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*CLOSTRIDIUM BOTULINUM* IN HONEY PRODUCTION  
WITH RESPECT TO INFANT BOTULISM

Mari Nevas

ACADEMIC DISSERTATION

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## ANTILLE JA VILJALLE

Kypsyminen ja kasvaminen on sitä,  
että opin liikkumaan  
onnen ja epäonnen vaihtumisen tahdissa.  
Kypsyminen on  
alati toistuvaa liikettä  
helposta vaikeaan,  
ylhäältä alas,  
eteenpäin ja taas takaisin.  
En halua luovuttaa  
kun asiat eivät suju,  
enkä leijua ulottumattomiin  
kun olen onnellinen.

Kypsyminen tarkoittaa,  
että odotan itseltäni kaikkea  
mihin kykenen,  
että panen liikkeelle kaikki voimavarani  
saavuttaakseni sen.  
En enempää enkä vähempää.

Ulrich Schaffer

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## ABBREVIATIONS

<b>AFLP</b>	amplified fragment length polymorphism
<b>AP-PCR</b>	arbitrarily primed polymerase chain reaction
<b>ATCC</b>	American Type Culture Collection
<b>a<sub>w</sub></b>	water activity
<b>BoNT</b>	botulinum neurotoxin
<b>BTH</b>	black tar heroin
<b>CBI</b>	<i>Clostridium botulinum</i> isolation agar
<b>CDAD</b>	<i>Clostridium difficile</i> associated diarrhoea
<b>CI</b>	confidence interval
<b>DNA</b>	deoxyribonucleic acid
<b>dNTP</b>	deoxynucleotide triphosphate
<b>ELISA</b>	enzyme-linked immunosorbent assay
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>ES</b>	EDTA-sodium lauroyl sarcosine buffer
<b>ESP</b>	EDTA-sodium lauroyl sarcosine proteinase K solution
<b>EYA</b>	egg yolk agar
<b>GBS</b>	Guillain-Barré syndrome
<b>HMF</b>	hydroxymethylfurfural
<b>HPLC</b>	high-performance liquid chromatography
<b>MLD<sub>50</sub></b>	mouse 50% lethal dose
<b>MPN</b>	most probable number
<b>mRNA</b>	messenger ribonucleic acid
<b>NCFA</b>	Nordic Committee on Food Analysis
<b>NCTC</b>	National Collection of Type Cultures
<b>OR</b>	odds ratio
<b>PCR</b>	polymerase chain reaction
<b>PFGE</b>	pulsed-field gel electrophoresis
<b>PIV</b>	Tris-NaCl buffer
<b>RAPD</b>	randomly amplified polymorphic DNA
<b>Rep-PCR</b>	repetitive element sequence-based PCR
<b>RFLP</b>	restriction fragment length polymorphism
<b>RT-PCR</b>	reverse transcription PCR
<b>SF</b>	supernatant filtration
<b>SIDS</b>	sudden infant death syndrome
<b>SNARE</b>	soluble N-ethylmaleimide-sensitive factor attachment protein receptors
<b>TE</b>	Tris-HCl, EDTA buffer
<b>TPGY</b>	tryptone-peptone-glucose-yeast extract broth
<b>UPGMA</b>	Unweighted Pair-Grouping Method of Averages



## ABSTRACT

The autopsy specimens of a suddenly deceased 11-week-old boy were studied with multiplex polymerase chain reaction (PCR), and *Clostridium botulinum* group I type B was detected in the intestinal contents and in the vacuum cleaner dust of the patient's household. Genetic similarity of these isolates was demonstrated with pulsed-field gel electrophoresis (PFGE) typing and randomly amplified polymorphic DNA (RAPD) analysis, confirming that dust can act as a vehicle for infant botulism, resulting in sudden death. This was the first reported case of infant botulism in Finland.

To evaluate the prevalence of *C. botulinum* in honey, a test protocol for reliable PCR-based detection of spores was developed. The inhibiting effects of honey were overcome by using a supernatant filtration method of in the preparation of samples before enrichment and PCR.

PFGE analysis was optimized to study *C. botulinum* group I isolates, and applied to analyse the diversity of 55 strains of *C. botulinum* types A, AB, B and F. A total of nine restriction enzymes were tested, from which *SacII*, *SmaI* and *XhoI* were chosen for the cluster analysis of the 55 strains. Of these three enzymes, *SacII* was shown to be superior for analysis of *C. botulinum* group I isolates. *C. botulinum* group I was found to be heterogeneous, and in the majority of cases, PFGE enabled discrimination between individual strains of types A and B. However, certain clustering of type B strains was observed, and type F strains were located in a single cluster.

A total of 190 samples from honey sold in Finland were studied by PCR to determine whether *C. botulinum* spores of types A and B were present. Spores of *C. botulinum* were detected in 8 of the 114 Finnish (7%) and in 12 of the 76 imported honey samples (16%). In addition, 294 honey samples produced in Denmark, Norway and Sweden were studied using multiplex-PCR for the presence of *C. botulinum* type A, B, E and F spores. The prevalence of *C. botulinum* showed a significant variation between Denmark, Norway and Sweden, the proportions of positive samples in each country being 26%, 10% and 2%, respectively. Type B was most frequently detected. The 24 strains isolated were confirmed to be proteolytic and were analysed with PFGE using restriction enzyme *SacII*. Twelve different PFGE patterns were produced. Three clearly distinguishable clusters containing more than three strains were noted, one of which included strains isolated in both from Denmark and Norway.

Factors influencing *C. botulinum* contamination in the honey production environment were evaluated in a three-year survey. A total of 1168 samples from 100 apiaries and related facilities in Finland were analysed for the presence of *C. botulinum* types A, B, E and F using multiplex-PCR. Production methods and environmental factors were registered using a questionnaire and personal observation. *C. botulinum* was shown to

be common throughout the whole honey production chain, with the type most frequently detected being group I type B. A PFGE analysis of 202 group I type B isolates suggested the existence of different genetic lineages. Only six PFGE profiles were observed, two of which predominated, covering 96% of the isolates. The high prevalence of *C. botulinum* in soil and in samples associated with beeswax suggests the accumulation of soil-derived botulinal spores in wax. In addition, based on the most significant bivariate Spearman's rank order correlations, production hygiene and extent of production were shown to have significant influences on the prevalence of botulinal spores in extracted honey. According to a logistic multivariable regression model, the presence of hand-washing facilities in the extraction room had a significant impact on decreasing the number of spores.

