although *C. botulinum* type E is believed to be a weak competitor, the organism was able to grow and produce toxin in seal *igunaq* inoculated with spore levels as low as 10 CFU per gram and aged within an aerobic atmosphere under abuse temperatures. The presence of an aerobic atmosphere in unsealed plastic containers and skin pouches favoured the growth of aerobic and facultative anaerobic bacteria as shown with the high APC counts observed throughout the storage periods of Exp. Nos. 1 and 2, reaching levels as high as 10^{10} CFU/g. The presence of aerobic spoilage microorganism such as *Pseudomonas aeruginosa* can enhance the growth of *C. botulinum* by scavenging oxygen and increasing the pH (Quortrup and Sudheimer, 1943). With the low levels of carbohydrate that are present in meat, non-proteolytic *C. botulinum* type E would also benefit from spoilage bacteria capable of metabolizing proteins into small peptides and amino acids that can be used as an energy source by vegetative cells of *C. botulinum* type E.

A layer of oil was formed as a result of fat decomposition during storage and was particularly evident in *igunaq* preparations aged in clear plastic containers. This oil

layer acted as a film barrier reducing the oxygen transmission rate to the deeper parts of the *igunaq* preparations, thus providing suitable anaerobic conditions for the germination of spores present under the oil layer. Degradation of amino acids by anaerobic and facultative anaerobic bacteria during putrefaction produces NH_3 and amines and in fresh meat increases the pH to the alkaline range (Ray, 1996). The surface pH values of most *igunaq* preparations aged in unsealed containers and seal skin pouches were also in the lower alkaline range, providing adequate pH conditions for the growth of *C. botulinum* type E. However, at 20°C, the surface pH of all *puurtaq* preparations and some *igunaq* aged in unsealed plastic containers was in the acidic range. This might be explained by the presence of active microbial populations at 20°C that are able to use oxygen in surface layers of *igunaq* and metabolize proteinaceous compounds using different biochemical pathways (aerobic respiration). Meat decay characterized by the growth of aerobic and facultative anaerobic bacteria, as well as molds, also likely influenced the surface pH of the *igunaq* preparations by oxidizing amino acids into their respective keto acids. Molds are known to grow well in warm and humid environments and their presence was particularly abundant in some *igunaq* preparations incubated at 20°C and stored for long periods of time (data not shown), which probably contributed to the slow reduction in pH of these *igunaq* preparations. Nevertheless, the pH of the seal *igunaq* was not sufficiently low at any sampling time during the storage period to prevent growth and toxin production of *C. botulinum* type E.

All basic growth parameters, including the oxido-reduction potential, measured in unsealed plastic containers and in *puurtaq* preparations, remained within the growth range for *C. botulinum* type E during six weeks of storage at 4, 10, and 20°C, respectively, despite the fact that both types of containers were opened bi-weekly.

According to the headspace oxygen measurements, the ambient air surrounding the *igunaq* preparations was able to flow through the suture of the skin pouches and maintain a continuous aerobic headspace. Even a continuous gas exchange through the cheese cloth covering the *igunaq* preparations aged in plastic containers failed to prevent the growth of *C. botulinum* type E and toxin production. These results are in agreement with others who showed that food packaged under a modified atmosphere containing 20% oxygen did not prevent toxigenesis (Lambert *et al.*, 1991).

The monitoring of the E_h in the three *igunaq* preparations revealed that all ORP curves followed a similar trend and remained negative for at least 42 days, whether they were incubated at 4, 10, or 20°C, respectively, and exposed to air. Even the presence of air in plastic containers covered with cheese cloth did not significantly influence the E_h in seal *igunaq* as the E_h remained below –275 mV for up to 28 days before slowly increasing to –190 mV at day 42. Thus, opening traditional or modern-type containers to allow air to flow inside, would not be an adequate method to increase the E_h in seal *igunaq* preparations. Spores of *C. botulinum* type E can germinate in the presence of air, and outgrow and divide in broth maintained at a E_h as high as + 198 mV (Ando and Iida, 1970). Below such a E_h level, the enzymatic activities of spores germinating in broth are capable of reducing the E_h to negative levels necessary for post-germination development. Even the natural positive E_h (+ 112 to + 202 mV) of fresh herring could not prevent botulinum toxin production in vacuum fish products challenged with a spore level as low as 10 CFU per gram and incubated at 10°C after eight days of storage (Huss *et al.*, 1979). The reduction in ORP in herring flesh throughout the storage period was attributed to the presence of spoilage bacteria able to strongly reduce the E_h environment to negative levels suitable for spore germination and growth of vegetative cells of *C. botulinum* type E.

Although the E_h environment was adequate for the growth of *C. botulinum* type E, toxin production was not detected in any *igunaq* samples incubated at 4°C and stored up to 84 days. These results are in agreement with previous inoculation studies in salmon and tilapia fillets (Garcia *et al.*, 1987; Reddy *et al.*, 1996, 1997b), cold and hot smoked trout fillets (Dufresne *et al.*, 2000b), pasteurized and unpasteurized crab meat (Solomon *et al.*, 1977; Harrison *et al.*, 1995), and cooked meat products (Lindström *et al.*, 2001), which all showed that storage at 4°C was effective in preventing toxin production of *C. botulinum* group II strains under various packaging conditions. Comparison of data between seal *igunaq* and various fish and meat inoculation studies is, however, not appropriate as several factors influencing the growth and toxin production of *C. botulinum* type E differed among the studies, including the initial microbial flora, types and levels of inoculum, type and size of food matrix, packaging type and heat treatment. The lack of type E toxin production at 4°C in each seal *igunaq* experiment is probably due to the presence of resident competing psychrotrophic microflora in seal meat, reflecting the bacterial population of the coastal environment. These results were consistent with those of other researchers who suggested that the psychrotrophic microflora contributed in preventing toxin production in fish packaged under vacuum or a modified atmosphere at 4°C storage (Reddy *et al.*, 1996; Reddy *et al.*, 1997b). Garcia and Genigeorgis (1987) showed that increasing the level of the initial microbial flora of fish significantly decreased the risk of toxigenesis. Elimination of the competing vegetative cells through thermal processing would, however, allow spores of *C. botulinum* group II to germinate, grow and better compete in the food environment. Thus, toxin production was reported in inoculated heated or pasteurized meat and seafood products such as beef stew (Schmidt *et al.*, 1961), lamb chumps (Moorhead and Bell, 1999), crawfish (Lyon and Reddmann, 2000), crab meat (Cockey and Tatro,

1974), and turkey (Genigeorgis *et al.*, 1991), with lag times ranging from 27 to 130 days despite the fact they were stored at 4°C under various packaging conditions. However, growth and toxigenesis of *C. botulinum* type E was also observed in vacuum-packed fresh catfish and cold-smoked rainbow trout stored at 4°C without any preheating process (Hyytiä, *et al.*, 1997, 1999c; Reddy *et al.*, 1997a). This highlights the uniqueness of each food product, and the importance of conducting challenge studies under their specific production and storage conditions.

The inoculation doses or spore inoculum had no effect on the length of the lag phase of *C. botulinum* type E toxigenesis in seal *igunaq*, while storage temperature was clearly identified as a risk factor in this study. The lack of an observed effect of spore inoculum (10^1 to 10^3 CFU/g) on the length of the lag phase could be attributed to the sampling design of the challenge studies, which was set at relatively long sampling intervals, every week or bi-monthly basis. In contrast, several inoculation studies of packed fish (Huss *et al.*, 1979; Garcia *et al.*, 1987; Garcia and Genigeorgis, 1987; Hyytiä *et al.*, 1997) and crab meat (Cockey and Tatro, 1974) challenged with different levels of non-proteolytic *C. botulinum* types, B, E, and F spores and sampled at selected times (days) showed that spore inoculum affected the length of the lag phase. Unlike the previous studies, Meng and Genigeorgis (1993) found that the spore load had a non-significant effect on the length of the lag phase of *C. botulinum* types B and E toxins in cooked turkey and chicken breast. This was due to the low variability in time to toxicity results observed at extreme temperatures with any of the spore inoculum used in the study.

The time to toxin production for non-proteolytic *C. botulinum* types B, E, and F in broths and various foods have been summarized by Betts and Gaze (1995), who found that the length of the lag phase of type E toxin could be as short as 15 days in vacuum-

packaged fresh salmon tissue homogenates stored at 4°C (Garcia and Genigeorgis, 1987) and 3 days in salmon fillets stored under modified atmospheres at 12°C (Garcia *et al.*, 1987). None of the *igunaq* preparations incubated at 4°C showed type E toxicity within 42 to 84 days of storage, but an increment of only 6°C, i.e., a temperature abuse of 10°C, resulted in toxin production within 14 days, regardless of the type of container or spore inoculum. A small increase in incubation temperature can significantly increase the growth rate of *C. botulinum* type E and toxin production, for example, the generation time could decrease from 42 to 7.19 h when the temperature increases from 5 to 10°C (Lund and Notermans, 1993). However, unexpectedly, in our study, two *igunaq* preparations were found positive for type B botulinum toxin after 84 and 42 days of storage period, despite the fact that they were inoculated with 10^2 and 10^3 spores of *C. botulinum* type E per gram, respectively. The ability of the non-proteolytic type B strains to compete, grow and produce toxin in fishery products among a spore inoculum composed of 4 non-proteolytic type B, 5 type E, and 4 non-proteolytic type F was previously demonstrated in three different inoculation studies where only type B toxin was detected in toxic samples (Ikawa, 1986; Garcia and Genigeorgis, 1987; Garcia *et al.*, 1987).

Contamination of negative control samples has been previously reported in fresh salmon tissue homogenate (Garcia and Genigeorgis, 1987) and packed cooked meat products (Lindström *et al.*, 2001), but not in inoculated samples. The presence of non-proteolytic type B strains of *C. botulinum* (not confirmed by isolation) in two inoculated *igunaq* samples is puzzling because: 1) No botulism outbreaks other than type E have never been reported in Nunavik or other regions of the Canadian Arctic; 2) only one case of botulism in Canada, from northern British Columbia, was caused by a non-proteolytic type B strain in a home-smoked salmon (CDWR, 1984); 3) type B strains

have not been isolated from the Arctic coastal environment. The origin of the naturally-contaminated seal meat with *C. botulinum* type B strains was a bearded seal that was butchered at high tide on a coastal rock located at the mouth of the Koksoak River, in which type E spores are highly prevalent and abundant throughout the entire length of the river. In spite of this, none of the negative controls used in Exp. No. 3 or in the other experiments of this study, were positive for type E botulinum toxin. However, from 50 cultures of 50-g meat samples collected from the same bearded seal, only two samples were found toxic for mice at an approximate background level of about 1 spore of *C. botulinum* per kg of meat, which was 10 times higher than the levels previously reported in fresh meat of domestic animals (Hauschild, 1989).

Most botulism incidents in Alaska for the 1990-2000 period have occurred between the spring and fall with a marked peak in July (Sobel *et al.*, 2004). Similarly in Nunavik, most botulism incidents reported for the period 1985-2005, occurred during the same months of the year, with a higher peak in August (Austin, unpublished data). To avoid temperature abuse, particularly during the warmer months, and to ensure the safety of seal *igunaq* products, all preparations should be aged in a walk-in refrigerator equipped with a safety thermostat and a temperature-recording system. The purchase of walk-in refrigerators is relatively inexpensive and would allow the Inuit from Northern villages of Nunavik and other regions of the Canadian Arctic to safely age their *igunaq* preparations under controlled temperatures, while maintaining a traditional Inuit practice for future generations. To complete the food safety assessment, further challenge studies should be conducted using large preparations of traditional and modern-type seal *igunaq*, inoculated with low levels of spores of *C. botulinum* non-proteolytic type B and type E, and aged under storage conditions of walk-in refrigerators.

CHAPTER 9

RISK PROFILE OF FOODBORNE BOTULISM FROM EATING AGED MARINE MAMMAL MEAT (*IGUNAQ*) IN NUNAVIK

9.1. Introduction

Human botulism has been recorded in the Nunavik region since 1964 and still remains a serious concern in spite of continuous public health education and the increased awareness of the disease among the Inuit (Grondin *et al.*, 1996). Based on the last two epidemiological reviews on botulism, there have been 32 reported outbreaks of botulism in Nunavik from 1964 to 1984, primarily associated with the consumption of marine mammals (86.7%) (Dolman, 1974; Hauschild and Gauvreau, 1985). A total of 77 cases were involved with 13 deaths for an overall mortality-case ratio of 16.9%. The disease has not been reviewed since 1985, but Health Canada's statistics indicate that the Nunavik region had the highest rate of botulism in Canada during the late 1990s (Austin, 1996; Austin *et al.*, 1997, 1999).

These reports revealed that 57.9% of outbreaks in Canada between 1995 and 1997 occurred in the Nunavik region, involving 29 cases and no deaths. The region had an overall incidence of botulism averaging approximately 10 cases per annum, for a total population of 8700 during this period. When expressed as cases per 100,000 population, the Nunavik region had among the highest reported incidence of botulism in the world, with approximately 112 cases/100,000 per year. All of these outbreaks were caused by the ingestion of traditional marine mammal foods containing BoNT/E, especially aged seal products (81.8%). These data indicate that marine mammals are the main vehicles of transmission of type E botulism in Nunavik. The risk of exposure to BoNT/E toxin through the consumption of aged marine mammals has not been estimated in Nunavik, or in other regions of the Canadian Arctic. As an initial step prior to conducting a quantitative risk assessment, the objective of this study was to develop a risk profile of human type E botulism from eating aged marine mammals in Nunavik using updated epidemiological data from the period 1985 to 2005.

9.2. Methodology

Epidemiological data. An extensive review of the literature and unpublished reports from 1985 to 2005 were conducted and data were organized and analyzed to provide an updated descriptive epidemiology of type E botulism in the Canadian Arctic. Sources of information included the Botulism Reference Service's database on laboratory-confirmed cases of botulism, publications of the Canada Communicable Disease Report, the EpiNorth newsletter, and various provincial and territorial public health reports. The data selected for analysis included: type of botulism, number of clinical cases, number of deaths, age of patients, transfer to an intensive care unit, implicated food, laboratory analysis, month and year of the outbreak, and the name of the Northern village.

Risk profiling. The establishment of a risk profile is an early step in the risk evaluation process performed during the microbial risk management (MRM) of a food safety problem (Codex, 2007). A risk profile allows managers to describe the problem and its context in a concise form. Risk profiling consists of gathering the current state of knowledge on the food-hazard combination of concern, the public health problem, the characteristics of the food from production to consumption, the risk assessment needs, the potential management options and various other elements that would facilitate MRM activities (Codex 2007). The information contained in this risk profile is organized and presented in a format similar to several risk profiles prepared for the New Zealand Food Safety Authority, including a risk profile conducted on *Clostridium botulinum* in ready-to-eat smoked seafood (ESR, 2006), which followed the procedures for the conduct of a qualitative risk assessment as described by Codex (1999).

9.3 Hazard identification

9.3.1. Hazard identification: the organism

Botulinum neurotoxins types A, B, E, and F are protein toxins able to cause botulism in humans and are produced by *C. botulinum* group I and group II and some strains of *C. butyricum* and *C. barati*. Since all reported outbreaks of human botulism in Nunavik have been caused by type E neurotoxin produced by *C. botulinum* type E in native foods, the risk profile will be conducted only on this organism.

Nomenclature. *C. botulinum* is a ubiquitous Gram-positive, spore-forming anaerobic rod-shaped bacterium. The organism can be divided into four physiological groups I to IV and subdivided into seven serotypes designated A to G. *C. botulinum* type E belongs to group II based on physiological differences and 16S ribosomal RNA gene sequencing (Collins and East, 1998).

The organism. Strains of *C. botulinum* type E are able to produce BoNT/E, the single known virulence determinant of this organism. Ingestion of pre-formed toxin causes disease in humans, fish, birds, and mammals.

Growth. The maximum growth rate for *C. botulinum* type E occurs at 35°C (Ohye and Scott, 1957) but the organism can grow as low as 3°C (Schmidt *et al.*, 1961) and as high as 45°C (Hobbs, 1981). Under otherwise optimal conditions, the minimum pH for growth of *C. botulinum* type E is pH 5 (Emodi and Lechowich, 1969). The outgrowth of spores of *C. botulinum* type E is inhibited by 4.9% sodium chloride, regardless of the incubation temperature or strains. In addition, not all strains are inhibited when 100 and 200 ppm sodium nitrite are added into media (Emodi and Lechowich, 1969). The

organism grows under anaerobic conditions and can tolerate the presence of oxygen. The lower a_w limit for growth is 0.965, but the growth rate can vary significantly among strains of *C. botulinum* type E growing at the same incubation temperature and a_w level (Ohye *et al.*, 1966).

Survival. Spores of *Clostridium* species are metabolically dormant and highly resistant to environmental stresses allowing them to survive for periods of thousands, or even of millions years in special niches (Setlow, 2005; Gould, 2006). Based on studies conducted with *B. subtilis*, spores are far more resistant than vegetative cells to UV radiation, desiccation, multiple cycles of freezing, wet heat, dry heat, γ-radiation and toxic chemicals (Setlow, 2005).

Inactivation. Spores of *C. botulinum* group II can be inactivated by thermal processing in less than 0.1 min (*D* value) at 100°C (Austin and Smith, 2006) or 0.6-1.25 min at 80°C (Collins and East, 1998), but some *C. botulinum* group II type B strains have *D* values as high as 32 min at 80°C (Scott and Bernard, 1982).

Sources. Spores of *C. botulinum* type E are ubiquitous and can be found in soil substrates, freshwater and marine sediments. The spores are particularly prevalent in intestinal contents of fish from aquatic environments rich in type E spores (Johannsen, 1963).