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by Roman numerals I to V:

- I Nevas, M., Lindström, M., Virtanen, A., Hielm, S., Kuusi, M., Arnon, S.S., Vuori, E., and Korkeala, H. 2005. Infant botulism acquired from household dust presenting as sudden infant death syndrome. *J Clin Microbiol* 43: 511-513.
- II Nevas, M., Hielm, S., Lindström, M., Horn, H., Koivulehto, K., and Korkeala, H. 2002. High prevalence of *Clostridium botulinum* types A and B in honey samples detected by polymerase chain reaction. *Int J Food Microbiol* 72: 45-52.
- III Nevas, M., Lindström, M., Hielm, S., Björkroth, K.J., Peck, M.W., and Korkeala, H. 2005. Diversity of proteolytic *Clostridium botulinum* strains, determined by a pulsed-field gel electrophoresis approach. *Appl Environ Microbiol* 71: 1311-1317.
- IV Nevas, M., Lindström, M., Hautamäki, K., Puoskari, S., and Korkeala, H. 2005. Prevalence and diversity of *Clostridium botulinum* types A, B, E and F in honey produced in the Nordic countries. *Int J Food Microbiol* 105: 145-151.
- V Nevas, M., Lindström, M., Hörman, A., Keto-Timonen, R., and Korkeala, H. 2006. Contamination routes of *Clostridium botulinum* in the honey production environment. *Environ Microbiol* In press.

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# 1. INTRODUCTION

Nearly two hundred years ago, German physician and poet Justinus Kerner described the symptoms of a fatal illness, "sausage poisoning", caused by inadequately boiled sausages (Kerner 1820). Seventy-five years later, the cause of the illness was discovered by Emilie Pierre Marie van Ermengem to be a toxin produced by a bacterium named *Bacillus botulinus* (van Ermengem 1897). The bacterium, later renamed *Clostridium botulinum*, is anaerobic and able to produce the most potent biological toxin known, botulinum neurotoxin, which causes the paralyzing illness botulism. The classical form of botulism, foodborne botulism, follows the ingestion of food containing preformed neurotoxin. In the 20<sup>th</sup> century, three other manifestations of botulism have been recognized: wound botulism, infant botulism and intestinal botulism (Shapiro *et al.* 1988). Next was the discovery of iatrogenic botulism, which is the result of an adverse reaction following injections of botulinum toxin for therapeutic or cosmetic purposes (Bakheit *et al.* 1997, Tugnoli *et al.* 2002, Ferreira *et al.* 2004b).

Infant botulism was not diagnosed until 1976 (Midura and Arnon 1976, Pickett *et al.* 1976), but it is the main form of botulism in the United States today (CDC 2004). Unlike foodborne botulism, infant botulism is an infectious disease affecting only infants younger than one year. Due to the immaturity of intestinal microflora, the spores carried into the intestinal canal may germinate and produce toxin *in vivo*. The course of the illness can vary from a prolonged constipation to sudden death of an infant, but several child-dependent predisposing factors are known to influence susceptibility to infection (Arnon *et al.* 1981, 1982). The most frequent causes of infant botulism are *C. botulinum* group I types A and B (Midura 1996). Infant botulism is a rare disease; however, the number of cases may be underestimated due to a variety of applicable differential diagnoses such as sepsis, different neurological disorders and sudden infant death syndrome (SIDS) (Johnson *et al.* 1979, Midura 1996). Thus far, no cases of infant botulism have been reported in Finland, and the possible implication of *C. botulinum* in the Finnish cases of SIDS has not been evaluated.

Honey was the only food item associated with infant botulism until 2005, when a suspected case of infant milk formula-derived infant botulism was described (Brett *et al.* 2005b). Health authorities in several countries have mandated or advised the use of labels on honey jars stating that honey should not be fed to infants of less than one year of age. However, about 50% of the reported infant botulism cases in Europe are still related to feeding honey to infants (Aureli *et al.* 2002). In the United States, the number of cases due to honey feeding has been significantly decreased to less than 10% as a result of public awareness (Schechter 1999).

*C. botulinum* is ubiquitous in nature, and honey has also been shown to occasionally contain *C. botulinum* spores (Sugiyama *et al.* 1978, Huhtanen *et al.* 1981, Nakano *et al.* 1990, Criseo *et al.* 1993). Spores do not germinate or produce toxin in honey, but they may be present for an indefinite period of time, as a heating process efficient enough to destroy the spores would inevitably degrade the taste and structure of honey. Although *C. botulinum* is known to be present in honey produced in several countries, the lack of data on contamination routes of *C. botulinum* spores in honey results in no prevention measures being available to decrease the level of spores during honey production.

No studies on *C. botulinum* in Finnish honey or honey imported to Finland have been conducted, and thus, no information on the possible risk for infant botulism posed by honey in Finland exists. To analyse a large number of honey samples, molecular detection techniques, such as PCR, should be optimized for this sample type, permitting a more rapid and cost-effective analysis than the mouse bioassay. In addition, to compare the genetic diversity of *C. botulinum* group I isolates related to honey production, an efficient typing method for group I strains should be developed and the overall diversity of group I strains evaluated. The data on genetic diversity of *C. botulinum* isolates could be applied to uncover the contamination routes of *C. botulinum* in the honey production environment.

## 2. REVIEW OF THE LITERATURE

### 2.1 *Clostridium botulinum*

#### 2.1.1 Classification and characteristics

*C. botulinum* is a Gram-positive, anaerobic, rod-shaped bacterium of 0.5-2.4 µm in width and 1.6-22.0 µm in length, with oval, subterminal spores that usually cause the cell to swell (Cato *et al.* 1986). The most unique feature of *C. botulinum* strains is the production of botulinum neurotoxin (BoNT), one of the most potent naturally occurring toxins known.

Seven serotypes (A to G) of *C. botulinum* are recognized on the basis of the different antigenic properties of their respective toxins. *C. botulinum* strains are divided into four distinct phenotypic groups (I-IV) based on biochemical properties (Table 1). Strains of group I are proteolytic types A, B and F, with an optimal growth temperature near human body temperature. Their growth below 10°C is unlikely (Smelt and Haas 1978). Group II strains of types B, E and F do not produce proteolytic enzymes and are psychrotrophic (Lynt *et al.* 1982, Hatheway 1993). Group III organisms, types C and D, have variable proteolytic activity, whereas group IV type G strains are proteolytic and differ from the other types by a lack of lipase production on egg yolk-containing agar plates. Type G has been re-designated *Clostridium argentinense*. The strains causing the majority of human botulism cases belong to groups I and II, while group III strains are the most frequent causes of botulism in animals. In addition to *C. botulinum* strains, *Clostridium butyricum* and *Clostridium baratii* are known to produce BoNT types E and F, respectively (Hall *et al.* 1985, McCroskey *et al.* 1986, Suen *et al.* 1988, Paisley *et al.* 1995, Fencia *et al.* 2002).

Table 1. Characterization of *Clostridium botulinum* groups I-IV.

Property	Group			
	I	II	III	IV
Neurotoxin type	A, B, F	B, E, F	C, D	G
Optimal growth temperature	35-40°C	18-25°C	40°C	37°C
Proteolysis	+	-	-/(+)	+
Lipase production	+	+	+	-
Spore heat resistance	1.23/112°C <sup>a</sup>	0.6-1.25/ 80°C <sup>a</sup>	0.1-0.9/ 104°C <sup>a</sup>	0.8-1.12/ 104°C <sup>a</sup>

<sup>a</sup> Example of the D-value at a certain temperature.

### 2.1.2 Botulinum neurotoxin

All botulinum neurotoxins (BoNTs) are dichain peptide molecules with a molecular mass of approximately 150 kDa. The molecule is initially produced in the bacterial cytosol as an inactive single polypeptide, which is released after bacterial lysis (Verastegui *et al.* 2002). The released molecule, called progenitor toxin, is composed of neurotoxin and a non-toxic component with or without hemagglutinin activity (Oguma *et al.* 1999). The inactive polypeptide is cleaved by endogenous or exogenous peptidases to produce two polypeptide chains, a heavy chain (H-chain, 100 kDa) and a light chain (L-chain, 50 kDa). The two chains are linked by a disulphide bond, the integrity of which is essential for the internalization and thus toxicity of the molecule (Sugiyama 1980, de Paiva *et al.* 1993). Genes coding BoNTs are localized either in chromosome; types A, B, E and F, in phage DNA; types C<sub>1</sub> and D (Eklund *et al.* 1971, Inoue and Iida 1971, Eklund *et al.* 1972) or in plasmid; type G (Zhou *et al.* 1995). In the case of *C. botulinum* types C<sub>1</sub> and D, the type of toxin (C<sub>1</sub> or D) is not determined by the host cell, but by phages, which contain the structural genes for the toxin (Oguma *et al.* 1973).

Type C strains have been hypothesized to produce toxin type C<sub>1</sub>, toxin type C<sub>2</sub> or toxin type D, and type D strains are thought to produce both type C and D toxins (Sugiyama 1980). Of these, toxin type C<sub>2</sub> is a cytotoxin differing from the neurotoxins produced by *C. botulinum* (Ohishi *et al.* 1980). However, Oguma *et al.* (1980, 1981) have shown that there is a homology among type C<sub>1</sub> and D toxins that is sufficient to allow a partial neutralization of both toxins by a serum with antigens against only one of the toxins. Hence, type C and D strains have been postulated to produce only one type of neurotoxin, depending on the infecting phage (Oguma *et al.* 1997).

Several reports introduce strains that are dual toxin producers: type AB (Fujinaga *et al.* 1995), type Ab (Franciosa *et al.* 1997, Kobayashi *et al.* 2003), type Af (Gimenez and Ciccarelli 1978, Fernández *et al.* 1986), type Ba (Hatheway *et al.* 1981) and type Bf (Hatheway and McCroskey 1987, 1989, Smith *et al.* 1989, Barash and Arnon 2004). Capitalized letters refer to predominating toxin types. The presence of unexpressed toxin genes has also been reported (Franciosa *et al.* 1994, 2004a). Within the same serotype, strain-dependent differences in the potency of the toxin produced have been observed (Hatheway *et al.* 1981, Kozaki *et al.* 1998). With types C and D, the strain dependent differences are partly due to the toxin production being controlled by bacteriophages (Oguma and Iida 1979).

### 2.1.3 Prevalence in the environment

Spores of *C. botulinum* are widely distributed in soil and sediment, as reported in several surveys (Table 2). The overall prevalence of *C. botulinum* in soil and sediment is 20%, according to the data presented in Table 2. According to previous surveys, types A and B seem to predominate in soil, with a particularly high prevalence of type A in samples from Argentina (Ciccarelli and Gimenez 1981, Lúquez *et al.* 2005). Types C and E prevail in samples from aquatic environments (Table 2). Type G is the most rarely detected, having been reported only in the soils of Argentina (Ciccarelli and Gimenez 1981, Lúquez *et al.* 2005) and Switzerland (Sonnabend *et al.* 1987).

## 2.2 Detection, quantification and typing of *Clostridium botulinum*

### 2.2.1 Conventional methods for detection and isolation of *Clostridium botulinum*

In describing the difficulty of isolating *C. botulinum*, the following remark by Smith (1977) still applies: “Primarily, this difficulty (of isolation and toxin identification) arises because no single procedure, no single medium is best for strains of all toxin types and cultural varieties of *C. botulinum*. Consequently, the procedures adopted and the media used represent compromises.” The anaerobic nature of *C. botulinum* sets further demands on laboratory work with this bacterium, and the presence of non-toxigenic *C. botulinum*-like strains disturbs the culture of *C. botulinum* (Lindström and Korkeala 2006).

The most widely used media for enrichment of *C. botulinum* are non-selective broths, such as Robertson’s medium, other broths containing meat extract or chopped meat (NCFA 1991b, CDC 1998), or tryptone-peptone-glucose-yeast extract (TPGY) broth (Lilly *et al.* 1971, BAM 1998). The cultures are then streaked on a non-selective agar, such as blood agar (NCFA 1991b), egg yolk agar (EYA) (Dowell *et al.* 1977, Hatheway and McCroskey 1987) or modified McClung-Toabe EYA plates (CDC 1998). The enrichment broths should be incubated at 35-37°C for types A, B, C, D and F, or at 30°C for type E (NCFA 1991b). On blood agar plates, *C. botulinum* colonies are irregular, 1-6 mm in diameter and surrounded by  $\beta$ -haemolysis (Cato *et al.* 1986). A lipase reaction, typical of *C. botulinum* groups I and II, is observed on egg yolk-containing agars. When the analysis is directed towards the detection of spores, heat treatment of the enrichment broths should be applied to kill vegetative cells of other bacteria prior to incubation (CDC 1998). Alcohol treatment may also be used to select the spores (Koransky *et al.* 1978), especially when isolating less heat-resistant group II strains.



Table 2. Prevalence of *Clostridium botulinum* in soil of different countries.