9.3.2. Hazard identification: the toxin

Production. Type E botulinum toxin production is optimal at 20 to 25°C in culture (Gunnison *et al.*, 1935).

Stability in fish and marine mammal products. The 12S botulinum toxin or progenitor toxin produced by vegetative cells of *C. botulinum* type E during growth is stable for weeks at room temperature in spoiling fish, salted fish, and low-acid fish products (Huss and Petersen, 1980). The stability of activated toxin in fish depends on the fish species and salt concentration. The activated toxin was found stable in herring mince regardless of the salt concentration, but not in cod mince, where its activity was reduced after three weeks of storage in cod mince having 0-16% salt and no toxin could be detected after six weeks (Huss and Petersen, 1980). Botulinum toxins are known to tolerate freezing temperatures. A concentration of 1000 MLD per gram of BoNT/E was titrated from a rancid seal flipper (utjak) that has been frozen for two months (Dolman, 1974).

Inactivation. Heating temperatures maintained at 79°C for 20 min or at 85°C for 5 min could inactivate 10^5 MLD_{50} of types A, B, E and F botulinum toxins (Woodburn *et al.*, 1979b). Botulinum type E toxin is resistant to freezing and has been shown to retain its original toxicity after 264 days of storage at –15°C in salmon fluid extract (Yao *et al.*, 1973). More recently, an investigation of a botulism case that occurred in Kuujjuaq on November 2002 revealed that the suspect traditional inuit food (tingnak) was frozen for two years before consumption (Chapter 7). Irradiation cannot be used for detoxifying foods without affecting the organoleptic quality (Siegel, 1993).

9.3.3. Hazard identification: the food

Harvesting levels of marine mammals in Nunavik. The only harvesting data for ringed seals and bearded seals originated from a harvesting study was conducted during

the period from 1976 to 1980 (Research Committee on Native Harvesting, 1989). The estimates generated by the proportional projection method revealed that Nunavik had an average yearly harvesting level of 11,900 and 992 for ringed seals and bearded seals, respectively. These estimates are now 27 years out-of-date and may not reflect the current harvesting levels. Aging of the population, the availability of food cargo subsidiary programs, and the shift of traditional hunting practices to modern-type activities by the younger population, support a decrease in seal harvesting levels.

Harvesting data for walrus have been recorded by the regional organizations of Nunavik since 1974, but it is only up until 1986 where all 14 Northern villages participated in the program (DFO, unpublished data). Out of the 891 animals harvested during the 1986-1997 period, 58%, 27% and 15% respectively, were collected by hunters originating from the Hudson Strait, Hudson Bay and Ungava Bay regions. The average harvesting level was 74 walrus per year, ranging from 30 to 165. The harvesting levels varied extensively among the villages and within each year. For instance, an unusually high harvest of 41 animals was recorded by Kangiqsujuaq in 1986, then the harvest levels of this community remained under six animals until 1997. The low harvesting levels reported for Kuujjuarapik and Umiujaq indicate that walrus is not an important part of the diet as compared to Salluit and Ivujivik, which together accounted for almost 40% of the total harvest.

The harvest statistics for beluga whales in Nunavik were compiled for the years 1974 to 2000 by Lesage *et al.* (2001). The harvesting level of the beluga has been restricted since 1986, following the introduction of management plans to preserve certain populations that have declined. Based on the recent five-year quota system (1996-2000), allocating 250 beluga per year in Nunavik, an average of 285 whales were harvested with a range of 267 to 302 in Nunavik.

Modes of preparation of various types of marine mammal products. There are several traditional aged dishes produced from marine mammals, fish, and caribou, which have specific Inuit names that could vary depending on the Arctic regions where they were produced (Table 9.1). Some of these products may be sold in a country food store by local producers, but generally they are shared locally among family members and friends or with persons from other neighboring villages (inter-community trade). Aged or putrefied (advanced decomposition) meat products from marine mammals are called *igunaq* in Nunavik and are commonly produced from walrus and seal (Weetaluktuk, 1997). Typically, an *igunaq* preparation is made of marine mammal fat and meat which is aged for periods ranging from two to six weeks. Walrus or seal *igunaq* is usually prepared by placing meat and fat pieces, without any other ingredients into a traditional skin pouch (*puurtaq*) made from the harvested animal, or within a modern-type container such as a plastic bag, hard plastic container, wooden box, or galvanized barrel. The *puurtaq* preparations are usually stored in a cache on a flat rock and covered by pebbles, or under a gravel beach, whereas the modern-type preparations are stored in a shed, outside in a wooden box, or simply covered by a piece of plywood. Traditionally, some *igunaq* preparations included the intestines (and other internal organs), skull (including the brain), paws and flippers in addition to meat and fat tissues. The preparation of aged flippers and paws only is called *utjaq* and are still produced in Nunavik.

Dried meat or *nikku* can occasionally be prepared with marine mammal meat, particularly from beluga, but most commonly it is made with caribou meat in Nunavik. The meat is sliced into thin pieces that are dried on a grill. The skin of the beluga whale is called *muktuk* and is considered a food delicacy in Nunavik. The *muktuk* is usually consumed raw with a thin covering layer of attached fat.

Table 9.1 Characteristics of Inuit traditional foods in Nunavik

Traditional foods	Animal origin[a]	Preparation	Processing
Igunaq	**Seal**, **walrus**, beluga whale, caribou, fish	Meat and fat into skin pouch or modern-type containers	Aging
Misiraq	Seal, beluga whale	Fat into skin pouch or modern-type containers	Aging
Puurtaq	Seal	Meat and fat into seal skin pouch	Aging
Utjaq	Seal	Flippers and paws into skin pouch or modern-type containers	Aging
Muktuk[c]	Beluga whale	Skin	None
Nikku	**Caribou**, beluga whale	Meat	Drying

[a]Species in bold are more commonly used to make the traditional food product in Nunavik.
[b]Modern-type containers include plastic bags, hard plastic containers, wooden boxes, galvanized barrels, and glass containers.
[c]*Muktuk* is commonly eaten aged in Nunavut and Northwest Territories.

Similarly to *igunaq*, the *muktuk* can also be aged in modern-type containers and consumed, but this aged product is particularly popular in the Northwest Territories and Nunavut. The fat of marine mammals can also be aged without meat to produce oil (*misiraq*) that is used as a condiment. *Misiraq* is currently prepared with seal or beluga fat that is stored in a skin pouch, or a glass or plastic container for several weeks, until the soluble fat is separated from the tissue.

Production of seal *igunaq*. The interviews conducted with 20 *igunaq* producers from Nunavik (Chapter 4) also included specific questions on the production and consumption of seal *igunaq* by producers which are presented in this section and section 9.5. However, the data from this study should be interpreted with caution, since different units or scales were allowed for the estimation of the levels of production and consumption of seal *igunaq* by producers. Of nine interviewed producers, eight (89%) would preferably produce *igunaq* using bearded seal meat, of which five (56%) exclusively use bearded seal meat. The reasons for this preference was explained by two producers from Kuujjuaq, who stated that the difficulty with using ringed seal meat is that it ages too rapidly and becomes too tender. Of 16 producers of seal *igunaq*, 13 (81.3%) produce 1 to 2 pails or 40 to 100 lbs of seal *igunaq* per year. Two of these producers make only one or two *puurtaq* a year, while one producer from Kuujjuaq produce up to 45 gallons of seal *igunaq* annually (Chapter 4).

Native foods involved in type E outbreaks. Table 9.2 shows the native foods implicated in type E outbreaks in Nunavik and other Arctic regions of Canada for the last 20 years. The Nunavik region accounted for 70% of type E outbreaks that occurred in the Canadian Arctic between 1985 and 2005 (Figure 9.1). All type E outbreaks with

known food sources from Nunavik were associated with marine mammals and at least 81.4% of these concerned aged products from seal, beluga or walrus. More than half (52.1%) of the outbreaks were caused by food products of seal origin, while beluga was most often implicated (57.1%) in botulism outbreaks elsewhere in the Canadian Arctic (Figure 9.2). Aged meat (*igunaq*) and aged flippers (utjaq) were the most frequently implicated seal products in Nunavik with 64%, and this proportion is probably underestimated due to the different ways samples were submitted and/or recorded. Several outbreaks involving seal products were recorded in laboratory books and CCDR reports as seal meat only, or seal meat and *misiraq*, without indicating whether the meat was eaten raw fresh, frozen or aged. The diagnostic samples recorded as *misiraq* and meat may have originated from a single seal *igunaq* preparation, or from two different aged products, i.e., *misiraq* and *igunaq*. Thus, some outbreaks associated with *misiraq* may in fact have been the *misiraq* produced during the aging of *igunaq* that had been collected and submitted for testing without any meat samples.

Table 9.2. Foods implicated in botulism outbreaks of Nunavik and the rest of the Canadian Arctic, 1985-2005

Food types	Nunavik				Other regions of the Canadian Arctic			
	No. of outbreaks	No. of cases	No. of deaths	Toxin type	No. of outbreaks	No. of cases	No. of deaths	Toxin type
Seal								
Aged meat (*igunaq*) and/or flippers	16	32	0	E	2	6	2	E
Meat (unknown preparation)	6	11	2	E	2	3	1	E
Aged fat (*misiraq*)	1	3	0	E				
Misiraq and/or meat (unknown prep.)	2	2	0	E				
Walrus								
Aged meat (*igunaq*)	3	9	0	E	1	1	0	E

Table 9.2 (Continued). Foods implicated in botulism outbreaks of Nunavik and the rest of the Canadian Arctic, 1985-2005

Food types	Nunavik				Other regions of the Canadian Arctic			
	No. of outbreaks	No. of cases	No. of deaths	Toxin type	No. of outbreaks	No. of cases	No. of deaths	Toxin type
Beluga whale								
Aged *muktuk*	2	2	0	E	3	7	0	E
Muktuk (unknown preparation)					8	13	0	E
Aged meat (*igunaq*)	1	1	0	A				
Dried meat	1	3	0	E				
Meat (unknown preparation)					1	1	0	E
Aged fat (tingnak)	1	1	0	E				

Table 9.2 (Continued). Foods implicated in botulism outbreaks of Nunavik and the rest of the Canadian Arctic, 1985-2005

Food types	Nunavik				Other regions of the Canadian Arctic			
	No. of outbreaks	No. of cases	No. of deaths	Toxin type	No. of outbreaks	No. of cases	No. of deaths	Toxin type
Marine mammals (unknown)								
Aged meat (*igunaq*)	1	1	0					
Meat (unknown preparation)	1	1	0	E				
Aged fat (*misiraq*)	7	10	0	E	1	1	0	E
Igunaq and/or *misiraq*	1	1	0	E				
Fish								
Aged heads					1	1	0	E
Marinated and smoked					1	3	1	E
Unknown food type	5	5	0	E	1	4	1	E

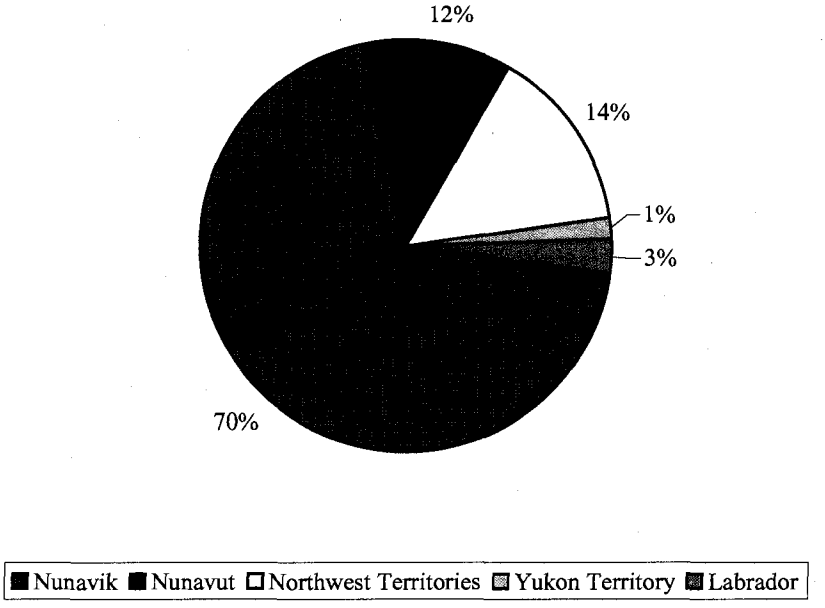


Figure 9.1. Geographical distribution of *C. botulinum* type E outbreaks in the Canadian Arctic 1985-2005.

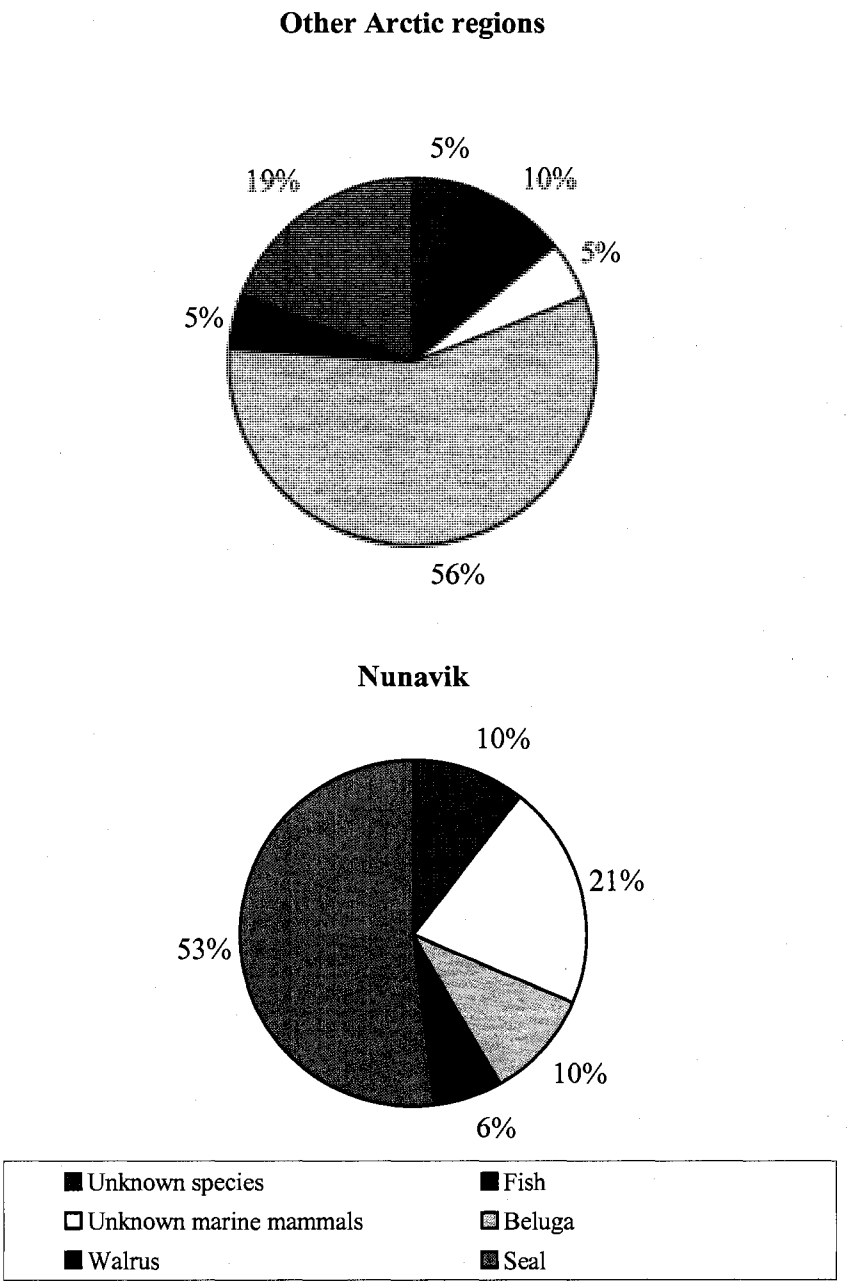


Figure 9.2. Proportion of various native foods implicated in *C. botulinum* type E outbreaks in Nunavik and other Canadian Arctic regions, 1985-2005.

9.4. Hazard characterization: adverse health effects

Clinical manifestation

Incubation. The clinical onset of type E botulism in northern regions usually occurs within 12-36 h following the ingestion of toxic foods (Wainwright, 1993), but can be as early as 6 h to as late as 10 days (Castrodale, 2005).

Gastrointestinal symptoms. Type E botulism is initially manifested by nausea and vomiting, diarrhea and abdominal pain. Constipation may occur later during the course of illness.

Neurological symptoms. Neurological symptoms usually follow gastrointestinal symptoms with cranial nerve deficits causing dysphagia, dysarthria, dry mouth, blurred vision, ptosis, dilated and fixed pupils, and diplopia occurring. Then, a symmetrical and descending flaccid paralysis causing weakness, fatigue, dyspnea and respiratory distress progressively develop.

Treatment. The clinical management of botulism cases essentially involves prompt administration of trivalent antitoxin (ABE) or bivalent types A and B and a monovalent type E antitoxin and provision of intensive care. Adverse reactions to botulinum antitoxin treatment are rare and were reported in 1.5% of confirmed or suspected cases in Alaska for the period from 1947 to 1985 (Wainwright *et al.*, 1988). Black and Gunn (1980) reported that 9% of treated cases have experienced hypersensitivity reactions in the United States between 1967 and 1977. However, less than 1% of cases which have received 2 to 4 fold lower doses of antitoxin than the previous years had serious

reactions (Sobel, 2005). In Canada, no hypersensitivity to horse serum antitoxin has been observed in the Inuit population (Hauschild, and Gauvreau, 1985).