Country	Sample	No. of positive samples/Total no. of samples (%)	Type and number of <i>C. botulinum</i> detected <sup>a</sup>	Reference
Argentina	Soil	244/722 (34)	A (159), B (47), F(11), Af(17), G(2), NS <sup>b</sup> (22)	Ciccarelli and Gimenez 1981
Argentina	Soil	472/2009 (23)	A(282), B(87), F(20), G(2), Af(17), NS(78)	Lúquez <i>et al.</i> 2005
Bangladesh	Aquatic sediment	5/12 (42)	CD(2), NS(7)	Huss 1980
Canada	Wetlands	124/326 (38)	C(124)	Wobeser <i>et al.</i> 1987
China	Soil	592/5901 (10)	A(254), B(289), C(20), D(26), E(41), F(2)	Gao <i>et al.</i> 1990
China	Coastal sediment	38/1405 (3)	A(2), C(18), D(12), E(5), F(1)	Gao <i>et al.</i> 1990
Costa Rica	Soil	7/30 (23)	A(1), B(1), C(5)	del Mar Gamboa <i>et al.</i> 1993
Czechoslovakia	Mud	11/26 (42)	C(11)	Neubauer <i>et al.</i> 1988
Denmark	Pond sediment	94/330 (28)	E(94)	Huss <i>et al.</i> 1974
Denmark	Marine sediment	194/212 (92)	E(194)	Huss 1980
Denmark	Soil	9/43 (21)	B(14), CD(1), NS(3)	Huss 1980
Falkland Islands	Sediment	1/3 (33)	B(1)	Smith <i>et al.</i> 1987
Faroe Islands	Aquatic sediment	2/63 (3)	E(2)	Huss 1980
Faroe Islands	Soil	0/35	ND <sup>c</sup>	Huss 1980
Finland	Aquatic sediment	85/125 (68)	E(85)	Hielm <i>et al.</i> 1998b
Finland and Baltic Sea	Marine sediment	78/110 (71)	E(78)	Hielm <i>et al.</i> 1998c
France	Sediment	1/25 (4)	E(1)	Fach <i>et al.</i> 2002
France	Mud	4/46 (9)	B(1), E(3)	Smith <i>et al.</i> 1977
Greenland	Marine sediment	18/21 (86)	E(31), NS(8)	Huss 1980
Iceland	Aquatic sediment	2/60 (3)	A(1), E(1)	Huss 1980
Iceland	Soil	1/40 (3)	B(1)	Huss 1980
Indonesian Waters	Sediment	11/592 (2)	A(2), B(4), C(2), D(3)	Suhadi <i>et al.</i> 1981
Italy	Cultivated and pasture soil	7/520 (1)	A(6), B(1)	Creti <i>et al.</i> 1990
Japan	Marine sediment	3/26 (12)	C(1), NS(2)	Venkateswaran <i>et al.</i> 1989
Japan	River soil	60/98 (61)	B(7), C(9), E(53)	Yamakawa and Nakamura 1992
Kenya	Soil	3/12 (25)	A(2), C(1)	Yamakawa <i>et al.</i> 1990b
Paraguay	Soil	4/17 (24)	A(1), C(1), F(2)	Yamakawa <i>et al.</i> 1990a

Table 2. Continued.

Country	Sample	No. of positive samples/Total no. of samples (%)	Type and number of <i>C. botulinum</i> detected <sup>a</sup>	Reference
South Orkney Islands	Aquatic sediment	0/43	ND	Smith <i>et al.</i> 1987
Spain	Wetland	1/58 (2)	ND	Contreras de Vera <i>et al.</i> 1991
Switzerland	Soil	18/41 (44)	A(5), B(15), C(1), F(1), G(5)	Sonnabend <i>et al.</i> 1987
Thailand	Coastal sediment	12/762 (2)	D(10), E(2)	Tanasugarn 1979
United Kingdom	Marine sediment	15/429 (3)	B(15)	Cann <i>et al.</i> 1968
United Kingdom	Lake/river mud	50/69 (72)	B(31), C(12), D(1), E(10), ND(2)	Smith and Moryson 1975
United Kingdom	Soil	1/25 (4)	B(1)	Smith and Moryson 1977
United Kingdom	Soil	15/60 (25)	B(9), C(3), D(3), E(1)	Smith and Milligan 1979
United Kingdom	Soil	10/174 (6)	B(6)	Smith and Young 1980
United Kingdom	Landfill sites	76/182 (42)	B(15), C(18), D(30), E(1), ND(12)	Ortiz and Smith 1994
United States	Soil	61/260 (23)	A(26), B(22), C(3), D(5), E or F(6) <sup>d</sup>	Smith 1978
United States	Pond sediment	26/467 (6)	C(26)	Marion <i>et al.</i> 1983
United States	Marine sediment	23/328 (7)	A(3), B(1), C(6), D(2), E(11)	Ward <i>et al.</i> 1967a
United States	Wetland sediment	16/18 (89)	C(16)	Williamson <i>et al.</i> 1999
United States	Wetland sediment	1144/2200 (52)	C(1144)	Sandler <i>et al.</i> 1993
United States	Marine sediment	12/335 (4)	A(1), B(2), C(3), D(2), E(4)	Ward <i>et al.</i> 1967b
United States, Alaska	Sediment of beach soil	68/154 (44)	E(44)	Miller 1975
United States, Alaska	Non-coastal soil	7/115 (6)	E(7)	Miller 1975
Zambia	Soil	3/46 (7)	B(1), D(2)	Karasawa <i>et al.</i> 2000

<sup>a</sup>More than one type may have been detected in the sample.

<sup>b</sup>NS, not specified. The toxin types were not possible to determine due to a low amount of toxicity.

<sup>c</sup>ND, not detected.

<sup>d</sup>Types E and F could not be distinguished from one another.

Group II strains have been reported to be sensitive to sulphamethoxazole-trimethoprim (Swenson *et al.* 1980), and thus, *Clostridium botulinum* isolation (CBI) agar, using cycloserine, sulphamethoxazole and trimethoprim as selective inhibitory agents, may be used to detect group I strains, and has been applied for isolating the bacteria from faeces (Dezfulian *et al.* 1981). However, certain group II type F and one type E strain were shown to grow on CBI medium (Dezfulian *et al.* 1981). By adding botulinal antibodies of types A, B or F into a medium containing the aforementioned antibiotic substances, Silas *et al.* (1985) introduced an agar differentiating between the three serotypes. However, cross-reactivity was noted, and thus, the method is not reliable for determining toxin type.

CBI and EYA media have been evaluated in direct plating of faecal samples to confirm infant botulism, and both media can be used to investigate stool suspensions of suspected cases of infant botulism, the success rate being higher for CBI than for EYA (Glasby and Hatheway 1985). CBI medium has also been further modified to a botulinum-selective medium (BSM). BSM agar contains similar amounts of the same inhibitory agents, but these are added to a simplified base, with only dehydrated heart infusion broth and thymidine phosphorylase in addition to egg yolk and agar, thus diminishing the cost of the medium (Mills *et al.* 1985). BSM agar was shown to provide better suppression of infant faecal flora and more rapid appearance of lipase-positive colonies (Mills *et al.* 1985).

The detection and isolation of *C. botulinum* in soil are complicated by many microbes, such as other *Clostridium* species, *Bacillus* spp. or *Streptococcus* spp. (Smith 1975, Sandler *et al.* 1998), which may inhibit growth and toxin production of *C. botulinum*. Competitive inhibition is also known to occur between different *C. botulinum* types and strains (Eklund *et al.* 2004).