Prognosis and long-term consequences. The availability of antitoxin therapy, and, more particularly, the development of modern intensive care with assisted ventilation, has contributed to decrease in the mortality rate to 3-5% (Sobel, 2005). Severe cases of botulism are debilitating conditions requiring close monitoring of forced vital capacity in intensive care units and assisted ventilation, if respiratory muscles become impaired. Early deaths are caused by a sudden decrease of the respiratory reserve followed by respiratory arrest. Respiratory complications may also develop and lead to death in some cases of botulism. Neurological disorders may last several weeks or months before patients recover from paralysis. Hospitalized patients often experience infectious complications, especially pneumonia and urinary tract infection (Barrett, 1991). Outpatient rehabilitation is often required after release from hospital. Even with administration of antitoxin, binding of botulinum toxin to presynaptic endplates is irreversible, causing irreparable damage. New endplates will eventually sprout from the axons and restore transmission of neural impulses to muscles and glands. The development of long-term sequelae associated with type E botulism has been poorly documented in the Arctic regions (Castrodale, 2005). There were reports of individuals being released from hospital after a number of weeks, while they were still experiencing symptoms such as headaches, impairment of taste and smell, constipation and weakness (Dolman, 1974), but the persistence of the symptoms and the impacts it had on patients at home, have not been assessed.

Groups at risk. Groups of all ages are susceptible to developing botulism when exposed to BoNT through foods, as the minimum lethal dose for humans is in the low nanogram range.

Immunity. Exposure to botulinum toxins does not provide immunity and protection for future episodes. It is believed that the concentration of toxin causing botulism, even in severe cases, is too low to induce an effective immunological response (Caya *et al.*, 2004). BRS has recorded cases where one individual has been intoxicated with BoNT/E more than once.

Serotypes causing disease. All botulism cases recorded in the Canadian Arctic since 1985 have been caused by strains of *C. botulinum* type E. Strains of types A, B, E, and more rarely F usually induce botulism in humans, and there are a few anecdotal cases that have been caused by strains of serotypes C and D (Demarchi *et al.*, 1958).

Situation in other circumpolar regions. Similar to the Ungava Bay region of Nunavik, type E botulism is endemic in the western and southern regions of Alaska. The disease is due to the ingestion of native foods contaminated with BoNT/E. However, an increasing number of type A and type B botulism cases related to non-commercial foods have been regularly reported since the 1970s. Type E botulism appears to occur more rarely in Greenland, despite the common consumption of seal meat (Pars *et al.*, 2001).

Dose-response for type E botulinum neurotoxin. The toxicokinetics of BoNT in humans, as well as the minimum dose causing disease, is unknown. The oral doses of BoNT causing symptoms and occasionally deaths from eating contaminated foods were

found to be as low as 0.1 to 1 μg (or 3,000 to 30,000 MLD_{50}) (Schantz and Johnson, 1992). These data have been used to model the dose-response relationship of bioterrorism scenarios involving the contamination of the food supply with various levels of BoNT (Wein and Liu, 2005).

The levels of toxin in sera of botulism patients are highly variable as some people might be severely ill and have no detectable toxins, while others with only mild symptoms can show detectable levels of toxin in their serum. The levels of toxins in blood are influenced by the amount of ingested and absorbed toxins through the intestinal wall, the time of sampling, the patient's immune response, and the rate of toxin absorption at the receptor site (Hauschild *et al.*, 1978). The level of BoNTs found in the sera of non-fatal cases is usually around 2 MLD per mL (Hauschild *et al.*, 1978). The highest level of BoNT/E reported in the sera of survivors was found to be 15 and 40 MLD per mL in two Inuits who ate contaminated walrus *igunaq* in February 1978 in Cape Dorset (Hauschild *et al.*, 1978). These high levels corresponded to 120,000 and 320,000 MLD of circulating toxin, respectively, and without prompt administration of antitoxin at the local nursing station followed by a rapid transfer to a hospital to obtain respiratory support, these patients would have probably died. The dose of type E toxin ingested by the Inuit patients could not be estimated since the remaining *igunaq* contained only 4 MLD, which was not representative of the level of toxin originally present in the *igunaq* they had consumed. During an outbreak investigation of type E botulism involving salmon eggs in Bella Bella, British Columbia in 1954, it was possible to estimate the level of toxin that induced the disease and/or caused deaths in two patients. This was based on the estimated amount of salmon eggs consumed and the repeated titration of toxins from the remnant salmon eggs ingested by the two patients (Dolman *et al.*, 1955). From knowing that the incriminated salmon eggs contained 6000

LD_{50}, it was roughly estimated that the survivor and the fatal case were orally exposed to 250,000 and 500,000 MLD, respectively. With one MLD representing 10 pg, these levels would correspond to human oral doses of 2.5 and 5 μg of BoNT/E causing illness and death, respectively.

Some information on the levels of BoNT/E present in native foods causing human botulism can be found from previous epidemiological investigations of type E outbreaks in Alaska and the northern regions of Canada. Table 9.3 summarizes the epidemiological findings associated with these outbreak, as well as those recently investigated by the BRS for which type E neurotoxin was titrated from suspect foods.

Table 9.3. Levels of BoNT/E titrated from foods causing outbreaks of botulism in Alaska and northern regions of Canada

Location	Year	Foodstuff implicated	BoNT/E levels in food (mouse MLD/g)	Cases	Fatalities	References
Point Hope, Alaska	1950	*Muktuk* (flipper)	4	5	0	Dolman, 1960
Bella Bella, British Columbia	1954	Salmon eggs	6000[a]	3	1	Dolman *et al.*, 1955
Nain, Labrador	1956	Utjak	600-1000	8	5	Dolman, 1960
Scammon Bay, Alaska	1959	Grey whale fluke	200-600	8	1	Dolman, 1960
Bella Bella, British Columbia	1961	Salmon eggs	40	4	0	Dolman, 1974
Masset, British Columbia	1963	Salmon eggs	>1000	2[b]	1	Dolman, 1974
Klemtu, British Columbia	1964	Salmon eggs	1500	1	0	Dolman, 1974
Kitimat, British Columbia	1967	Salmon eggs	1000	1	0	Dolman, 1974
Cape Dorset, Nunavut	1969	Aged seal meat	5	5	3[c]	Stuart *et al.*, 1970
Cape Dorset, Nunavut	1978	Walrus *igunaq*	4	2	0	BRS
Kuujjuaq, Nunavik	1988	Seal meat/*misiraq*	1600	1	0	BRS
Aupaluk, Nunavik	1998	Seal *igunaq*	250-2500	1	0	BRS

[a]The level of BoNT/E is expressed as mouse LD_{50} per gram.
[b]Both cases had eaten approximately 50 g of salmon eggs.
[c]It has been estimated that the three people who died ate between 300 and 1200 g of meat.

9.5. Exposure assessment

***Clostridium botulinum* type E in the coastal environment.** *C. botulinum* type E is not uniformly distributed along the Nunavik coast. The prevalence and levels of type E were higher in the coastal environment of Ungava Bay than in the Hudson Strait or Hudson Bay region of Nunavik. Approximately 88% of the shoreline soil samples collected near butchering sites along the Ungava Bay coast were positive for *C. botulinum* type E and contained between <6 to 1800 spores/kg (Table 3.2). In comparison, 50% of the shoreline soil samples from the coastal areas of Hudson Bay contained type E up to a maximum level of 15 spores per kg, while no spores were isolated from any samples collected from the Hudson Strait region. Most positive coastal rocks and seawater samples clustered in the southern part of Ungava Bay, near the mouth of the Koksoak and Whale rivers, with a prevalence rate of 18.8 and 27.8%, respectively (Table 3.2).

Other *C. botulinum* serotypes in the coastal environment. Two of the 29 (6.9%) positive shoreline soil samples collected in the coastal environment of Nunavik contained strains belonging to *C. botulinum* group I. Strains of *C. botulinum* type A and type B were isolated from shoreline soil samples of Hudson Bay and Ungava Bay, respectively (Table 6.2). No other coastal environment samples in this study tested positive for group I *C. botulinum*, although the detection method was biased toward the isolation of type E strains.

***Clostridium botulinum* type E in marine mammals.** The overall prevalence of *C. botulinum* type E in the intestinal contents of harvested seals from Nunavik was found to be low, i.e., 4.4% (Table 3.4). The overall prevalence of intestinal carriers when all

marine mammal species were combined, was 3.8% for Nunavik. None of the skin samples collected from seals harvested in Nunavik during the field survey were contaminated with spores of *C. botulinum* type E (Table 3.4).

Other *C. botulinum* serotypes in marine mammals. Two strains of *C. botulinum* type A were isolated from the intestinal contents of a bearded seal and a ringed seal from Nunavik, as well as a type B group I *C. botulinum* from the skin of a ringed seal (Table 6.1). Serotypes B or F belonging to *C. botulinum* group II were not found in any of the samples, although the detection method was biased toward the isolation of type E strains.

***Clostridium botulinum* type E in raw meat of marine mammals.** The prevalence and levels of *C. botulinum* type E in raw meat of marine mammals following harvesting and butchering is currently unknown. None of the raw seal meat samples collected from four seals during butchering were found positive for type E (Table 6.3). Based on meat obtained from two other bearded seals hunted in southern Ungava Bay and used for the inoculations studies, the background level of *C. botulinum* type E was estimated to be as low as <4 MPN per kg of meat. When 50 meat samples of 50 g each or 2.5 kg of meat from a single harvested bearded seal were tested for *C. botulinum* type E, only two enrichment broths yielded toxicity for an estimated level of approximately 1 spore per kg.

Other *C. botulinum* serotypes in raw meat of marine mammals. None of the other serotypes of *C. botulinum* were isolated from raw meat samples collected from four seals of Nunavik during butchering. However, a type B strain of *C. botulinum* group I

was isolated from a raw fat sample collected from one bearded seal and an unconfirmed serotype was demonstrated from a raw fat sample collected from a ringed seal.

***Clostridium botulinum* type E in ready-to-eat *igunaq*.** No food surveys have been conducted on the prevalence and level of *C. botulinum* type E in *igunaq* preparations consumed by the Inuit of Nunavik. Two of four separate *igunaq* preparations made with meat from two different seals were found positive for *C. botulinum* type E and botulinum type E neurotoxin during the field survey (Table 6.3). According to the producer, these two preparations were fit for human consumption.

Conclusion. The probability of contamination of raw marine mammal meat from the environment is higher for animals butchered from shoreline areas of southern Ungava Bay, particularly near the mouth of large rivers where the concentration of spores are higher. Despite the fact that the natural levels of *C. botulinum* type E in raw marine mammal meat may be as low as 1 spore per kg, large preparations of seal or walrus *igunaq* contain several kilograms of meat and fat tissues, thus increasing several fold the probability of this product being contaminated with spores. The shoreline environment is more heavily contaminated with spores of *C. botulinum* than intestinal contents or skin of marine mammals and likely contributes more frequently to the contamination of *igunaq* preparations.

Consumption of *igunaq* and other aged marine mammal products. Little data has been published on the consumption of various types of *igunaq* and other aged marine mammal products by Inuit populations of the Arctic. A food survey conducted with the Inuit from the Belcher Island, Nunavut showed that 14.7 and 41.2% of households

frequently eat aged bearded seal flippers and aged fat from ringed seal, respectively, but no data was generated on the consumption of seal or walrus *igunaq* (Wein *et al.*, 1996). In Nunavik, a survey was undertaken to determine the consumption profile of walrus *igunaq* in Tasiujaq following three consecutive walrus-related botulism outbreaks between July 2001 and February 2002 (Berthe *et al.*, 2002). All of the interviewed Inuit households (93%) from Tasiujaq consumed walrus *igunaq* during the preceding 13 months. The month of November followed by December and January were the months in 2001 in which the highest proportion of consumers of Tasiujaq had their first meal of walrus *igunaq*. The survey revealed that 31% of these households produced and consumed home-made walrus *igunaq*, and up to 55% of the surveyed occupants consumed walrus *igunaq* within the 13-month period, with an average of 6.2 *igunaq* meals since January 2001. These data clearly indicate that walrus *igunaq* remains a popular dish among the Inuits of Tasiujaq.

The profile of consumption of various types of *igunaq* varies among Northern villages of Nunavik. The Inuit population of Umiujaq generally does not produce or consume any *igunaq*, regardless of the marine mammal species, while populations of Aupaluk and Ivujivik usually do not produce seal *igunaq*, but rather prefer to make whale or walrus *igunaq*. It was recently estimated that 17% of the population of Nunavik aged between 18 and 74 years had eaten seal *igunaq* in 2004 with an average daily consumption of approximately 1.25 g per day or 37.5 g per month (Blanchet, unpublished data). Based on a survey conducted with 20 *igunaq* producers from five Northern villages of Nunavik in 2001-2002, the number of seal *igunaq* meals annually consumed by producers varied from 1 to 5 meals to more than 50 meals (Figure 9.3). At least 50% of them, particularly those from the Hudson Strait region, eat more than 30 meals of seal *igunaq* annually. Producers of seal *igunaq* from the Ungava Bay region,

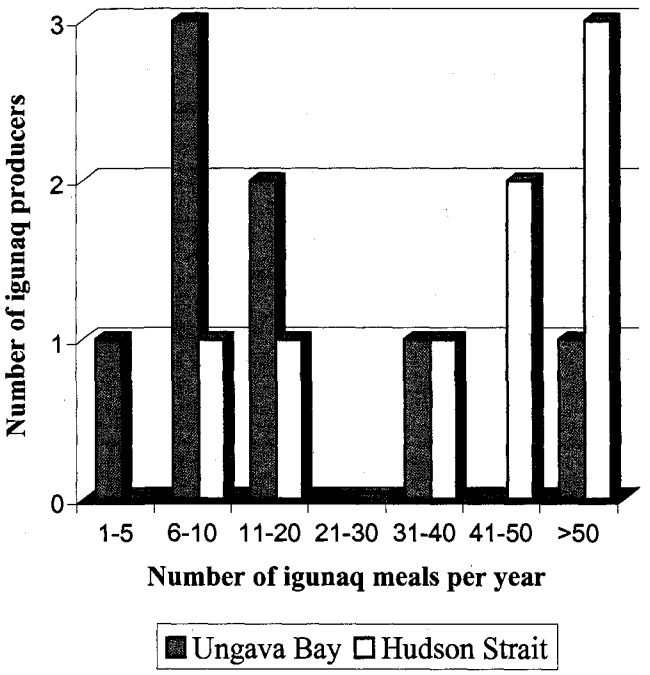


Figure 9.3. Annual consumption of seal *igunaq* meals by the producers from Ungava Bay and Hudson Strait regions.

including Tasiujaq, Kuujjuaq, and Kangiqsualujjuaq, generally eat less than 20 meals of seal *igunaq* annually. Based on a different survey, it was found that *igunaq* producers from Tasiujaq eat an average of 20 meals of walrus *igunaq* annually (Suppa and Proulx, 2002), indicating that this group of the population is at high risk of being exposed to botulinum toxins, especially those that like to eat various types of *igunaq*.

Food consumption data of *igunaq* producers probably reflects the consumption level of the population group who frequently consume *igunaq*. The number of meals and quantity of walrus *igunaq* consumed per meal generally increased with age (Suppa and Proulx, 2002). According to the Tasiujaq survey that last over 13 months, the age groups above 40 years consumed the highest number of meals on average, with more than 12 meals for those >60 years. All age groups greater than 1 year had reported eating walrus *igunaq* during the study period, with an overall average of 6.2 walrus *igunaq* meals. The individuals, aged between 15 to 59 years, consumed on average 0.5 to 1 cup (i.e., 250 mL) of walrus *igunaq* during the survey, while those <14 years or >60 years ate less than 0.5 cup. These data indicate that all age groups of the population of Tasiujaq, except infants, may be exposed to botulinum toxins through the consumption of contaminated walrus *igunaq*.

Qualitative estimate of exposure

Number of servings and serving sizes. The number of servings and serving size of *igunaq* have been poorly documented. Based on an outbreak involving five cases, including three fatalities, who consumed aged seal meat (not *igunaq*) from a non-eviscerated seal left outside for more than two months, it was possible to estimate the amount of meat consumed by the individuals (Stuart *et al.*, 1970). From a single meal of aged seal meat taken from a harp seal weighing approximately 135 kg, a minimum of

10 servings were shared. The servings ranged in size from less than 50 g to a maximum of 1200 g. Of the 10 persons who participated in the meal, six consumed less than 50 g and had either no symptoms or questionable symptoms, one ate 300 g and was symptomatic and recovered, and the remaining three who died either ate 600 or 1200 g.

Frequency of contamination. There have not been any surveys conducted on the prevalence of botulinum spores in marine mammal meat and related aged products consumed by the Inuit. One can expect a high frequency of contamination of meat when originating from marine mammals butchered in coastal areas rich in botulinum spores. The estimated concentration of spores in bearded seal meat used in our experiment was as low as 1 spore per kg, despite the fact that the animal was butchered at the mouth of the Koksoak River, an environment which is highly contaminated with *C. botulinum* type E spores. From a previous outbreak investigation in Cape Dorset in 1969 involving aged seal meat taken directly from a non-eviscerated seal left outside for a period of two months, one of four submitted aged seal meat samples were found toxic with 5 MLD per g (Stuart *et al.*, 1970). However, it was reported that one of the non-toxic samples contained at least 10^3 viable *C. botulinum* type E cells per g. Therefore, the frequency of contamination may have significantly differed depending on whether the meat is raw or aged. There is currently no data on the prevalence and level of contamination of *C. botulinum* type E in ready-to-eat (RTE) *igunaq*.