### **2.2.2 PCR-based detection of *Clostridium botulinum***

Due to *C. botulinum* usually being present in low numbers in naturally contaminated samples and forming highly resistant spores in aerobic conditions, PCR detection directly from sample material often fails and enrichment is required to obtain reliable results (Lindström and Korkeala 2006). To detect the *C. botulinum* serotypes most often associated with human botulism, several PCR methods based on the detection of one of the *bot* genes using specific oligonucleotide primers have been described for groups I and II (Fach *et al.* 1993, 1995, Ferreira *et al.* 1993, 1994, Szabo *et al.* 1993, Franciosa *et al.* 1994, Takeshi *et al.* 1996, Alsallami and Kotlowski 2001, Kimura *et al.* 2001, Craven *et al.* 2002). To decrease the detection time, Craven *et al.* (2002) developed primers for group I and II strains with similar amplification conditions, thus allowing the use of a single thermal cycler, although the serotypes are detected separately. The efficacy of detection was further enhanced by multiplex-PCR, by which the gene fragments of group I and II strains are

detected simultaneously in the same reaction mixture (Lindström *et al.* 2001). Applications of these PCR methods have been utilized in several studies to analyse clinical (Lindström *et al.* 2004), environmental (Szabo *et al.* 1994, Hielm *et al.* 1996), food (Fach *et al.* 1993, Szabo *et al.* 1994, Hyytiä *et al.* 1998, Mäde *et al.* 2000, Kimura *et al.* 2001) and faecal samples (Szabo *et al.* 1994, Dahlenborg *et al.* 2001).

Using degenerate primers for group I, II and IV strains (Aranda *et al.* 1997, Campbell *et al.* 1993, Fach *et al.* 1995), the presence of types A, B, E, F or G may be detected in a single PCR run, but with no discrimination of type. By using specific probes and performing a Southern or slot blot hybridization of the amplified products, the organism may be further detected at the serotype level (Campbell *et al.* 1993, Fach *et al.* 1995). PCR detection with degenerate primers may also be accompanied by high-performance liquid chromatography (HPLC) to determine the serotype according to the different peak profiles observed (Franciosa *et al.* 2004b).

Nested-PCR involves two sets of primers, used in two successive PCR runs. Compared with ordinary PCR, nested-PCR enhances the sensitivity and specificity of the detection, but is more time-consuming. Nested-PCR has been applied to detect *C. botulinum* type B in a case of infant botulism (Kakinuma *et al.* 1997) and types B, E and F from swine faecal samples, with types E and F being detected using the same set of primers (Dahlenborg *et al.* 2001).

A quantitative application of reverse transcription-PCR (RT-PCR) has been used to measure the level of toxin-encoding mRNA in *C. botulinum* types E (McGrath *et al.* 2000), and B (Lövenklev *et al.* 2004b), and to evaluate the effect of food preservatives on bacterial toxigenesis of *C. botulinum* types E (Sharkey *et al.* 2004) and B (Lövenklev *et al.* 2004a).

In addition to methods for detecting the typical causes of human botulism, PCR protocols for detecting types C and/or D (Szabo *et al.* 1993, Fach *et al.* 1996, Franciosa *et al.* 1996, Williamson *et al.* 1999, Nol *et al.* 2004) and type G (Fach *et al.* 1995) have also been developed.

### **2.2.3 Quantification of *Clostridium botulinum***

Enumeration of *C. botulinum* may be performed conventionally as a plate count (Hauschild and Hilsheimer 1977, Glasby and Hatheway 1985) or by the most probable number (MPN) method, which may be interpreted visually, with mouse bioassay or with PCR (Hielm *et al.* 1996). However, when using a non-selective enrichment medium, visual interpretation is limited to analysing pure cultures. MPN is based on subdividing a sample and detecting growth of a certain organism in one or more tubes in a series of tubes containing a known amount of sample material or sample dilutions. Unlike bacterial plate count, MPN provides

only an estimate of the bacterial count, and tends to yield a higher result than plate count (Peeler *et al.* 1992).

DNA-based methods have also been used for quantification of *C. botulinum*. Quantitative real-time PCR has been applied for analysis of type E (Kimura *et al.* 2001) and type A toxin genes in food (Yoon *et al.* 2005), and types A, B and E in samples associated with botulism cases (Akbulut *et al.* 2004).

#### **2.2.4 DNA-based typing of *Clostridium botulinum***

Genotyping of *C. botulinum*, to compare the genetic diversity of different strains provides a useful tool in epidemiological analysis. The basis of molecular typing is to produce and visualize a DNA fragment profile, a bacterial fingerprint, representing either the whole bacterial genome or specific sequences of the genome.

In pulsed-field gel electrophoresis (PFGE), the whole bacterial genome is cut using restriction enzymes. The method is applied to analyse *C. botulinum* group II strains (Hielm *et al.* 1998a, 1998b, Korkeala *et al.* 1998, Hyytiä *et al.* 1999b, Lindström *et al.* 2004), a few strains of group I type A (Lin and Johnson 1995, Franciosa *et al.* 2004a) and type A strains with a *botB* gene either expressed (type Ab) or unexpressed [type A(B)] (Franciosa *et al.* 2004a). However, the PFGE method has not been optimized for a large number of group I strains, and thus, no data are available on the genetic diversity of group I. PFGE provides a powerful tool for epidemiological analysis of botulism cases, as shown in previous studies (Korkeala *et al.* 1998, Lindström *et al.* 2004, Johnson *et al.* 2005).

Restriction fragment length polymorphism (RFPL) analysis has been used to differentiate several *Clostridial* strains, including group II *C. botulinum* types B and E (Broda *et al.* 2000) and type A (Franciosa *et al.* 2004a). In these applications, either PCR-amplified 16S rDNA gene (Broda *et al.* 2000) or PCR-amplified *botA* gene (Franciosa *et al.* 2004a) has been digested with restriction endonucleases.

Randomly amplified polymorphic DNA (RAPD) analysis, also referred to as arbitrarily primed PCR (AP-PCR), is based on using a single universal primer that hybridizes at multiple locations in the genome. Due to the random nature of the priming and amplification, the reproducibility is generally not high. RAPD has been applied for typing certain *C. botulinum* group I (Hyytiä *et al.* 1999a, Franciosa *et al.* 2004a) and group II strains (Hyytiä *et al.* 1999a). Hyytiä *et al.* (1999a) reported a higher discrimination of a few type E strains using RAPD than previous results obtained with PFGE (Hielm *et al.* 1998a), but overall the fingerprint profiles generated by PFGE were more discriminating and easier to interpret (Hyytiä *et al.* 1999a, 1999b). Franciosa *et al.* (2004a) observed a lower discriminatory power of RAPD compared with PFGE. Based on the results obtained with RAPD, the genetic diversity among group I strains is small (Hyytiä *et al.* 1999a). Due

to its low reproducibility and difficulties in interpreting results, RAPD might not be the best alternative for studying the genetic variability of a large number of strains. It may, however, be used in confirming results of epidemiological analysis, as the typeability of the analysed strains has been reported to be 100% (Hyytiä *et al.* 1999a, 1999b, Franciosa *et al.* 2004a). Unlike in RAPD, in repetitive element sequence-based PCR (rep-PCR), the primers are designed to hybridize with known conserved sequences found throughout the genome. However, the discriminatory power of rep-PCR is inferior to that of RAPD or PFGE (Hyytiä *et al.* 1999a).

Ribotyping, involving both restriction enzymes and specific hybridization probes, has been applied to analyse both group I and group II strains, and has proven useful for group- or species-level identification, but not for discriminating isolates at strain level (Hielm *et al.* 1999, Skinner *et al.* 2000).

The most recently applied method for analysis of *C. botulinum* group I and group II strains, amplified fragment length polymorphism (AFLP), combines the advantages of restriction enzyme digestion and selective amplification of fragments. AFLP provides high discrimination of *C. botulinum* and is thus suitable for typing at strain level (Keto-Timonen *et al.* 2005).

## 2.3 Detection of BoNT

### 2.3.1 Mouse bioassay

The method for detecting BoNT should provide a very high sensitivity, as toxin levels adequate to cause intoxication are extremely low. The only accepted standard method for BoNT detection is the mouse bioassay (Kautter and Solomon 1977, NCFA 1991a, CDC 1998). In this assay, mice are injected intraperitoneally with a sample eluate, which in case of suspected non-proteolytic strain is supplemented with trypsin. Mice are monitored for up to four days for the presence of typical symptoms of botulism, including muscle weakness and respiratory failure. When symptoms are noted, the mice are euthanized (NCFA 1991a). To determine the toxin type, neutralizing antibodies are injected with the toxic sample. Mice receiving the appropriate type of antitoxin remain asymptomatic (NCFA 1991a, CDC 1998).

The possibility of measuring toxin activity by local muscular paralysis was first described by Sugiyama *et al.* (1975). The characteristic flaccid paralysis of the muscle was observed after an intramuscular injection of a sublethal dose of type A toxin (Sugiyama *et al.* 1975). Two decades later, a non-lethal alternative to the mouse assay was developed by Sesardic *et al.* (1996), based on symptoms of paralysis of abdominal muscles after subcutaneous injection of a sublethal dose of toxin type A into the inguocrural region.

The conventional mouse assay has not, however, been replaced with the non-lethal assay as the standard method.

Several problems are associated with the mouse bioassay; in addition to being ethically debatable, it is time-consuming, laborious and expensive, requiring specially trained personnel and appropriate facilities for the animals.

### **2.3.2 Immunological methods**

In seeking alternatives to the mouse bioassay, several immunological methods have been developed for the detection of BoNTs. Despite the rapidity of these tests compared with the mouse bioassay, their capacity to replace the mouse assay is limited due to their inability to distinguish biologically active toxin from inactive toxin (Ekong 2000). The most widely used immunoassay is enzyme-linked immunosorbent assay (ELISA), which has a detection limit of approximately 10-100 mouse 50% lethal doses (MLD<sub>50</sub>) (Notermans *et al.* 1978, 1979, 1982, Shone *et al.* 1985, Goodnough *et al.* 1993, Poli *et al.* 2002, Ferreira *et al.* 2003, 2004a, Guglielmo-Virét *et al.* 2005). Similar sensitivity, but more rapid performance has been achieved with electrochemiluminescence (ECL) immunoassay for type B toxin (Guglielmo-Viret *et al.* 2005). The sensitivity of ELISA has been further enhanced using an immuno-PCR assay for type A (Wu *et al.* 2001) or PCR-ELISA for types A, B, E and F (Fach *et al.* 2002). A combination of ELISA and enzyme-linked coagulation assay (ELCA) has also proven to reach the sensitivity levels of the mouse assay (Doellgast *et al.* 1994), but due to its complexity it is not a viable alternative in routine analysis. In addition, a slot blot immunoassay, developed by Cadieux *et al.* (2005) for the detection of toxin type E in enrichment cultures, achieved a sensitivity of approximately 4 MLD<sub>50</sub>. ELISA-based applications have been used to analyse clinical (Dezfulian *et al.* 1984, Poli *et al.* 2002) and food samples (Shone *et al.* 1985, Ferreira *et al.* 2004a), and to study therapeutic preparations of type A toxin (Ekong *et al.* 1995). However, no systematic validation of any ELISA-based methods for detection of BoNT in clinical materials has been reported (Lindström and Korkeala 2006).

Commercial lateral-flow toxin detection kits to detect toxin types A, B and E are available and have been evaluated for their suitability in detecting toxins in foods (Sharma *et al.* 2005). However, the use of these tests is limited to presumptive analysis due to their relatively low sensitivity (Hörman *et al.* 2005, Sharma *et al.* 2005).

### **2.3.3 Endopeptidase assay**

The endopeptidase assay is based on the highly specific activity of BoNTs to cleave target peptides in presynaptic nerve endings. It resembles the mouse bioassay in that only

biologically active toxin is detected (Wictome *et al.* 1999a). The endopeptidase assay could thus provide a highly sensitive future alternative to the mouse bioassay as a standard method for toxin detection. A method combining ELISA with endopeptidase assay to detect type B neurotoxin in foods has been shown to be capable of detecting toxin at a concentration of 0.5 MLD<sub>50</sub> within 5 to 6 hours (Wictome *et al.* 1999a), and it has also proven to be efficient in detecting toxin in different food material (Wictome *et al.* 1999b). There is still a possibility for obtaining false-negative results, as discussed by Wictome *et al.* (1999b). Moreover, a mass spectrometric assay may be utilized to analyse the product peptides derived from endopeptidase activities of BoNT in order to detect BoNTs of types A, B, E and F (Barr *et al.* 2005) or A to G (Boyer *et al.* 2005). No cross-reactions have been observed between the different BoNTs, and multiplex analysis is allowed. The reported sensitivities have varied between 0.039 and 0.625 MLD<sub>50</sub> (Barr *et al.* 2005, Boyer *et al.* 2005).