Predicted contamination level in RTE *igunaq*. The level of spores in raw meat used for the production of *igunaq* of different marine species is unknown in the Canadian Arctic. It is therefore difficult to predict if some types of *igunaq* may have a higher probability of becoming toxic during the aging process. In theory, a single viable spore

is sufficient to produce toxic levels of botulinum toxin under suitable growth conditions. The frequency of occurrence of BoNT/E in *igunaq* preparations produced by a household or a local producer is currently unknown, as well as the proportion of preparations that is annually rejected by producers because they were found unfit for consumption. However, there is some information on the proportion of toxic *igunaq* that is produced by examining data from previous laboratory and epidemiological investigations of type E outbreaks. Berthe *et al.* (2002) found that of 25 to 30 walrus *igunaq* preparations produced in 2001 in Tasiujaq, three (or 10-12%) caused botulism. Of the 10 people who consumed some of these three toxic *igunaq* preparations, six developed symptoms, for an attack rate of 60%. The attack rates of type E botulism could be calculated for some outbreaks in the Canadian Arctic and ranged from 9.1 to 66.7% for the period from 1985-2005 (Table 9.4). In comparison with the total number of walrus *igunaq* meals consumed in Tasiujaq during the 2001 survey, the proportion of toxic *igunaq* meals was relatively low at 0.8% (6/730).

The proportion of positive *igunaq* samples within a lot can also be low while being very toxic. Two of 18 persons (11%) who consumed walrus *igunaq* at Cape Dorset in 1978 developed severe symptoms of type E botulism, and had toxin levels in their serum of 15 and 40 MLD per mL (Hauschild *et al.*, 1978). Of the 16 individuals who had no signs or symptoms of botulism, 13 had their sera tested and all 13 were negative for botulinum toxin. Of four separate seal *igunaq* preparations used to monitor internal temperature fluctuations in this study, two (50%) preparations became toxic after two months of storage between mid-July and mid-September (temperatures ranged from 2 to 23°C). The two toxic preparations were judged satisfactory for consumption by the local producer from Kuujjuaq. The proportion of toxic *igunaq* subsamples (50 g) from each of the two preparations was respectively 10% (1/10) and 100% (10/10),

Table 9.4. Attack rates of type E botulism from some outbreaks that occurred in the Canadian Arctic between 1985-2005

Date of outbreak	Region	Northern village	Food type	No. of participants	No. of cases	No. of deaths	Attack rate (%)
August 1985	Nunavut	Kimmirut	Walrus *igunaq*	11	1	0	9.1
August 1995	Nunavik	Tasiujaq	Walrus *igunaq*	15	5	0	33.3
June 1996	Nunavut	Umingmaktok	Aged fish heads	2	1	0	50.0
August 1997	Nunavut	Arviat	Whale meat	6	4	1	66.7
September 1997	Nunavik	Kangiqsualujjuaq	Seal *igunaq*	14	9	0	64.3
May 2000	Northwest Territories	Inuvik	*Muktuk*	3	1	0	33.3
November 2002	Nunavik	Kuujjuaq	Aged beluga fat	3	1	0	33.3

despite the fact that both preparations were subjected to similar temperature patterns during storage. Thus, these data indicate that the distribution of botulinum toxins in large *igunaq* preparations can be uneven and variable in concentration from one area to another. This may explain, in part, the large variation in attack rates of type E botulism observed in the Canadian Arctic.

Growth rate during aging. In Nunavik, the length of the aging process of seal *igunaq* is variable and may last only a week or more than 4 weeks, but generally lasts between 3 to 4 weeks (Chapter 4). The RTE *igunaq* is then kept cold or frozen for long-term storage. The growth rate of *C. botulinum* type E has not been fully investigated through inoculation studies. The concentration of *C. botulinum* cells in *igunaq* preparations inoculated with 10^3 spores per gram may increase by approximately 3 and 5 log CFU/g after two weeks of storage at 10 and 20°C, respectively (Chapter 8). The organism was also able to grow and produce toxin in seal *igunaq* inoculated with spore levels as low as 10 CFU per gram and aged within an aerobic atmosphere under the same abuse temperatures, despite the presence of an active microflora. However, the lack of type E toxin production at 4°C in all seal *igunaq* experiments is probably due to the presence of the resident competing psychrotrophic microflora in seal meat.

Heat treatment. In Nunavik, all types of *igunaq* preparations are usually eaten raw without any heat treatment. However, a number of type E outbreaks from the Canadian Arctic have been previously associated with seal meat, caribou meat or fish eaten parboiled (boiled for a short time to partially cook). The extent of this practice in the North, as well as the various time-temperature combinations employed, has not been documented.

Exposure summary. There is currently a lack of data on the frequency of contamination of raw marine mammal meat and RTE *igunaq* products with spores of *C. botulinum* type E. There is also no information on the frequency and levels of consumption of various types of *igunaq* at the population level in Nunavik. This lack of information makes the qualitative assessment of exposure difficult to estimate. Based on the relatively high level of production and consumption of walrus *igunaq* in Tasiujaq, one may suspect that the contamination level of *igunaq* products with spores is very low or the growth conditions leading to toxigenesis in walrus *igunaq* produced in this community of southern Ungava Bay are usually not optimal.

International context. *C. botulinum* type E was not found in intestinal tracts of 32 seals harvested from Alaska (Miller, 1975). The overall prevalence of intestinal carriers when all marine mammal species are combined was only 2.3%. However, similar to the Nunavik region, the distribution of spores in the coastal environment of Alaska followed the distribution pattern of type E botulism cases. Baseline surveys have not been conducted on the prevalence and levels of *C. botulinum* type E in marine mammal products consumed by the Inuit populations of Alaska or other circumpolar regions. A single survey at Bristol Bay, Alaska revealed that more than half of the families from four Yupik villages eat fermented foods, but no quantitative data are available on the number and sizes of various fermented meals consumed annually by the local populations (Shaffer *et al.*, 1990). In Greenland, Huss (1980) showed that 86% of 21 marine sediments from the Greenland waters harboured *C. botulinum* type E spores. Although seal meat is the most frequently consumed traditional food on a daily basis in Greenland (Pars *et al.*, 2001), the proportion of fermented seal products consumed by the Inuit population remains unknown.

9.6. Risk characterization

Regardless of the serotypes involved, human botulism is considered a public health emergency that requires rapid medical intervention and triggers an epidemiological investigation to avoid further cases from occurring. Without prompt clinical management, the conditions of the patients may deteriorate, leading to respiratory arrest and death.

Adverse health effects in the Canadian Arctic

Incidence. The incidence rates of type E botulism between 1996 and 2004 were relatively high in the Canadian Arctic, particularly in the Nunavik region (Table 9.5). The incidence rate per 100,000 population ranged from 0 to 126 cases per year in Nunavik. The mean incidence rate for this region was 51.6 cases annually per 100,000 population, which was 9 times higher than the rate for the entire Canadian Arctic or more than 2000 times the rate for Canada as a whole. Thus, the Inuit population from the Nunavik region is at far greater risk of being exposed to BoNT/E than any other population in Canada.

Age groups older than 30 years are more often affected than younger age groups (Figure 9.4). Of the 73 type E botulism cases reported in Nunavik between 1985-2005, 79.5% and 90.4% were older than 40 and 30 years, respectively. Botulism has not been reported in children less than 10 years of age in Nunavik, but two cases aged 1-4 years were recorded in other Arctic regions. The median age of botulism cases from Nunavik calculated in this review was 51 years and 39 years for cases originating from other regions of the Canadian Arctic. The gender ratio of male and female botulism cases was found to be similar in both areas, i.e., it is 0.9 and 1.0 in Nunavik and other regions of the Canadian Arctic, respectively.

Table 9.5. Rates of type E botulism in Canada from 1996 to 2004

Year	Rate per 100,000 (number of cases)[a]		
	Nunavik	**Canadian Arctic**	**Canada**
1996	91.8 (8)	8.4 (9)	0.041 (12)
1997	126.2 (11)	14.8 (16)	0.060 (18)
1998	45.9 (4)	3.7 (4)	0.013 (4)
1999	34.4 (3)	4.7 (5)	0.020 (6)
2000	0.0 (0)	0.9 (1)	0.003 (1)
2001	20.8 (2)	3.7 (4)	0.026 (8)
2002	72.7 (7)	6.4 (7)	0.022 (7)
2003	41.5 (4)	3.6 (4)	0.013 (4)
2004	31.1 (3)	2.7 (3)	0.009 (3)

[a]Rates between 1996 and 2000 and rates between 2001 and 2004 were calculated based on 1996 and 2001 census data, respectively for Nunavik region, whereas annual population estimates were used for Canada and other regions of the Canadian Arctic. Source of census and intercensal data can be found at: http://www.statcan.ca.

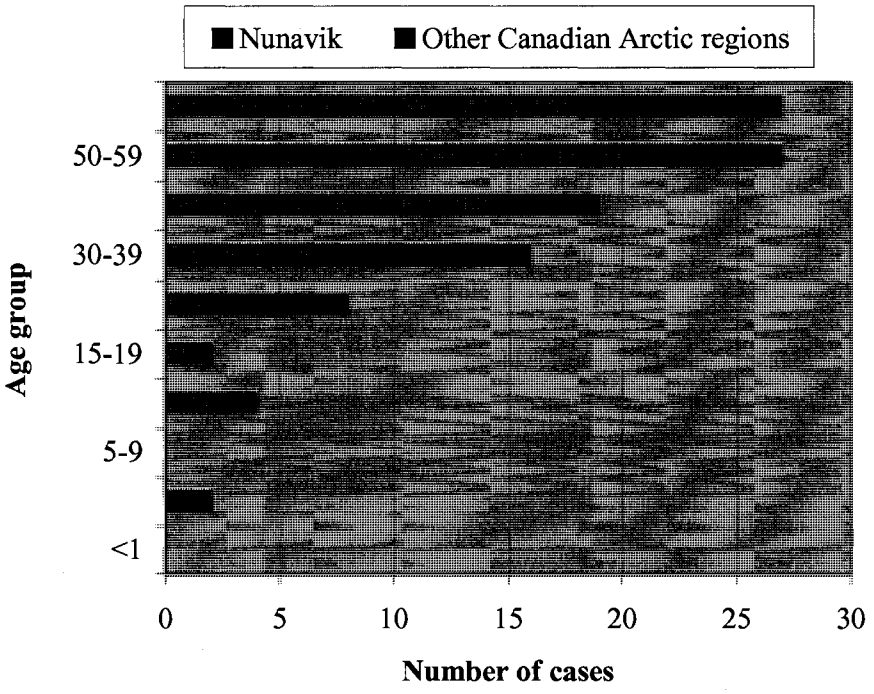


Figure 9.4. Age distribution of cases of foodborne type E botulism in the Canadian Arctic between 1985 and 2005.

Clinical consequences of botulism. Over 90% of the botulism cases in Nunavik between 1995 and 2003 required hospitalization, and more than 40% of them were transferred to southern hospitals due to the severity of their respiratory conditions (Proulx, 2004). Table 9.6 shows that for the 2001-2005 period, the rates of intensive care in southern provincial medical centres reached 50% in Nunavik and in other regions of the Canadian Arctic. Prior to 1965, the fatality rate was above 50% and decreased to 14% for the 1981-84 period in Canada (Hauschild and Gauvreau, 1985). The overall fatality rate of type E botulism for the 1985-2005 period was 2.5% in Nunavik and 12.2% within the rest of the Canadian Arctic. The fatality rate has remained at 0% in Nunavik since the 1991-95 period, but the rate of admissions to intensive care units in southern hospitals increased from 26.1 to 50%.

Serotypes causing diseases. Most (97%) botulism incidents reported in the Canadian Arctic during the period from 1985 to 2005 were laboratory confirmed, with the vast majority (98.5%) being caused by type E strains of *C. botulinum*. However, a single case of botulism from Sanikiluaq in Nunavut in 2001 was associated with the isolation of *C. botulinum* type A in the gastric fluid. However, botulinum toxin was not detected in any of the clinical specimens. The isolation of *C. botulinum* type A was previously observed in the gastric fluid of a case from Tasiujaq, Nunavik in 1996, which was caused by BoNT/E. It is likely that C. botulinum type A spore had been ingested, but BoNT/A had not caused the illness of the patient from Sanikiluaq.

Case-control studies and risk factors. There have not been any case-control studies conducted for the investigation of large type E botulism outbreaks in the Canadian Arctic.

Table 9.6. Intensive care hospitalisations and fatality rates of type E botulism in the Canadian Arctic for the period from 1985 to 2005

Period	Nunavik						Other Canadian Arctic regions					
	No. of incidents	No. of cases	ICU[a]	ICU rate	No. of deaths	Fatality rate (%)	No. of incidents	No. of cases	ICU	ICU rate	No. of deaths	Fatality rate (%)
1985-90	9	16	3	18.8	2	12.5	7	12	0	0	3	25
1991-95	13	23	6	26.1	0	0	7	18	3	16.7	1	5.6
1996-00	12	26	10	38.5	0	0	6	9	3	33.3	1	11.1
2001-05	13	16	8	50	0	0	2	2	1	50	0	0
Overall	47	81	27	33.3	2	2.5	22	41	7	17.1	5	12.2

[a]ICU: Intensive Care Unit. These cases were transferred to provincial medical centres located in southern regions due to the severity of their conditions.

Previous epidemiological studies have identified the food types and the modes of preservations of the foods frequently associated with type E botulism in the Canadian Arctic (Dolman, 1974; Hauschild and Gauvreau, 1985). Aged marine mammal and fish were by far the predominant vehicles of type E botulism prior to 1985, and remains the food types responsible for the great majority of type E botulism in Canada. The occurrence of type E botulism in the Canadian Arctic appears to be biphasic, reflecting the periods by which *igunaq* is most often prepared and freshly consumed. Although *igunaq* or aged marine mammal products are eaten throughout the year, they are usually prepared during mid-summer and fall, and later during early spring, which corresponds to the periods of the year where hunting of marine mammals is more intensive. For the period from 1985 to 2005, the highest number of cases recorded in the Canadian Arctic was during the months of August and September with 25 and 19%, respectively. In Nunavik, 81% of botulism cases occurred between the months of July and November with August, September and November being the highest peak months (Figure 9.5). Temperature is a critical factor that influences growth and production of *C. botulinum* type E toxin in *igunaq* preparations and any temperatures above 4°C may be abusive during the aging process.

Adverse health effects in other circumpolar regions

Incidence. Fifty-eight botulism incidents involving 103 persons occurred in Alaska between 1990 and 2000 for an incidence rate of 1.9 cases per 100,000, approximately 190 times the rate for the United States (US) (Sobel *et al.*, 2004). Most of the incidents were caused by *C. botulinum* type E (84%), but an increasing number (14%) were related to type B botulism. Apart from Alaska, there were no other reports pertaining to

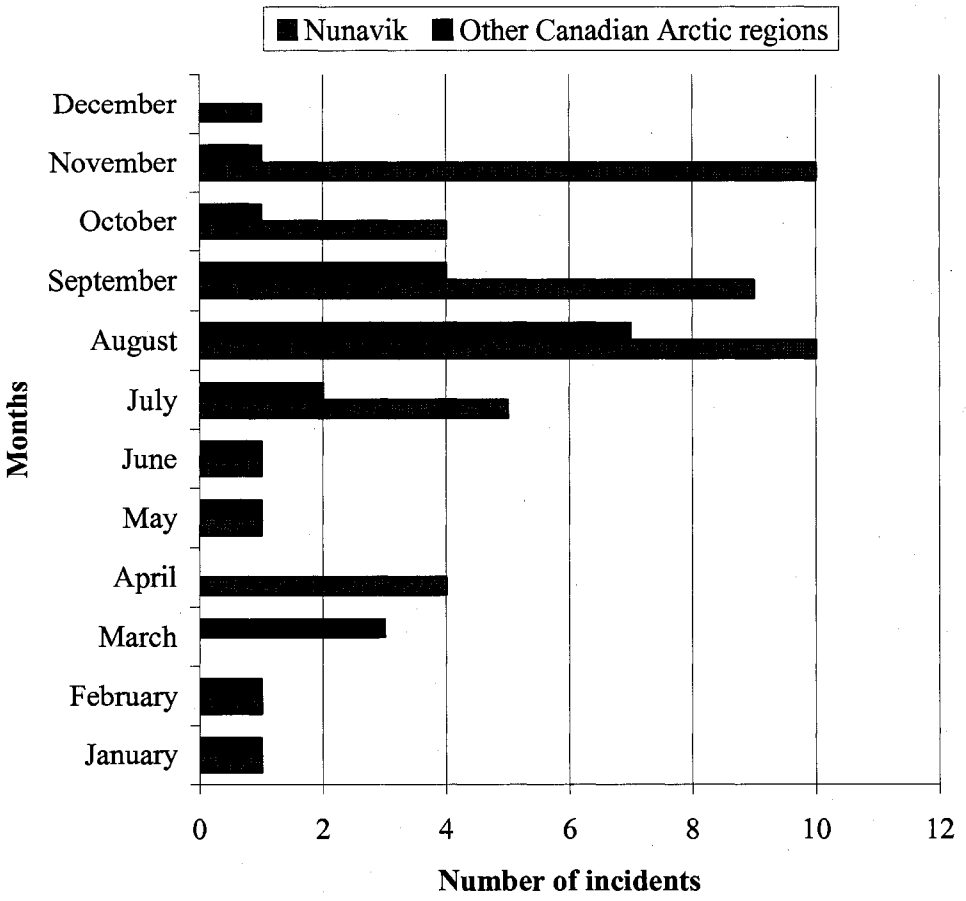


Figure 9.5. Monthly distribution of foodborne type E botulism incidents in the Canadian Arctic between 1985 and 2005.

the occurrence of type E botulism associated with the consumption of marine mammals in circumpolar regions.