## 2.4 Botulism

### 2.4.1 Pathogenesis and symptoms

Human botulism is most typically caused by *C. botulinum* groups I and II. Toxic doses for humans, estimated from the doses given to monkeys, are very low, i.e. approximately 1 ng/kg body weight for parenterally given toxin types A and B (Gill 1982). Type C has been reported to have caused botulism in a single case of infant botulism (Oguma *et al.* 1990), but no reports exist on *C. botulinum* type D intoxication in man. One study describes the isolation of type G organism and its toxin from human autopsy specimens (Sonnabend *et al.* 1981); however, no confirmation of clinical botulism type G in humans or in animals has been published. Varying ability of the different serotypes to cause disease may depend on their ability to cross gut epithelial cells; toxins of serotypes A and B seem to cross the gut epithelial cells more efficiently than type C toxin (Simpson 2004). Speculation has centred around whether differences in binding on the surface of nerve endings have an effect on the efficacy of C, D and G toxins (Black and Dolly 1986a, 1986b), and according to a recent survey, the properties of the receptor for BoNT/D do seem to differ from those of the other BoNTs (Tsukamoto *et al.* 2005), which may affect its pathogenicity in humans. In *In vitro* studies, type C BoNT has been shown to produce a blockade of neuromuscular transmission in human muscle, whereas type D did not affect muscle function (Coffield *et al.* 1997).

The pathogenesis of *C. botulinum* toxin is similar regardless of the route of exposure. Progenitor toxin ingested with foods passes through the stomach and is absorbed from the upper part of the small intestine. Survival through the stomach is dependent on the



non-toxic components, which make the progenitor toxin highly resistant to pepsin and to the low pH of gastric juice (Ohishi *et al.* 1977). The non-toxic components may also play a role in absorption of the toxin from the small intestine (Oguma *et al.* 1999). The dissociation of progenitor toxin is supposed to take place in lymphatic vessels, and the toxin reaches target cells via lymphatic and blood vessels (Oguma *et al.* 1999). At neuromuscular junctions, the H-chain binds the molecule to the specific receptors on the membranes of nerve endings. Thereafter, the L-chain causes neurotoxic effects inside the neuronal cell by acting as a zinc-dependent endoprotease to cleave components of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex in the nerve cell, thus blocking the acetylcholine-containing vesicles from fusing with the terminal membrane of the motor neuron (Montecucco and Schiavo 1994, Schiavo *et al.* 2000, Simpson 2004). Acetylcholine release is inhibited in a dose-dependent way, and the blocking results in symptoms often referred to as the 4Ds: dysphagia, dry mouth, diplopia, dysarthria (Hughes *et al.* 1981). These symptoms appear symmetrically, and the ocular effects, which include blurred vision, ptosis and double vision, are usually the first to be noticed (Konig *et al.* 1975, Cherington 1998). In foodborne botulism, the symptoms may be preceded by gastrointestinal symptoms such as vomiting and constipation (Hatheway 1995). According to Varma *et al.* (2004), based on 706 botulism patients studied, the most common symptoms on admission to hospital were fatigue (90% of patients), muscle weakness (89%) and difficulty in swallowing (81%), whereas the predominant findings on physical examination were ophthalmoplegia, ptosis and slurred speech. Because BoNT does not penetrate the blood-brain barrier, the symptoms are limited to peripheral cholinergic nerve endings.

Recovery from botulism begins with formation of a network of new nerve sprouts, which are responsible for regeneration of muscle contractions (de Paiva *et al.* 1999). In a later stage of the recovery, the functional ability of the original nerve terminal is restored and the new nerve sprouts are eliminated (de Paiva *et al.* 1999).

Because of the high potency of BoNT, the amount of toxin adequate to cause symptoms of botulism is lower than the amount of toxin needed to induce an immune response, and thus, repeated botulism in a patient can occur (Beller and Middaugh 1990). However, antibodies to BoNT type A have been reported to develop in patients treated with botulinum toxin for dystonia (Jankovic and Schwarz 1991).

#### **2.4.2 Forms of the disease**

Four different forms of botulism have been recognized; foodborne, wound, infant and intestinal (Shapiro *et al.* 1998). The oldest form is foodborne intoxication, which occurs following ingestion of food containing preformed neurotoxin. Because it was originally

associated with food poisoning from meat products, the bacterium received the name *botulinum*, derived from *botulus*, the Latin word for sausage (Erbguth and Naumann 1999). Foodborne botulism is the most common form of botulism worldwide (Hatheway 1995, Aureli *et al.* 1999, Brett 1999, Galazka and Przybylska 1999, Kuusi *et al.* 1999, Therre 1999, Varma *et al.* 2004). The formation of toxin results from contamination of food with *C. botulinum* spores, which in the case of the highly heat-resistant spores of group I are able to germinate and produce toxin in inadequately processed foods especially home-made foods (Hauschild 1993). Group II strains pose a safety risk mainly in industrial foods, which are processed with mild heat treatments, allowing the survival of less heat-resistant spores (Lindström *et al.* 2006). Hermetic sealing ensures extended shelf-lives of products, but also enables the growth of botulinum spores. As the use of salt and other preservatives is limited, the microbiological safety relies on refrigerated storage, the temperature of which is often above the reported minimal growth temperature for *C. botulinum* group II (Lindström *et al.* 2006). The main factors limiting growth and toxin formation of *C. botulinum* in foods are temperature, pH, water activity, redox potential, food preservatives and competitive microflora (Kim and Foegeding 1993). After ingesting a relatively large amount of toxin, the symptoms may develop within a few hours, but the typical incubation time varies from 12 to 71 h (Arnon *et al.* 2001). In the intestinal lumen, BoNT binding sites are distributed across the apical surface of intestinal epithelial cells, and after endocytosis the toxin is transferred across the cell, being released on the basolateral surface. This occurs within approximately 20-30 min (Ahsan *et al.* 2005).

Wound botulism was first described in 1951 (Davis *et al.* 1951), although the potential of *C. botulinum* to cause botulism as a consequence of a wound infection was discussed as early as in the 1920s (Edmonson *et al.* 1920, Starin *et al.* 1925). Wound botulism results from BoNT production *in vivo*, after colonization of injured tissue with *C. botulinum*. As few as 10 spores of *C. botulinum* type A are able to induce wound botulism in mice (Dezfulian and Bartlett 1985). Wound botulism has been associated with wounds from trauma, surgery, injection drug use or sinusitis resulting from cocaine sniffing (CDC 1998). *C. botulinum* type A is the most common type in infections (Weber *et al.* 1993, Werner *et al.* 2000). During the last few years the number of wound botulism cases has increased drastically due to drug users injecting mainly black tar heroin (BTH) subcutaneously or intramuscularly (Werner *et al.* 2000, Brett *et al.* 2004, 2005a). BTH is typically heated in a spoon to dissolve the heroin in a weak solution of citric acid (Brett *et al.* 2004). Repeated injections of citric acid decreases the aerobicity of the muscle (Brett *et al.* 2004), and heating may cause the activation of spores carried in heroin (Werner *et al.* 2000, Brett *et al.* 2004).