Contributions to outbreaks and incidents. In Alaska, 37 and 42% of laboratory-confirmed cases for the 1947-1985 period were associated with marine mammals and fish, respectively (Wainright *et al.*, 1988). Seal and whale products accounted for 27 and 17% of these cases, respectively, followed by salmon eggs with 15%. The authors showed that 62% of implicated foods were aged ("fermented"), 23% dried, 8% frozen, and 8% in oil or salted. The aging process was traditionally done by placing foods in a pit in the ground overlaid with the skin of animals or woods. Food was protected from the weather conditions by a covering of moss. All outbreaks of botulism recorded in Alaska between 1990 and 2000 were also caused by non-commercial foods, predominantly of aquatic origin (Table 9.7). Traditional aged marine mammal products accounted for 51% of the incidents, of which 44% were *igunaq* from various marine mammals and 56% were seal *misiraq*. Fermented fish and fish eggs contributed to 43% of the incidents. From 11 type E outbreaks reported in Greenland between 1984 and 1989, seven were associated with seal meat, and three with sheep meat (Hauschild, 1993).

Case-control studies. There have not been any previous case-control studies investigating large type E botulism outbreaks in Alaska. However, to assess the risk factors related to food preparation, Shaffer *et al.* (1990) conducted a survey which included native foods not associated with botulism outbreaks from three affected and three unaffected villages of Bristol Bay, Alaska. The survey revealed that the

Table 9.7. Botulism incidents in Alaska, 1999-2000[a]

Type of food	No. incidents	No. of cases
Fermented marine mammals	11	21
Seal oil	14	20
Fermented fish	14	28
Fermented fish eggs	7	18
Mixed ingredients	3	5
Total	49	92

[a]Sobel *et al.*, (2004).

preparation methods varied from one producer to another with regards to the type of fermentation chambers, ingredients, and duration of fermentation.

Risk assessments and other international activities. No risk assessment studies have estimated the risk of contracting botulism from eating aged marine mammal meat. Hauschild (1982) assessed the risk of botulism hazards from cured meat products and compared the probability of outgrowth and toxigenesis of various meat products containing different concentrations of brine and salt on a quantitative basis. In addition, a risk profile on *C. botulinum* in ready-to-eat smoked seafood in sealed packaging was recently developed in New Zealand, and the risk to consumers from these products was found to be very low (ESR, 2006).

Secondary transmission. Botulism is not contagious and there have been no reports of person-to-person transmission of botulism.

Qualitative estimate of risk. The qualitative estimate of the risk of developing *C. botulinum* type E botulism from eating aged marine mammal in the Nunavik region is difficult to determine without a qualitative estimate of exposure. However, based on recent field and inoculation studies conducted with aged seal meat, it is possible to provide insights on specific elements that will influence the risk. The current post-harvesting methods of cutting, butchering and handling of marine mammals in Nunavik are carried out in the field directly on the shoreline environment along the islands and the continental coasts. These aquatic habitats naturally harbour various microbial populations, including dormant spores of *C. botulinum* type E. This traditional procedure is usually performed on the shoreline without a physical barrier and

inevitably will result in the bacterial contamination of meat and other tissues that come into contact with water, coastal rocks or shoreline sediments. Thus, the risk of contamination depends on the level of spores encountered in the shoreline environment and the meat handling practices used by hunters to minimize contamination.

Use of the sediment bed at low tide or shoreline areas with high concentrations of spores in marine sediments, surface water or surface of shoreline rocks exposed to tidal water, would inevitably lead to the contamination of meat due to the current meat handling practices. Any handling activities enhancing contact of meat with the surface of the coastal rocks or seawater from these environments, would increase the probability of contaminating the meat. It appears that high-risk meat handling practices such as placing meat onto rock surfaces while butchering, or rinsing meat with surface water, are commonly used by hunters of Nunavik. Thus, one could reasonably guess that the risk of contamination would be high for raw meat derived from seals butchered on the shoreline areas of southern Ungava Bay where the levels of spores in the aquatic environment is relatively high. However, the extent of contamination of raw meat remains to be determined in future studies.

Based on inoculation studies with seal meat, the probability of *C. botulinum* type E toxigenesis during aging increased for seal *igunaq* preparations incubated above 4°C, regardless of the spore levels or preparation methods. The preparation and aging process of seal *igunaq* involves the use of traditional or modern-type containers and storage areas that vary extensively from one producer to another, but all producers claimed that the incubation is performed at cold temperatures similar to refrigeration temperatures. The variation in temperature inside various types of *igunaq* preparations during aging is unknown under field conditions, but it is likely influenced by the external temperature. The data loggers introduced inside four large *igunaq* preparations made of seal meat and

fat mixed inside plastic containers and stored outside during a two-month period, from July to September, revealed that the inside temperature varied from 2 to 23°C, with an average of 10°C. These data indicate that the preparations were temperature abused and two of them (50%) were found to be unfit due to the presence of BoNT/E. According to the data loggers, the temperature of these preparations remained above 5°C until mid-September. Thus, the probability of *C. botulinum* type E toxigenesis in *igunaq* would increase for preparations aged during the summer months when the external temperature rises above 4°C. Without any control measures being implemented to inhibit the growth of *C. botulinum* type E or to destroy spores or BoNTs during the processing of *igunaq* or prior to consumption, one could expect the continuous sporadic occurrence of type E botulism from eating toxic *igunaq* that has been prepared during the warmer months.

Risk Categorization. The severity of type E botulism varies greatly among outbreak-related cases from minimal symptoms or mild botulism, to severe respiratory impairment requiring mechanical ventilation (Wainwright *et al.*, 1988). The proportion of cases in whom botulinum toxin was demonstrated and required respiratory support is relatively high, varying from 49 to 66% (Wainwright *et al.*, 1988; Barrett, 1991). The hospitalisation stay was more than two days in 91% of the cases studied in Alaska between 1973 and 1986, and significantly increased for those who received intubation (Barrett, 1991). The mean naso-tracheal intubation time was 8.6 days, as compared to 15.5 days for patients who received a tracheostomy. Although the fatality rate has remained at 0% in Nunavik since the 1991-95 period, the rate of admissions to intensive care units in southern hospitals increased from 26.1 to 50% (Table 9.6). Based on the severity of the disease and the prolonged hospitalisation of cases requiring respiratory support, type E botulism should be placed in the highest severity category.

The overall incidence rate of type E botulism in Nunavik attributable to aged marine mammal meat for the period from 1996 to 2004 was high, with 33 cases per 100,000 population. At least 69% of these outbreaks were due to the consumption of seal *igunaq*, followed by walrus *igunaq*, and unidentified aged marine mammal meat. All these outbreaks occurred in the Northern villages of Ungava Bay, of which 81.3% clustered in the southern villages, i.e., Kuujjuaq, Tasiujaq and Kangiqsualujjuaq. According to the categories for risk profiles used by the New Zealand Food Safety Authority (ESR, 2006), this incidence rate is ranked in the second highest incidence category (rate range of 10-100).

9.7. Risk management information

Relevant food controls. Hunting and processing of marine mammals in Nunavik is part of traditional subsistence activities which are not subjected to regulations or safety standards. There are no slaughter activities involving federal or provincial inspection, or meat processing activities for the purpose of commercializing marine mammal meat products in Nunavik and elsewhere in the Canadian Arctic. The US CDC, in collaboration with the Alaska State Department of Health Social Services, has recommended methods for aging native foods (Sobel *et al.* 2004; Castrodale, 2005). These methods consist of using a traditional grass-line hole in the ground to allow air circulation around foods, and storing food preparations at temperatures below 3°C during the aging process to prevent toxin production. Similar to Alaska, for Nunavik, it was recommended by Weetaluktuk (1997) to avoid: i) the use of plastic bags; ii) the presence of stagnant air around foods during aging and; iii) preparing *igunaq* during the warmer months, particularly in the Ungava Bay region where the incidence of botulism is high. However, based on the results of the challenge studies (Chapter 8), the type of containers employed does not influence the time to toxicity of seal *igunaq*, but rather the storage temperature influences the lag phase.

Consumers. In Alaska, it is recommended to boil aged or fermented native foods prior to consumption, but this control measure changes the taste of *igunaq* products and is not currently used in the Nunavik region.

Economic Costs. An economic study based on six incidents has shown that the mean cost per botulism incident was over $70,000 or $7,200 per case in the Canadian Arctic and northern coastal British Columbia (Todd, 1988). Based on the annual average

number of cases in these regions, the author estimated the annual cost of botulism in Canada at $576,000 or $2,076,000, when the value of lost lives was included. This figure is in 1988 dollars and is not adjusted for inflation.

Table 9.8. Summary of the risk categorization for *C. botulinum* type E in aged marine mammal meat

Food/hazard combination	Severity[a]	Incidence[b]	Trade importance
C. botulinum type E in aged marine mammal meat	1	2	Inter-community[c]

[a]Severity category 1 has >5% of cases that experience severe outcomes resulting from exposure to the hazard. This can be estimated from the proportion of hospitalisations, long term illness, and deaths.
[b]According to ESR (2006), the "incidence is an estimate of the proportion of the foodborne disease rate due to an individual hazard, that is transmitted by a single food or food group". The incidence category 2 corresponds to a rate range of 10 to 100 cases per 100,000.
[c]The level and extent of the inter-community trade of native foods among Northern villages of Nuvavik has been poorly documented. Suppa and Proulx (2002) reported from a survey conducted in Tasiujaq that at least eight suppliers of *igunaq* for this village originated from Aupaluk, Quaqtaq, Kangiqsujuaq and also from villages in Nunavut.

9.8. Conclusions

Description of risks to Nunavik consumers

Risks associated with aged marine mammal meat. The incidence rate of type E botulism attributable to aged marine mammal meat in Nunavik is very high, with greater than 30 reported cases per 100,000 population in 2004. All cases reported for the period from 1996 to 2004 were clustered in Ungava Bay, particularly in the southern villages, which had a three-fold greater incidence of botulism. These results reflect the higher risk of contamination of marine mammal meat from the shoreline environment of this region, which harbours higher levels of spores than the Hudson Bay and Hudson Strait regions. As the processing of marine mammals is carried out in the field where the sanitary conditions are uncontrolled, it is expected that contamination of marine mammal meat butchered in the shoreline areas of Ungava Bay will continue to occur if significant changes in processing and handling practices are not made. In addition, the risk of contracting type E botulism from eating aged marine mammal meat will likely not decrease in future years if an effective means of controlling the growth of *C. botulinum* type E during the aging process is not implemented.

Risks associated with other foods. In Nunavik, various other aged marine mammal products have also been implicated in previous type E outbreaks, with seal *misiraq*, aged *muktuk* and dried beluga meat accounting for 27% of identified suspect foods between 1996 and 2004. In contrast, most outbreaks from other regions of the Canadian Arctic have mainly involved aged beluga whale products, mostly aged *muktuk*.

Quantitative risk assessment. This risk profile has identified which gaps need to be filled in order to perform a comprehensive quantitative risk assessment of type E

botulism in Nunavik. The prevalence and levels of *C. botulinum* type E spores in raw meat of seal and other marine mammals used for the production of aged products in different regions of Nunavik is currently unknown. The knowledge of the month in which the botulism outbreaks occur are now routinely recorded, but not the initial start time and duration of the aging process. This information would provide insights on the probability of toxigenesis during the various times of the year in which the *igunaq* are produced. Monitoring data on the variation of temperature inside various *igunaq* products prepared by producers would provide even more precise estimates of the probability of *C. botulinum* type E toxigenesis in these products. Finally, a food survey assessing the preference of the population for various types of *igunaq* and the corresponding number of meals and serving size consumed, is needed to better estimate the risk of exposure for various age groups.

Commentary on risk management options. Given the severity of the disease and the current incidence rate of type E botulism in Nuanvik, it is urgent to define management options and recommend the appropriate actions to be taken. The risk of contamination of marine mammal meat during the butchering process performed under field conditions is high, and it would be difficult to reduce the risk without significantly changing current processing and handling practices. Education of the Inuit population on how to minimize field contamination of marine mammal meat during the butchering process and prevent growth and toxin production of *C. botulinum* type E during aging, should as a minimum, target the igunaq producers and all consumers who frequently eat igunaq. This would particularly include those over 30 years of age for which more than 90% of the type E botulism cases reported in Nunavik between 1985-2005, have been associated. To eliminate contamination of butchered meat from the shoreline

environment, a hunter would be required to butcher the animals inside a processing plant where the hygienic conditions could be controlled. However, this option is logistically not practical for those who hunt at very far distances from the village. An option that could significantly minimize the risk of contamination from the environment would be to use frozen raw meat derived from animals that have been hunted and butchered on the ice. Producers of *igunaq* could freeze a number of lots of raw marine mammal meat and fat and use these for the preparation of *igunaq* at a later time. However, this would not eliminate the potential risk of contamination from the intestinal tract, although based on the prevalence data, the risk would be relatively low.

Boiling aged marine mammal meat products is not an acceptable option for consumers who like the traditional taste of this delicacy and hence efforts should be directed at controlling growth of *C. botulinum* type E to prevent development of toxigenesis from accidental contamination of raw meat during the post-harvesting stages. Based on inoculation studies, low storage temperature during the aging process of seal meat can prevent production of BoNT/E in *igunaq*, regardless of the inoculum doses or preparation methods. Low storage temperatures should therefore be considered as an option to control the growth of *C. botulinum* type E. Installation and use of large walk-in refrigerators with controlled thermostats would allow community members to safely age large preparations of *igunaq* and other native foods at constant low storage temperatures.

CHAPTER 10

GENERAL DISCUSSION, CONCLUDING REMARKS, AND FUTURE RESEARCH

Human botulism, or qasunniq in Inutittut (Weetaluktuk, 1997), occurs sporadically in the Canadian Arctic affecting primarily the Inuit population, which is considered at the highest risk for botulism in Canada (Dolman, 1974; Hauschild and Gauvreau, 1985). The disease is foodborne and usually occurs following ingestion of aged marine mammal meat (*igunaq*) containing preformed BoNT/E. The traditional Inuit dishes have been the predominant food type associated with type E botulism outbreaks in Canada (Austin and Dodds, 2000) and Alaska (Wainwright *et al.*, 1988). However, little was known about the contamination sources and transmission routes of *C. botulinum* type E in raw materials used in the preparation of these traditional dishes, which are considered food delicacies by the Inuit population.

The field surveys have revealed that *C. botulinum* type E is not encountered in all coastal areas used by hunters for post-harvesting activities of hunted seals in Nunavik. The geographical distribution of type E spores predominantly clustered in Ungava Bay region and closely follows the spatial distribution of type E botulism outbreaks. Up to 88% of shoreline soil samples from the coastline environment of Ungava Bay were positive, a region where botulism is endemic, accounting for 96% of the outbreaks in Nunavik since 1985. In comparison, in the northern villages of the Hudson Strait region where no outbreaks have been recorded during the same period, no shoreline soil samples collected near butchering areas contained spores. The drainage basins of the rivers flowing into Ungava Bay are vast and more rich in vegetation than those linked to Hudson Strait. Thus, the amount of spores originating from the rivers draining into Hudson Strait may be lower due to the relatively smaller land mass and/or the lower proportion of wetlands in the drainage area. To confirm this hypothesis, further field studies would be required to estimate the prevalence and levels of spores in these river

systems and compare the estimates with those observed in the Koksoak River or Whale River.

Similarly, *C. botulinum* type E was more prevalent along the southern and western coastlines of Alaska, near villages where botulism outbreaks were reported, but not along the northern coast (Miller, 1975). The prevalence of spores was found to be very low in marine sediments of Iceland and the Faroe Islands, but not in Greenland waters, where 86% of marine sediments harboured *C. botulinum* type E spores. In contrast, type E botulism is relatively rare in Greenland. Since 1967, only nine incidents have been linked to the consumption of marine mammals, with one incident due to the ingestion of a putrefied sperm whale and seven linked to seal meat (Müller and Thomsen, 1968; Hauschild, 1993). Seal meat is the most popular traditional food consumed by Greenlandic Inuit, i.e., up to 20% of respondents in a survey eat seal meat on a daily basis (Pars *et al.*, 2001). However, little information exists on the modes of preparation of seal meat in this region, and whether the meat is aged into *igunaq* like the Inuit of Alaska and the Canadian Arctic.

The surveys also showed that the levels of spores in shoreline environments varied between the three main regions of Nunavik. The concentrations of spores in shoreline soil was highly variable, with median concentrations of 5 and 23 spores/kg in samples collected along the coast of Hudson Bay and Ungava Bay, respectively. However, the levels of spores were found to be particularly high in the coastal areas of southern Ungava Bay where the levels increased to 270 to 1,800 spores/kg at the mouths of the Koksoak and Whale rivers. This is the first report assessing the level of *C. botulinum* type E spores in Arctic waters. In Scandinavia, the mean spore count of *C. botulinum* type E in seashore samples of Finland was found to be relatively high at 500 spores/kg (Hielm *et al.*, 1998c), and in Danish waters, the levels reached as high as

240,000 spores/kg in closed shallow fiords (Huss, 1980). The highest concentration of spores in Nunavik waters was found in the marine portion of the Koksoak River, with more than 5400 spores/kg. To reduce the risk of contamination of meat during butchering, quantification of spores in shoreline soils near butchering areas could be used to identify coastal areas that should be avoided for post-harvesting activities of marine mammals.

C. botulinum type E was present in most sediment samples from the mouth to the origin of the Koksoak River over a distance of 137 km, including its tributaries and the in-land soil 25 to 100 meters distant from the tributaries. Spores were isolated from 100% of marine sediments and terrestrial soil samples and from 94% of freshwater sediments. These results contrast with the survey of the Fox River, the main tributary of Green Bay, Lake Michigan, where only 4% of bottom and shoreline soil samples were positive for *C. botulinum* type E (Bott *et al.*, 1968). Overall, 17.7% of river and lake samples near Green Bay contained spores and all inland soils beyond 175 feet from the bay were negative. This lead the authors to conclude that the very high level of spores encountered in Green Bay (100-1000 spores/g) resulted from the multiplication of the organism *in situ*. In contrast, *C. botulinum* type E was demonstrated in the lower and upper reaches of four rivers in the central part of Japan, with detection rates of 33 to 82% (Yamakawa and Nakamura, 1992). The organism was also detected from mountainous soil at 1000 to 1700 m above sea level where two of these rivers originate, suggesting that the organism was of telluric rather than aquatic origin. Whether the Arctic strains of *C. botulinum* type E were of telluric or aquatic origin in Nunavik is academic. However, the survey revealed that the Koksoak River is an abundant source of *C. botulinum* type E spores which contribute to the pool of spores in the marine portion of the river and its dispersion in the coastline environment.

The use of PFGE as a microbial source tracking (MST) method to track endogenous and extraneous sources of contamination of seal meat at the butchering site revealed the presence of a heterogeneous population of *C. botulinum* type E in the coastal environment. Previous PFGE and RAPD typing have shown that extensive genetic biodiversity exists among type E isolates from fish and aquatic environments (Hielm *et al.*, 1998b; Hyytiä *et al.*, 1999b). The finding of multiple PFGE subtypes of *C. botulinum* type E in seal *igunaq* No. 4 that were all different from the isolates that were recovered from the mud near the seal where it was butchered, illustrates the difficulty in tracking the source in the aquatic environment (Chapter 6). At least 11 different subtypes of *C. botulinum* type E and a type B strain were recovered from this area by analyzing up to three colonies per sample. It has been suggested that between 1000 and 2000 isolates of *Escherichia coli* had to be ribotyped in order to develop an adequate host origin database (Jenkins *et al.*, 2003). Clearly, typing more environmental isolates of *C. botulinum* type E should be done to develop environmental source databases to allow source tracking of spores in seal meat.

Phenotypic and genotypic methods are currently being developed and evaluated to track sources of bacteria in water and foods (Meays *et al.*, 2004). Seven protocols including PFGE, ribotyping, and PCR-based methods have been compared with regards to their reproducibility and ability to predict the sources of contamination of *Escherichia coli* (Stoeckel *et al.*, 2004). PFGE was the only method which correctly classified all test replicates to host species. Comparison of fingerprinting methods for typing *C. botulinum* group II strains (Chapter 5), showed that both the automated ribotyping and PFGE procedures were reproducible, as demonstrated with the analysis of replicate samples. While RAPD analysis produced the highest number of different DNA fragment patterns, the technique was not sufficiently reproducible to adequately

link the outbreak strains, even when only the reproducible fragments were selected, as previously done by others (Hyytiä *et al.*, 1999a,b). The duplicate RAPD samples did not consistently produce identical fingerprints from run-to-run, even with the use of Ready-To-Go™ RAPD kits which minimize differences in PCR reagent concentrations. Automated ribotyping using EcoRI had poor discriminatory ability, as most fingerprints displayed only two fragments, limiting its use for epidemiological investigations. However, PFGE was discriminatory and reproducible, allowing recognition of outbreak strains among unrelated type E strains (Leclair *et al.*, 2006). Molecular characterization of type E outbreak strains isolated from 24 botulism incidents that occurred in the Canadian Arctic from 1995 to 2004, showed that several distinct subtypes of *C. botulinum* were involved in these incidents and they were not limited to a cluster of subtypes (Chapter 7). These results were expected, following the finding of a genetically diverse population of *C. botulinum* type E in the Koksoak River and the coastal environment of Nunavik, where most of the incident isolates of the Canadian Arctic originated. However, some subtypes of *C. botulinum* type E have been incriminated in more than one botulism incident within the same region. Some of these strains causing botulism in the northern villages of the southern Ungava Bay, were found in the sediments of large rivers and even in inland locations south of Kuujjuaq.

The identification of the contamination routes during butchering activities was not possible using PFGE as a microbial source tracking method, due to the low number of raw meat and raw fat products yielding isolates of *C. botulinum* type E. The natural background level of *C. botulinum* type E in raw seal meat is very low, i.e., below 1 spore/kg following post-harvesting activities (Chapter 6). However, using data obtained from the questionnaire with *igunaq* producers (Chapter 4), the environmental surveys (Chapter 3) and the field observations of meat handling practices (Chapter 6), it was

possible to identify the sources and potential contamination routes of seal meat with *C. botulinum* type E at the butchering site (Figure 10.1). The probability of contamination of seal meat likely depends on the location of the butchering site in the field, and the meat handling practices used by the hunter. The marine portions of the Koksoak and Whale rivers of southern Ungava Bay, were shown to contain the highest concentrations of spores in sediments. Butchering marine mammals directly on a river bed at low tide would inevitably lead to the contamination of meat with spores of *C. botulinum* type E. Despite the fact that most butchering activities are carried out on a flat coastal rock at high tide, several contamination routes were identified and involved contact of the butchered meat with the surface of the coastal rocks or seawater in various ways (Figure 10.1).

In theory, a single viable spore is needed to outgrow and induce toxicity, in meat under favourable growth conditions (Hauschild, 1982). Due to the low level (nanogram range) of BoNT causing illness and death in humans (Arnon *et al.*, 2001), low levels of contamination of seal meat in the field would represent a risk to public health if the meat was to be aged under conditions which allowed for the growth of *C. botulinum* type E. Inoculation studies conducted with seal *igunaq* aged under different experimental conditions, showed that the preparation methods as well as air circulation, have no influence on the toxigenesis of *C. botulinum* type E (Chapter 8). Preparations of seal iguaq inoculated with a low spore load (10 spores/g) and aged in traditional seal skin pouches open bi-weekly or in plastic containers covered with cheese cloth to allow for the exposure to air, became toxic within two weeks of storage at 10°C. However, using a temperature of 4°C during the aging process, prevented the development of BoNT/E in seal *igunaq*, regardless of the spore load or preparation method.

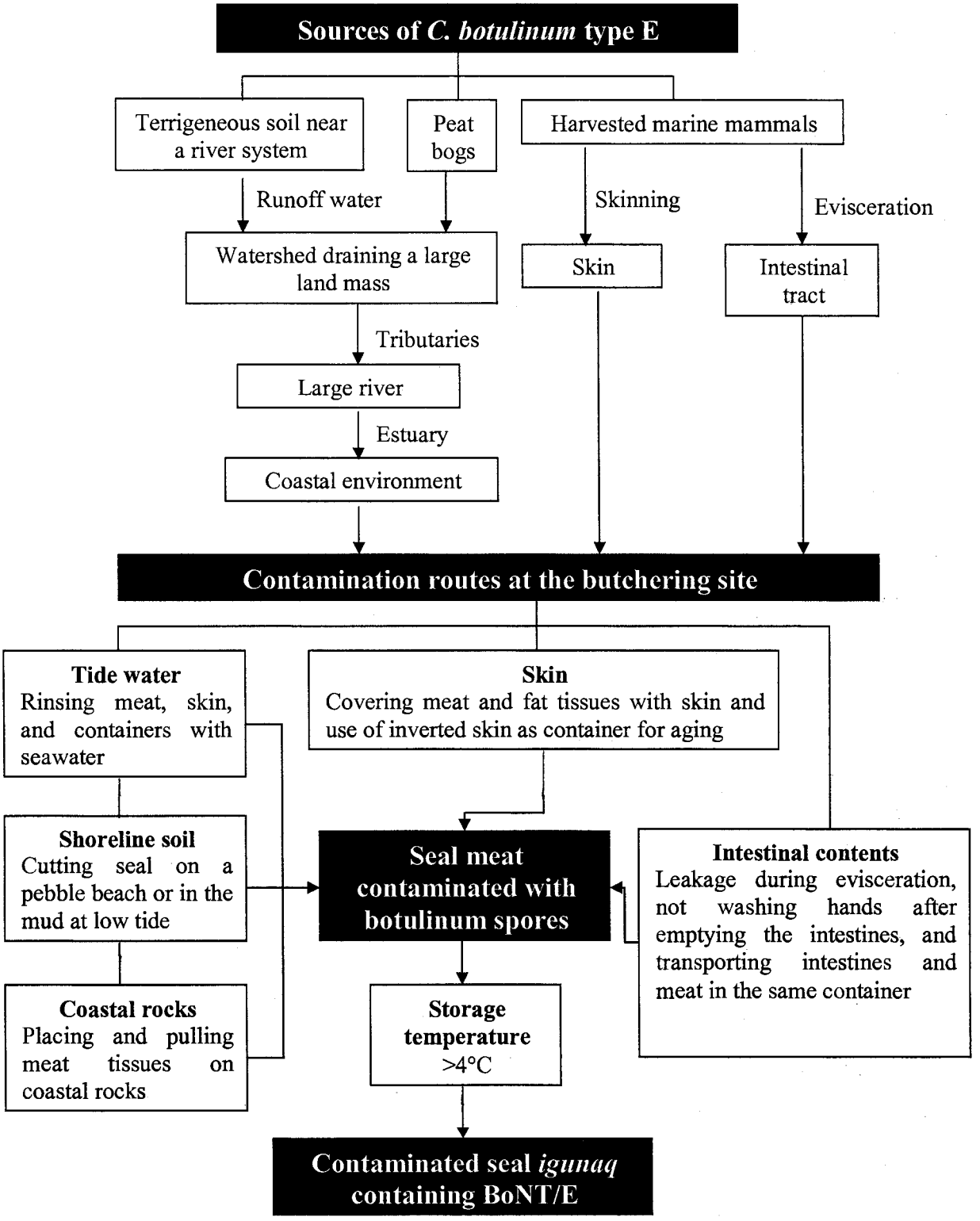


Figure 10.1. Sources and contamination routes of *C. botulinum* type E in seal meat at the butchering site.

These results were consistent with those of other researchers who found 4°C storage to be effective in preventing toxin production in fish packaged under vacuum or a modified atmosphere (Reddy *et al.*, 1996, 1997b; Dufresne *et al.*, 2000b). However, further challenge studies are required to validate these results using large preparations of seal *igunaq*, including the evaluation of the internal temperature profile. It is critical that the internal temperature of the traditional or modern-type *igunaq* preparations is constantly maintained at 4°C, or lower, during the entire aging period.

Since it is not possible to eliminate the contamination of seal meat with spores of *C. botulinum* type E with the current meat handling practices used by hunters in the field, refrigeration storage temperatures represent the only effective measure to control the growth and toxin production of *C. botulinum* type E in seal *igunaq* and to reduce the incidence rate of type E botulism in the Nunavik region. The probability of *C. botulinum* type E toxigenesis in *igunaq* is particularly high for preparations aged during the summer months when the external temperature rises above 4°C, as was observed with the use of temperature dataloggers in Kuujjuaq between mid-July to mid-September (Chapter 8). Data from the risk profile (Chapter 9) showed that for the period from 1985 to 2005, 81% of the botulism cases in Nunavik occurred between the months of July and November, with August, September, and November being the highest peak months. Type E botulism associated with *igunaq* prepared and aged during the warmer months (Chapter 9) is likely to continue to occur in Nunavik if control measures are not implemented to inhibit the growth of *C. botulinum* type E during the aging process, or to destroy BoNTs prior to consumption.

Food surveys conducted in the Arctic regions have shown that seal meat is frequently consumed among the Inuit of Greenland (Pars *et al.*, 2001) and Belcher Island (Wein *et al.*, 1996). However, little information has been published on the use

and preference for aged marine mammal products in any Arctic Inuit population. It has been estimated that 14.7 and 41.2% of households from Belcher Island frequently eat aged bearded seal flippers and aged fat from ringed seals, respectively, but no data has been generated on the consumption of seal or walrus *igunaq*. To quantitatively estimate the risk of type E botulism from eating aged marine mammal products in Nunavik, further studies are needed to determine the preference of the Inuit population for various types of *igunaq*, along with the corresponding number of meals and serving sizes consumed. In addition, the prevalence and levels of *C. botulinum* type E spores in raw meat of marine mammals used for the production of aged products in different regions of Nunavik remain to be estimated. Quantitative risk assessment (QRA) methodology can then be used to organize and analyze traditional and scientific data generated from this research to estimate the probability of developing human botulism following ingestion of different *igunaq* preparations. The QRA methodology has been previously applied to model the human health risk from foodborne pathogens associated with the ingestion of food processed in the industry (Cassin *et al.*, 1998). Future studies may involve the development of mathematical models to predict human exposure and estimate the risk of botulism associated with the ingestion of aged marine mammal products in the Canadian Arctic.

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APPENDICES

Table A1. Characteristics of shoreline soils surrounding the Nunavik coast

Sample ID	Region	Northern village	Location	Latitude	Longitude	Substrate composition	Detection of spores	MPN count[a] (Number/kg)
BL-300	Hudson Bay	Kuujjuaraapik	Boat Opening	RM[c]	RM	Sand/pebbles	Positive	<6
BL-301	Hudson Bay	Kuujjuaraapik	Kajurtuit Point	55°55'336'	76°51'.710'	Sand	Positive	<6
BL-302	Hudson Bay	Umiujaq	Sikutaaluk Bay	56°09'.500'	76°33'.700'	Sand/pebbles/shells	Negative	NA
BL-303	Hudson Bay	Umiujaq	Le Goulet	56°09'.320'	76°34'.685'	Sand	Positive	15
BL-304	Hudson Bay	Inukjuak	Qikiqtaruqtatuq	RM	RM	Sand/pebbles	Positive	13
BL-305	Hudson Bay	Inukjuak	Harrison Island	58°23'.776'	78°06'.276'	Sand/pebbles	Positive	<6
BL-306	Hudson Bay	Inukjuak	Satujaaq	58°24'.650'	78°11'.790'	Sand/shells	Negative	NA
BL-307	Hudson Bay	Puvirnituq	Hotel Beach	RM	RM	Pebbles	Positive	<6
BL-309	Hudson Bay	Puvirnituq	Big Finger Pointe	60°00'.799'	77°19'.902'	Sand/pebbles	Positive	13
BL-310	Hudson Bay	Akulivik	Coastline	60°46'.826'	78°13'.007'	Pebbles	Negative	NA
BL-311	Hudson Bay	Akulivik	Coastline	61°14'.374'	77°47'.167'	Sand/pebbles	Negative	NA
99SO-01	Hudson Bay	Ivujivik	Aulatsivik	RM	RM	Sand/pebbles	Negative	NA
99SO-02	Hudson Bay	Ivujivik	Aulatsivik	RM	RM	Sand/pebbles	Negative	NA

Table A1 (Continued). Characteristics of shoreline soils surrounding the Nunavik coast

Sample ID	Region	Northern village	Location	Latitude	Longitude	Substrate composition	Detection of spores	MPN count[a] (Number/kg)
99SO-03	Hudson Bay	Ivujivik	Aulatsivik	RM	RM	Sand/pebbles	Negative	NA
99SO-04	Hudson Strait	Ivujivik	Ivujivik Bay	RM	RM	Sand/pebbles	Negative	NA
99SO-05	Hudson Strait	Ivujivik	Ivujivik Bay	RM	RM	Sand/pebbles	Negative	NA
99SO-06	Hudson Strait	Ivujivik	Ivujivik Bay	RM	RM	Sand/pebbles	Negative	NA
99SO-07	Hudson Strait	Ivujivik	Erik cove	RM	RM	Sand/pebbles	Negative	NA
99SO-08	Hudson Strait	Ivujivik	Erik cove	RM	RM	Sand	Negative	NA
99SO-09	Hudson Strait	Ivujivik	Erik cove	RM	RM	Sand/pebbles	Negative	NA
99SO-10	Hudson Strait	Ivujivik	Sakkayat	RM	RM	Sand	Negative	NA
99SO-11	Hudson Strait	Ivujivik	Sakkayat	RM	RM	Sand/pebbles	Negative	NA
99SO-12	Hudson Strait	Ivujivik	Sakkayat	RM	RM	Sand/pebbles	Negative	NA
BL-314	Hudson Strait	Ivujivik	Ivujivik Bay	RM	RM	Pebbles	Negative	NA
BL-315	Hudson Strait	Ivujivik	Coastline	62°22'.016'	78°02'.141'	Pebbles	Negative	NA
BL-312	Hudson Strait	Salluit	Qaqaluatyutuaq	62°11'.269'	75°45'.832'	Sand/pebbles	Negative	NA

Table A1 (Continued). Characteristics of shoreline soils surrounding the Nunavik coast

Sample ID	Region	Northern village	Location	Latitude	Longitude	Substrate composition	Detection of spores	MPN count[a] (Number/kg)
BL-316	Hudson Strait	Salluit	Sagluk Island	RM	RM	Pebbles	Negative	NA
BL-317	Hudson Strait	Kangiqsujuaq	Kangiarajuk	61°32'.891'	71°42'.452'	Sand	Negative	NA
BL-319	Hudson Strait	Kangiqsujuaq	Kangiarajuk	61°32'.891'	71°42'.452'	Pebbles	Negative	NA
3	Hudson Strait	Kangiqsujuaq	Kangiarajuk	RM	RM	Sand	Negative	NA
4	Hudson Strait	Kangiqsujuaq	Kangiarajuk	RM	RM	Sand	Negative	NA
5	Hudson Strait	Kangiqsujuaq	Kangiarajuk	RM	RM	Sand	Negative	NA
BL-320	Hudson Strait	Quaqtaq	North of peninsula	61°00'.041'	69°28'.129'	Pebbles	Negative	NA
BL-321	Ungava Bay	Kangirsuk	Payne River	RM	RM	Sand/pebbles	Positive	<6
BL-322	Ungava Bay	Kangirsuk	Payne River	RM	RM	Sand/pebbles/mud	Positive	15
BL-323	Ungava Bay	Aupaluk	Village beach	RM	RM	Sand	Positive	7
BL-324	Ungava Bay	Aupaluk	Village beach	RM	RM	Sand	Negative	NA
BL-325	Ungava Bay	Tasiujaq	Leaf Bay	58°44'.086'	69°49'.919'	Mud	Positive	23
BL-326	Ungava Bay	Tasiujaq	Leaf Bay	58°42'.207'	69°41'.835'	Mud/sand	Positive	110

Table A1 (Continued). Characteristics of shoreline soils surrounding the Nunavik coast

Sample ID	Region	Northern village	Location	Latitude	Longitude	Substrate composition	Detection of spores	MPN count[a] (Number/kg)
BL-327	Ungava Bay	Kangiqsualujjuaq	North of peninsula	58°50'.716'	66°23'.174'	Sand	Positive	<6
BL-328	Ungava Bay	Kangiqsualujjuaq	Kaugaq Island	58°56'.176'	66°28'.876'	Sand	Negative	NA
BL-329	Ungava Bay	Kuujjuaq	Mouth of Koksoak Riv.	58°31'.970'	68°07'.773'	Mud	Positive	270
BL-330	Ungava Bay	Kuujjuaq	Mouth of Koksoak Riv.	58°33'.133'	68°11'.097'	Sand/pebbles	Positive	7
KR-44	Ungava Bay	Kuujjuaq	Mouth of Koksoak Riv.	58°29'.748'	68°10'.406'	Mud/sand	Positive	740
KR-42	Ungava Bay	Tasiujaq	Tasiujaq Islands	59°06'.807'	68°56'.246'	Mud	Positive	7
KR-36	Ungava Bay	Tasiujaq	Diana Bay	RM	RM	Mud/sand	Positive	15
KR-35	Ungava Bay	Kuujjuaq	Mouth of Whale River	RM	RM	Mud	Positive	1800
KR-19	Ungava Bay	Kuujjuaq	Big Island	58°17'.589'	67°40'.046'	Mud	Positive	130
KR-29	Ungava Bay	Kuujjuaq	Big Island	58°17'.589'	67°40'.045'	Mud	Positive	1800

[a]Based on mouse bioassay results.
[b]NA: not applied.
[c]Recorded on a 1:250,000 map.

Table A2. Characteristics of sediment and terrestrial soils collected in the Koksoak River area

Sample ID	Sample type	Location	Latitude	Longitude	Substrate composition	Detection of spores	MPN count[a] (Number/kg)
BL-329	Marine sediment	Mouth of Koksoak River	58°31'.970'	68°07'.773'	Mud	Positive	270
BL-330	Marine sediment	Mouth of Koksoak River	58°33'.133'	68°11'.097'	Sand/pebbles	Positive	7
KR-44	Marine sediment	Mouth of Koksoak River	58°29'.748'	68°10'.406'	Mud/sand	Positive	740
KR-15	Marine sediment	K. River, downstream of Kuujjuaq	58°10'.061'	68°20'.418'	Mud	Positive	200
KR-23	Marine sediment	K. River, downstream of Kuujjuaq	58°23'.024'	68°12'.876'	Mud	Positive	>5400
KR-24	Marine sediment	K. River, downstream of Kuujjuaq	58°14'.892'	68°18'.337'	Mud	Positive	150
KR-38	Marine sediment	K. River, downstream of Kuujjuaq	58°18'.257'	68°13'.731'	Mud	Positive	110
KR-46	Marine sediment	K. River, downstream of Kuujjuaq	58°15'.154'	68°14'.177'	Mud	Positive	90
KR-51	Marine sediment	K. River, downstream of Kuujjuaq	58°26'.192'	68°11'.709'	Mud	Positive	3100
KR-18	Freshwater sediment	Lake, east of mouth of K. River	58°29'.748'	68°10'.406'	Mud	Positive	15
KR-26	Freshwater sediment	Tributary, downstream of Kuujjuaq	58°09'.711'	68°17'.601'	Sand	Negative	NA[b]
KR-40	Freshwater sediment	Tributary, downstream of Kuujjuaq	58°09'.211'	68°20'.354'	Pebbles	Positive	40
KR-13	Freshwater sediment	Tributary upstream of Kuujjuaq	57°53'.036'	69°09'.049'	Sand	Positive	1400

Table A2 (Continued). Characteristics of sediment and terrestrial soils collected in the Koksoak River area

Sample ID	Region	Location	Latitude	Longitude	Substrate composition	Detection of spores	MPN count[a] (Number/kg)
KR-20	Freshwater sediment	Tributary upstream of Kuujjuaq	57°56'.027'	68°57'.828'	Sand/pebbles	Positive	130
KR-22	Freshwater sediment	Tributary upstream of Kuujjuaq	57°56'.027'	68°57'.828'	Sand	Positive	570
KR-37	Freshwater sediment	Tributary upstream of Kuujjuaq	57°53'.036'	69°09'.049'	Sand	Positive	40
K02-01-S	Freshwater sediment	Koksoak River, dock	RM[c]	RM	Sand/pebbles	Positive	7
K02-02-S	Freshwater sediment	Tributary upstream of Kuujjuaq	RM	RM	Sand	Positive	7
K02-03-S	Freshwater sediment	Tributary upstream of Kuujjuaq	RM	RM	Sand/pebbles	Positive	23
K02-04-S	Freshwater sediment	Tributary upstream of Kuujjuaq	RM	RM	Sand	Positive	46
K02-05-S	Freshwater sediment	K. River, upstream of Kuujjuaq	RM	RM	Sand	Positive	26
K02-06-S	Freshwater sediment	K. River, upstream of Kuujjuaq	RM	RM	Sand	Positive	46
K02-07-S	Freshwater sediment	K. River, upstream of Kuujjuaq	RM	RM	Sand	Positive	<6
K02-08-S	Freshwater sediment	Caniapiscau River	RM	RM	Sand	Positive	35
KR-25	Terrestrial soil	ca. 25 m from KR-20	57°56 .027	68°57 .828	Sand	Positive	3100

Table A2 (Continued). Characteristics of sediment and terrestrial soils collected in the Koksoak River area

Sample ID	Region	Location	Latitude	Longitude	Substrate composition	Detection of spores	MPN count[a] (Number/kg)
KR-27	Terrestrial soil	ca. 25 m from KR-22	57°56 .027	68°57 .828	Sand	Positive	90
KR-43	Terrestrial soil	ca. 25 m from KR-13	57°53 .036	69°09 .049	Sand	Positive	57
KR-50	Terrestrial soil	ca. 25 m from KR-37	57°53 .036	69°09 .049	Sand	Positive	1400
K02-02-T	Terrestrial soil	ca. 100 m from K02-02-S	RM	RM	Sand	Positive	5400
K02-03-T	Terrestrial soil	ca. 100 m from K02-03-S	RM	RM	Sand	Positive	20
K02-04-T	Terrestrial soil	ca. 100 m from K02-04-S	RM	RM	Sand	Positive	110
K02-05-T	Terrestrial soil	ca. 100 m from K02-05-S	RM	RM	Sand	Positive	30
K02-06-T	Terrestrial soil	ca. 100 m from K02-06-S	RM	RM	Sand	Positive	140
K02-07-T	Terrestrial soil	ca. 100 m from K02-07-S	RM	RM	Sand	Positive	86
K02-08-T	Terrestrial soil	ca. 100 m from K02-08-S	RM	RM	Sand	Positive	<6

[a]Based on mouse bioassay results.
[b]NA: not applied.
[c]Recorded on a 1:250,000 map.

Table A3. Field and laboratory quality control samples tested between batches of surface swabs, shoreline soil and seawater samples cultured for *Clostridium botulinum* type E

Sample ID	Sample type	Region	Village	Sample size	Spike (spores)	Enrichment broth cultivation techniques		
						UN-SPGYT[d]	Alc-SPGYT	H60-SPGYT
DL-260	Seawater	Hudson Bay	Puvirnituq	2 L	2000	Positive	Positive	Positive
BL-308	Shoreline soil	Hudson Bay	Puvirnituq	300-500 g	500	Positive	Positive	ND[a]
MJ-416	Rock surface	Hudson Bay	Puvirnituq	Sponge	500	Positive	Positive	ND
DL-267	Seawater	Hudson Strait	Kangiqsujuaq	2 L	2000	Positive	Positive	Positive
BL-318	Shoreline soil	Hudson Strait	Kangiqsujuaq	300-500 g	500	Positive	Positive	Positive
MJ431/432	Rock surface	Hudson Strait	Kangiqsujuaq	Sponge	500	Positive	Positive	ND
DL-281	Seawater	Ungava Bay	Kuujjuaq	2 L	2000	Positive	Positive	Positive
BL-331	Shoreline soil	Ungava Bay	Kuujjuaq	300-500 g	500	Positive	Positive	ND
MJ459/460	Rock surface	Ungava Bay	Kuujjuaq	Sponge	500	Positive	Positive	ND

Table A3 (Continued). Field and laboratory quality control samples tested between batches of surface swabs, shoreline soil and seawater samples cultured for *Clostridium botulinum* type E

Sample ID	Sample type	Sample size	Spike (spores)	Enrichment broth cultivation techniques		
				UN-SPGYT[c]	Alc-SPGYT	H60-SPGYT
Internal QCS	GPB[b]	100 mL	10	Positive	Positive	ND[a]
Internal QCS	GPB	100 mL	10	Positive	Positive	ND
Internal QCS	GPB	100 mL	10	Positive	Positive	ND
Internal QCS	GPB	100 mL	10	Positive	Positive	ND
Internal QCS	GPB	100 mL	10	Positive	Positive	ND
Internal QCS	GPB	100 mL	10	Positive	Positive	ND
Internal QCS	GPB	100 mL	10	Positive	Positive	ND
Internal QCS	GPB	100 mL	10	Positive	Positive	ND

[a]ND: not done.
[b]GPB: Gelatine-phosphate-buffer.
[c]SPGYT: special peptone-peptone-glucose-yeast extract plus trypsin.

Table A4. Laboratory quality control samples tested during the analysis of seal skin and intestinal contents cultured for *C. botulinum* type E

Sample ID	Sample type	Region / river	Village	Sample size	Spike (spores)	Enrichment broth cultivation techniques		
						UN-SPGYT[b]	Alc-SPGYT	H60-SPGYT
IK-24-02	Skin	Hudson Bay	Inukjuak	15.9-16.7 g	10	Negative	Negative	Positive
IK-15-02	Skin	Hudson Bay	Inukjuak	7.4-14.7 g	10	Positive	Positive	Positive
IK-15-02	Skin	Hudson Bay	Inukjuak	7.6-15.8 g	10	Positive	Positive	Positive
IK-15-02	Skin	Hudson Bay	Inukjuak	10.9-18.5 g	10	Positive	Positive	Positive
01SE-18[a]	Intestinal content	Hudson Strait	Kangiqsujuaq	5.9 g	10	Positive	Positive	Positive

[a]The intestinal content samples were cultured in cooked meat medium and not in SPGYT broth.
[b]SPGYT: special peptone-peptone-glucose-yeast extract plus trypsin.

B. ASSESSMENT OF SOURCES OF ENVIRONMENTAL CONTAMINATION OF SEAL *IGUNAQ* WITH *CLOSTRIDIUM BOTULINUM* SPORES

Questionnaire intended for seal igunaq makers

Background

Human botulism still sporadically affects people from Nunavik. The disease usually occurs following the ingestion of seal *igunaq* or seal misiraq contaminated with the botulism toxin. The sources of contamination of seal meat and blubber remain unknown. However, a study conducted in the coastline environment of Alaska reported that the bacteria producing the botulism toxin can be detected in seawater, beach soil, marine sediments, salmon gills and intestinal contents of marine mammals. The Nunavik Research Centre, in collaboration with the Nunavik Regional Board of Health and Social Services and the Botulism Reference Service of Health Canada, has begun a study to determine the distribution and levels of the bacteria in the Nunavik coastal environment where ringed seals and bearded seals are commonly butchered. The following questionnaire has been designed to determine if one or several of these sources of bacteria could be involved in the contamination of seal meat and seal blubber during butchering and preparation of seal products into *igunaq* and misiraq.

IDENTIFICATION OF INTERVIEWEE

Community: _____

Name of seal *igunaq* maker: _____

Address: _____

Phone number: _____

Age of the *igunaq* maker: _____

Years of experience: _____

Name of the hunter: _____
(if an elder does not hunt anymore)

Date of the interview: _____

Length of the interview: _____

SECTION A - GENERAL INFORMATION

1- What are the different types (seal, walrus, fish etc.) of *igunaq* you make?

2- What is the percentage of each type on an annual basis?

3- What is the percentage of ringed versus bearded seal *igunaq*?

4- In average, how many pails of *igunaq* you usually produce in a year?

5- In average, how many meals of *igunaq* you consume in a year?

1-5 6-10 11-20 21-30 31-40 41-50 more than 51

6- In your opinion, what is the percentage of the community that regularly eats seal *igunaq*?

7- Is seal *igunaq* consume by all age groups of the community?

Yes □ No □

If yes, what would be the proportion of children, teenagers, adults and elders from your community that consume regularly seal *igunaq*?

Comments on section A

SECTION B - CHARACTERISATION OF THE BUTCHERING SITES

1- Do you have a preferred site for butchering bearded and ringed seals?

Yes □ No □

If yes, what is the name and location of the site?

_____

_____

2- On what type of ground surface do you usually butcher ringed and bearded seals?

Flat rock □ Pebble beach □ Sandy beach □ Gravel beach □ Others □

_____

_____

3- Is the butchering site covered at high tide (below water line)?

Yes □ No □

4- Is the butchering site usually covered with one or more of these surface materials?

Soil □ Sand □ Small gravel □ Mud □ Slime □ Others □ (Buyak)

_____

_____

5- Is this particular site commonly used by other hunters?

Yes □ No □

Comments on section B

_____

_____

_____

_____

_____

_____

SECTION C - SOURCES OF CONTAMINATION DURING BUTCHERING

i) Contamination with beach soil

1- Do you usually put the meat and blubber temporarilly on the ground while waiting for a suitable container?

Yes □ No □

2- Could the meat or blubber become dirty with soil while butchering?

Yes □ No □

If yes, what is the extent (parts of animal)?

_____
_____
_____

3- Could the skin become dirty with soil when you pull the animal on the shore?

Yes □ No □

If yes, what is the extent (parts of animal)?

_____
_____
_____

4- Do you take any precautions or materials (e.g. tarp) to reduce contact of the carcass with beach soil?

_____
_____
_____

5- Could the meat and blubber become in direct contact with the inner surface of the boat?

Yes □ No □

If yes, could you describe the conditions?

_____
_____

ii) Contamination with seawater

1- Do you usually rinse the meat or carcass (without skin) in seawater after butchering?

Yes □ No □

If yes, what is the reason?

_____
_____
_____

2- Do you usually rinse the skin (hide) in seawater to wash off the dirt after butchering?

Yes □ No □

3- Do you usually rinse the flippers in seawater to wash off the dirt after butchering?

Yes □ No □

4- What type of container do you use to carry the meat and blubber?

_____
_____
_____

5- Do you usually clean the meat's container with seawater?

Yes □ No □

6- Do you put the rear and front flippers in the meat's container?

Yes □ No □

If no, how do you carry them?

_____
_____
_____

iii) Contamination with the intestinal contents

1- Could part of the intestinal contents fall into the carcass when you tear off the intestines from the anus?

Yes □ No □

If yes, do you wash off the intestinal contents from the carcass?

Yes □ No □

If yes, how do you wash the carcass?

_____
_____
_____

2- Do you usually wash your hands with seawater after you emptied the intestines?

Yes □ No □

3- Do you put the emptied intestines in the meat's container?

Yes □ No □

If no, how do you carry them?

_____
_____
_____

Comments on section C

_____
_____
_____
_____
_____
_____
_____
_____
_____

SECTION D - SOURCES OF CONTAMINATION DURING PREPARATION AND FERMENTATION OF *IGUNAQ*

1- Do you have a preferred site for ageing your *igunaq* preparation?

Yes □ No □

If yes, what is the location?

_____

_____

2- What is the type of container you usually use to make *igunaq*?

Puurtaq □ Plastic bag □ Plastic container □ Wooden box □ Others □

_____

_____

_____

3- Does the hide used to make *puurtaq* (skin bag) is usually rinsed in seawater?

Yes □ No □

4- Does the inside of the *puurtaq* remain wet when the meat and blubber are stuffed in?

Yes □ No □

5- Does the skin usually inverted so the hairs are inside the *puurtaq*?

Yes □ No □

6- Do you include the guts in the *igunaq* preparation?

Yes □ No □

If yes, are they pre-cooked?

Yes □ No □

7- What other parts of the animal do you use as ingredients?

8- How do you cover or protect your *igunaq* preparation from the environment?

Covered by rocks □ Under gravel beach □ Shed □ Others □

9- Does the surface water usually seep in the *puurtaq* when it is covered by rocks?

Yes □ No □

10- Does the underground water usually seep in the *puurtaq* when it is buried under gravel?

Yes □ No □

11- Do you usually observe the presence of soil particles during inspection of *igunaq*?

Yes □ No □

Comments on section D

SECTION E- DESCRIPTION OF *IGUNAQ* RECEIPIES AND EXPERIENCE OF *IGUNAQ* MAKERS

1- Would you participate in a study where we would test your *igunaq* preparation for botulism before and after the fermentation process?

Yes □ No □

2- What months of the year do you usually make seal *igunaq*?

3- What are the common ingredients?

4- What are the different types of containers?

5- What are the different covers or protections from the environment?

6- How long do you usually incubate the preparation?

7- Is there any air circulation inside the preparation?

8- What would be your estimation of the temperature inside the preparation?

9- How often do you monitor your *igunaq*?

10- When do you know the *igunaq* is ready to eat?

11- What are the signs of a bad preparation?

12- Have you ever thrown away a bad preparation?

Yes □ No □

If yes, what do you think was the cause of a bad preparation?

13- Have you ever had botulism?

Yes □ No □

If yes, were you hospitalised?

Yes □ No □

Was your *igunaq* the source of the sickness?

Yes □ No □

14- Have you used other *igunaq* preparation methods

Yes □ No □

If yes, was there a method you would **not** recommend?

15- How can we prevent botulism in Nunavik?

Comments on section E

RÉSUMÉ

Daniel Leclair, DVM, PhD
Veterinarian-Microbiologist

Areas of expertise: Food microbiology, zoonotic diseases and wildlife toxicology
Languages: French and English, both written and spoken

EDUCATION

2000-2008 **Doctorate in Microbiology and Immunology**, Faculty of Medicine, University of Ottawa.
Thesis title: **Molecular epidemiology and risk assessment of human botulism in the Canadian Arctic**

1992-1994 **Master of Science in Veterinary Pathology et Microbiology with specialisation in toxicology**, Faculty of Veterinary Medicine, Université de Montréal. Postgraduate scholarship - FCAR.
Thesis title: **Évaluation de la variation biologique des profils de porphyrines dans les excrétions urofécales de Canards de Pékin juvéniles par chromatographie liquide à haute performance**

1985-1989 **Doctorate of Veterinary Medicine,** Faculté de médecine vétérinaire, Université de Montréal. Member in good standing of the Ordre des médecins vétérinaires du Québec.

EMPLOYMENT

2006-present **Food Safety Division, Canadian Food Inspection Agency,** Ottawa
Antimicrobial Resistance Coordinator

- Coordination of the abattoir component of the Canadian Integrated Antimicrobial Surveillance Program
- Design and management of national microbiological baseline surveys in food-producing animals and meat products

2003-2006 **Food Directorate, Health Products and Food Branch, Health Canada,** Ottawa
Emergency Preparedness Coordinator

• Participate in the drafting and editing of policies, plans, procedures and other documents pertaining to emergency preparedness and response and business continuity planning for the Food Directorate;
• Participate as a core member in the activities of the chemical, biological, radiological, and nuclear (CBRN) Research and Technology Initiative laboratory Biological cluster for the Bureau of Microbial Hazards of the Food Directorate;
• Participate in the design, evaluation, observation or play of national and international tabletop exercises on deliberate contamination of the food supply by CBRN agents;
• Participate in various departmental and interdepartmental working groups and committees on emergency preparedness and response;
• Participate in various national and international workshops, forums and meetings pertaining to emergency preparedness and response against bioterrorism.
• Vice-Chairman of the Botulism Reference Service for Canada.

2000-2003 **Nunavik Research Centre,** Kuujjuaq (work conducted in the laboratories of Health Canada, Ottawa)
Project coordinator - Endemic foodborne botulism in Nunavik (Northern Quebec): a research initiative for risk assessment and risk management of human botulism in the Canadian Arctic

•Field surveys on the occurrence and levels of *Clostridium botulinum* type E in coastal and fluvial environments, and marine mammals harvested in Nunavik;
•Molecular subtyping of environmental, food and clinical isolates of *C. botulinum* group I and group II;
•Inoculation studies on *C. botulinum* type E in traditional and non-traditional seal meat preparations aged under different temperatures and atmosphere conditions;
•Qualitative risk analysis of human botulism from eating aged marine mammal meat.

1994-2000 **Nunavik Research Centre,** Kuujjuaq
Wildlife veterinarian

•Field and laboratory research on heavy metals and infectious diseases such as brucellosis, toxoplasmosis, and trichinellosis in traditional Inuit foods and in the aquatic environments of Nunavik;
•Management of the pathology lab as well as necropsy and tissue examination of fish, birds, terrestrial and marine mammals for disease investigations;
•Training and supervision of staff of the diagnostic service for the detection of *Trichinella* larvae in walrus meat for food safety;
•Active participation in the Nunavik rabies control program;

•Member of the Nunavik Nutrition and Health Committee which deals with public health issues related to nutrition, contaminants and infectious diseases.

1991-1994 **Consultant**

Project coordinator - Wildlife toxicology projects

•Medical monitoring of Lesser Scaup *(Aythya affinis)* exposed to PCBs from eating contaminated zebra mussels *(Dreissena polymorpha)* from Lake Erie and Montreal's harbour (McGill University).

•Inspections of ringed seal (*Phoca hispida*) and caribou (*Rangifer tarandus*) carcasses harvested by Inuit hunters from Umiujaq and Quaqtaq for disease investigation and toxicological studies (Nunavik Research Centre).

•Investigation into the effects of methylmercury exposure in a juvenile osprey (*Pandion haliaetus*) population from the La Grande Hydroelectric Complex by the analysis of urinary biomarkers (Université de Montréal).

•Feasibility study on the use of farmed mink (*Mustela vison*) as an animal model for the investigation of the effects of chronic methylmercury exposure on urinary biomarkers (Université de Montréal).

•Pilot study on the use of different methods for the study of the ecology and health of an osprey (*Pandion haliaetus*) population from La Grande Hydroelectric Complex exposed to methylmercury (Environment Canada).

1989-1990 **Montreal's and St-Hubert's exotic bird and animal hospitals**

Veterinarian in exotic medicine

•Medicine and surgery on small mammals, birds, reptiles et amphibians.

ACADEMIC EXPERIENCE

Leclair D. Infectious agents in country foods. Wildlife Diseases and Food Safety – Emerging Issues in 2001. Oral presentation given for a course at the Canadian Cooperative Wildlife Health Centre, University of Saskatoon, Saskatchewan, February 28-March 2, 2001.

Leclair D. Wildlife and drinking water. Wildlife Diseases and Food Safety – Emerging Issues in 2001. Oral presentation given for a course at the Canadian Cooperative Wildlife Health Centre, University of Saskatoon, Saskatchewan, February 28-March 2, 2001.

Lecturer – Clinical toxicology (MMV 2030, 1 credit). Université de Montréal, Faculté de médecine vétérinaire, 1994.

Assistant – Laboratory work in biochemistry (PAA 1140, 1 credit). Université de Montréal, Faculté de médecine vétérinaire, Saint-Hyacinthe, 1993.

SCIENTIFIC PUBLICATIONS

Dixon, B. R., L. J. Parrington, M. Parenteau, D. Leclair, M. Santín, and R. Fayer. 2008. *Giardia duodenalis* and *Cryptosporidium* spp. in the intestinal contents of ringed seals (*Phoca hispida*) and bearded seals (*Erignathus barbatus*) in Nunavik, Quebec, Canada. J. Parasitol. Accepted.

Dubey, J. P., T. Quirk, J. A. Pitt, N. Sundar, G. Velmurugan, O. C. H. Kwok, D. Leclair, R. Hill, and C. Su. 2008. Isolation and genetic characterization of *Toxoplasma gondii* from racoons (*Procyon lotor*), cats (*Felis domesticus*), striped skunk (*Mephitis mephitis*), black bear (*Ursus americanus*), and cougar (*Puma concolor*) from Canada. J. Parasitol. 94: 42-45.

Rodrigue J., L. Champoux, D. Leclair, and J.-F. Duchesne. 2007. Cadmium concentrations in tissues of willow ptarmigan (*Lagopus lagopus*) and rock ptarmigan (*Lagopus muta*) in Nunavik, Northern Québec. Environ. Pollut. 147: 642-647.

Kirkwood, J., A. Ghetler, J. Sedman, D. Leclair, F. Pagotto, J. W. Austin, and A. A. Ismail. 2006. Differentiation of group I and group II strains of *Clostridium botulinum* by focal plane array fourier transform infrared spectroscopy. J. Food Prot. 69: 2377-2383.

Leclair, D., F. Pagotto, J. M. Farber, B. Cadieux, B. Blanchfield, and J. W. Austin. 2006. Comparison of DNA fingerprinting methods for use in the investigation of type E botulism outbreaks in the Canadian Arctic. J. Clin. Microbiol. 44: 1635-1644.

Rodrigue J., R. McNicoll, D. Leclair, and J.-F. Duchesne. 2005. Lead concentrations in Ruffed Grouse, Rock Ptarmigan, and Willow Ptarmigan in Québec. Arch. Environ. Contam. Toxicol. 49: 97-104.

Leclair, D., L. B. Forbes, S. Suppa, J.-F. Proulx, and A. A. Gajadhar. 2004. A preliminary investigation on the infectivity of *Trichinella* larvae in traditional preparations of walrus meat. Parasitol. Res. 93: 507-509.

Leclair D., L. B. Forbes, S. Suppa, and A. A. Gajadhar. 2003. Evaluation of a digestion assay and determination of sample size and tissue for the reliable detection of *Trichinella* larvae in walrus meat. J. Vet. Diagn. Invest. 15: 180-183.

Lapointe J.-M., D. Leclair, C. Mesher, and A. Balachandran. 2002. Cross Canada Disease Report (Quebec) - Screening for chronic wasting disease in caribou in northern Quebec. Can. Vet. J. 43: 886-887.

Proulx J.-F., J. D. Maclean, T. W. Gyorkos, D. Leclair, A. K. Richter, B. Serhir, L. B. Forbes, and A. A. Gajadhar. 2002. Novel prevention program for trichinellosis in inuit communities. Clin. Infect. Dis. 34: 1508-1514.

Gall D., K. Nielsen, L. B. Forbes, W. Cook, D. Leclair, S. Balsevicius, L. Kelly, P. Smith, and M. Mallory. 2001. Evaluation of the fluorescence polarization assay and comparison to other serological assays for detection of brucellosis in cervids. J. Wild. Dis. 37: 110-118.

Mikaelian I., D. Leclair, and J. Inukpuk. 2001. Adenocarcinoma of the small intestine in a ringed seal from Hudson Bay. J. Wild. Dis. 37: 379-382.

St-Louis R., S. de Mora, E. Pelletier, D. W. Doidge, D. Leclair, I. Mikaelian, and D. Martineau. 1999. Hepatic butyltin concentrations in beluga whales (*Delphinapterus leucas*) from the St. Lawrence estuary and Northern Quebec, Canada. Appl. Organo. Chem. 14: 218-226.

Proulx J.-F., D. Leclair, and S. Suppa. 1998. Preventing trichinosis in Nunavik. Makivik Magazine 48: 18-22.

Chamberland G., N. Larivière, D. Bélanger, D. Leclair, L. Vermette, and J-S. Blais. 1995. Improved method for the extraction and quantitative determination of porphyrins in tissues, urine and feces. Toxicol. Environ. Chem. 48: 183-194.

TECHNICAL REPORTS

Leclair D. 2005. Molecular epidemiology and risk assessment of human botulism in the Canadian Arctic. Progress Report No.4., Nunavik Research Centre, Makivik Corporation, Kuujjuaq, Quebec. 12 p.

Leclair D., L. B. Forbes, S. Suppa, and A. A. Gajadhar. 2003. Evaluation of a sampling procedure and digestion assay for the detection of *Trichinella* larvae in walrus meat. Report QUE-009-STK, Nunavik Research Centre, Makivik Corporation, Kuujjuaq, Quebec. 23 p.

Leclair D., L. B. Forbes, S. Suppa, and A. A. Gajadhar. 2002. Infectivity of *Trichinella* larvae in aged walrus meat. Report 12-355, Nunavik Research Centre, Makivik Corporation, Kuujjuaq, Quebec. 24 p.

Leclair D. 2002. *Clostridium botulinum* in the arctic environment. Progress Report No.2., Nunavik Research Centre, Makivik Corporation, Kuujjuaq, Quebec. 11 p.

Leclair D., and D. W. Doidge. 2001. Seroprevalence survey for *Toxoplasma gondii* in arctic wildlife from Nunavik. Progress Report 12-349, Nunavik Research Centre, Makivik Corporation, Kuujjuaq, Quebec. 4 p.

Leclair D. 2001. Occurrence of *Clostridium botulinum* spores in the Nunavik coastline environment. Progress Report 12-397A, Nunavik Research Centre, Makivik Corporation, Kuujjuaq, Quebec. 8 p.

Leclair D. 2001. Assessment of the baseline levels of metals in the aquatic environment of Crater Lake, Deception, Puvirnituq and Vachon rivers. Report 12-331, Nunavik Research Centre, Kuujjuaq, Quebec 77 p.

de Mora S. J., R. St-Louis, D. W. Doidge, D. Leclair, E. Pelletier, I. Mikaelian, and D. Martineau. 1999. Butyltin contamination in Beluga Whales (*Delphinapterus leucas*) from the St. Lawrence Estuary and Northern Quebec. Synopsis of research conducted under the 1998/99 Northern Contaminants Program, Indian and Northern Affairs Canada.

Doidge D. W., D. Leclair, and G. Berthe. 1997. Investigation of the bacteriological status of Quugaapik at the North edge of Umiujaq town site. Nunavik Research Centre, Makivik Corporation, Kuujjuaq, Quebec.

Leclair D., and D. W. Doidge. 1997. Investigation of brucellosis in harvested seals in Nunavik. Report 12-285, Nunavik Research Centre, Makivik Coporation, Kuujjuaq, Quebec.

Leclair D., and D. W. Doidge. 1995. Detection of *Trichinella* larva in walrus meat harvested by the Sallumiut in fall 1994. Kuujjuaq Research Centre, Makivik Corporation, Kuujjuaq, Quebec.

Leclair D. 1993. Étude exploratoire sur l'application de méthodes de cueillette de renseignements sur l'écologie et la santé d'une population d'aiglons balbuzards (*Pandion haliaetus*) nichant dans le secteur ouest du Complexe hydroélectrique de la Grande. Wildlife Canadian Service, Environment Canada.

POSTER AND ORAL PRESENTATIONS

Leclair D., J. W. Austin, J. Farber, B. Cadieux, and B. Blanchfield. Toxicity of aged seal meat challenged with *Clostridium botulinum* type E. Poster presented at the Federal Food Safety and Nutrition Research Meeting, Ottawa, Ontario. Octobre 4-5, 2004.

Leclair D., J. W. Austin, J. Farber, B. Cadieux, F. Pagotto, and B. Blanchfield. Comparison of ribotyping, RAPD and PFGE for the subtyping of *Clostridium botulinum* group I and group II strains. Poster presented at the Annual Poster Day of the Biochemistry,

Microbiology, and Immunology Department of the University of Ottawa, Ottawa, April 23, 2003.

Leclair D., J. W. Austin, J. Farber, B. Cadieux, F. Pagotto, and B. Blanchfield. Foodborne botulism in Nunavik: Epidemiology, ecology and risk factors. Oral presentation given at the seminar series of the Bureau of Microbial Hazards of Health Canada, Ottawa, Ontario, March 12, 2003.

Parenteau M., L. Parrington, D. Leclair, J. Bussey, and B. Dixon. Simultaneous detection of *Giardia* cysts and *Cryptosporidium* oocysts in harvested seals from Nunavik, Canada, by dual-colour flow cytometry. Poster presented at the Health Canada Research Forum: From Science to Policy, Ottawa, Ontario, November 18-19, 2002.

Leclair D., J. W. Austin, J. Farber, B. Cadieux, and B. Blanchfield. Use of thiourea for subtyping untypeable *Clostridium botulinum* type E strains by pulsed-field gel electrophoresis. Poster presented at the Federal Food Safety and Nutrition Research Meeting, Guelph, Ontario, September 15-17, 2002.

Parenteau M., L. Parrington, D. Leclair, J. Bussey, and B. Dixon. Development of a dual colour flow cytometric method for the simultaneous detection of *Giardia* cysts and *Cryptosporidium* oocysts. Poster presented at the Federal Food Safety and Nutrition Research Meeting, Guelph, Ontario, September 15-17, 2002.

Leclair D. Risk assessment of human trichinellosis from eating marine mammal meat in Nunavik, Canada. Oral presentation given at the seminar series of the Bureau of Microbial Hazards of Health Canada, Ottawa, Ontario, May 16, 2002.

Leclair D., J. W. Austin, B. Blanchfield, J. Farber, B. Cadieux, and S Suppa. Distribution of *Clostridium botulinum* type E in northern Quebec. Poster presented at the World of Microbes, Xth International Congress of Bacteriology and Applied Microbiology, Paris, France, July 27- August 1, 2002.

Leclair D., J. P. Dubey, D. W. Doidge, S. Suppa, C. Mesher, and P. May. Serological monitoring of *Toxoplasma gondii* in the George River and Leaf River caribou herds. Poster presented at the 9th North American Caribou Workshop, Kuujjuaq, Quebec, April 23-27, 2001.

Leclair D., J. W. Austin, B. Blanchfied, and J. Farber. Molecular epidemiology of foodborne botulism in Nunavik, Canada. Poster presented at the 44th annual meeting of the Canadian Federation of Biological Societies, Ottawa, Ontario, June 20-23, 2001.

Forbes L. B., D. Leclair, A. A. Gajadhar and W. B. Scandrett. *Trichinella* larvae in walrus meat, as significant food safety hazard. Oral presentation given at the World Association for the Advancement of Veterinary Parasitology, Stresa, Italy, August 26-30, 2001.

Proulx J.-F., D. Leclair, and S. Gordon. Trichinellosis and its prevention in Nunavik, Quebec, Canada. Poster presented at the 11th International Congress on Circumpolar Health, Harstad, Norway, June 4-9, 2000.

Forbes L. B., D. Leclair, and J.-F. Proulx. A diagnostic system to identify walrus meat infected with *Trichinella* larvae. Poster presented at the World Association for the Advancement of Veterinary Parasitology, Copenhagen, Denmark, August 15-19, 1999.

Forbes L. B., D. Leclair, G. Falk, and A. A. Gajadhar. Characterization of *Trichinella* larvae recovered from walruses in the Canadian arctic. Oral presentation given at the 43 Meeting of the American Association of Veterinary Parasitologists, Baltimore, Maryland, July 25-28, 1998.