Differing from foodborne intoxication, infant botulism is an infection resulting from ingestion of spores of *C. botulinum*, which may germinate, grow and form neurotoxin in the

intestinal lumen (Arnon 1980). A more detailed description of infant botulism is given in section 2.5. In rare cases, intestinal colonization and toxin production have been reported to occur in adults or in children older than one year (McCroskey and Hatheway 1988, Kobayashi *et al.* 2003).

In addition to the four recognized forms of botulism, a fifth manifestation of botulism, iatrogenic botulism, has recently been noted. Iatrogenic botulism is a rare consequence of injections of botulinum toxin for therapeutic or cosmetic purposes (Bakheit *et al.* 1997, Tugnoli *et al.* 2002, Ferreira *et al.* 2004b). Both oral and inhalation poisonings due to BoNT also appear to be possible (Park and Simpson 2003), although only one report exists of this kind of intoxication (Holzer 1962).

## 2.5 Infant botulism

### 2.5.1 Epidemiology and risk factors

The case definition of infant botulism used in the United States specifies infant botulism as an illness, consistent with the known paralysing action of botulinum toxin, in which *C. botulinum* toxin and/or organisms are identified in serum or faecal specimens (CDC 1997). Infant botulism, first described in late 1976 as a distinct clinical entity, is an infectious disease, caused by the absorption of botulinum toxin produced in the intestinal lumen of infants less than one year of age (Midura and Arnon 1976, Pickett *et al.* 1976). However, instead of being a new disease, there is evidence that already in 1931 a case of infant botulism was misdiagnosed as encephalitis (Arnon *et al.* 1979b). Based on the severity of the illness, infant botulism may be divided into three groups as follows: (1) mild, outpatient illness “failure to thrive”, (2) paralysis, needing hospitalization and (3) sudden unexpected death of an infant (Arnon *et al.* 1982).

The most typical age of onset of infant botulism is between two weeks and six months, and the median age is 10 or 12 weeks (Arnon *et al.* 1981, Morris *et al.* 1983, Aureli *et al.* 2002). Thilo *et al.* (1993) reported onset at an age of six days, but according to a recent report, in the earliest case of infant botulism the symptoms began at 38 hours of age (Barash *et al.* 2005).

The food item first identified as a source of *C. botulinum* spores was honey (Arnon *et al.* 1979a), and this remained the only food item associated with infant botulism cases until 2005, when a possible link to infant formula milk was reported in the UK (Brett *et al.* 2005b, Johnson *et al.* 2005). However, as samples from the infant's home were not analysed, it could not be ascertained that the formula had not been contaminated from the environment (Brett *et al.* 2005b).

Besides honey, dust is another significant vehicle for infant botulism (Arnon *et al.* 1979a). Disruption of the surface soil, a dusty or windy environment or a recent construction site may contribute to exposure to the spores, as discussed by Thompson *et al.* (1980).

Of the possible risk factors for infant botulism, breast-feeding has been the most widely debated. In several surveys, among hospitalized patients the number of breast-fed infants has been shown to be higher than the number of formula-fed infants (Arnon *et al.* 1982, Morris *et al.* 1983, Spika *et al.* 1989), thus giving the impression that breast feeding might predispose to infant botulism. However, the protective effect of breast feeding was noted by Arnon *et al.* (1982), who observed that the hospitalized breast-fed patients were significantly older ( $13.8 \pm 6.7$  weeks) than the formula-fed patients ( $7.6 \pm 2.9$  weeks), and that human milk protects from the most fulminant form of infant botulism; there were no sudden infant death syndrome (SIDS) -like cases of infant botulism among the breast-fed infants. Mothers' colostrum has been shown to contain antibodies to *C. botulinum* (Arnon *et al.* 1982), and differences in the intestinal microflora of infants depend mainly on the diet; the intestinal microflora of formula-fed infants contains significantly more *Clostridia* than that of breast-fed infants (Benno *et al.* 1984). Differences in intestinal microflora may induce more rapid germination and toxin production in formula-fed infants. Sibship order has a significant association with acquisition of the illness, as fewer first-born children acquire infant botulism (Arnon *et al.* 1981), but gender seems to have no impact on developing the infection (Arnon *et al.* 1981, CDC 2002, 2004).

A report on a possible "asymptomatic carrier" (Thompson *et al.* 1980) has been questioned by researchers who claim that based on studies of hundreds of samples *C. botulinum* is not part of the normal intestinal microflora of infants (Arnon *et al.* 1981, Stark and Lee 1982a, 1982b, Murrell *et al.* 1993). Since the possibility of asymptomatic carriers is small, and, as discussed by Hatheway and McCroskey (1987), the coincidence between the carrier state and a neuroparalytic illness mimicking botulism is likely rare, the chance of a false-positive confirmation of *C. botulinum* being detected in intestinal or faecal specimens would be slight.

Although very rare, foodborne botulism has been shown to be a possible cause of botulism in infants (Armada *et al.* 2003). In this case, the weakness is rapidly progressive. The proper diagnosis is needed to be able to withdraw the toxic food from the market and to prevent the possible spread of an epidemic of botulism.

### **2.5.2 Toxin types associated with infant botulism cases**

The majority of infant botulism cases are caused by *C. botulinum* group I types A and B (Midura 1996, CDC 2002, 2004), and the existence of both types in one patient has also

been documented (Douglas *et al.* 1980, Hatheway and McCroskey 1987). The high incidence of group I strains is presumably due to their optimal growth temperature being near human body temperature. However, all toxin types except type D have been detected or isolated from infants showing infant botulism-like symptoms. In four cases, the causative *C. botulinum* strain was discovered to be dual toxin-producer type BF, three of these cases occurring in the United States (Hatheway and McCroskey 1987, 1989, Barash and Arnon 2004) and one in the United Kingdom (Smith *et al.* 1989). The first and thus far the only case of infant botulism caused by *C. botulinum* type C was reported in Japan (Oguma *et al.* 1990). Although *C. botulinum* type G has not been fully established as a cause of either human or animal disease, Sonnabend *et al.* (1981) reported the occurrence of this type in intestines and type G toxin in the serum of an infant who had died unexpectedly and of unknown cause. Sonnabend *et al.* (1985) also detected organisms and toxins of types B, C and F in the intestinal samples of several suddenly deceased children.

Five cases of infant botulism caused by BoNT E-producing *Clostridium butyricum* have been reported in 1984-2002 in Italy (McCroskey *et al.* 1986, Fencia *et al.* 2002). The first described type F infant botulism case (Hoffman *et al.* 1982) was later discovered to be caused by *Clostridium baratii* (Hall *et al.* 1985), and BoNT F-producing *C. baratii* has also apparently been the causative organism in other cases of infant botulism (Paisley *et al.* 1995, Barash *et al.* 2005, Keet *et al.* 2005).

### **2.5.3 Clinical symptoms and diagnostics**

Epidemiological and clinical information suggests that the incubation period of infant botulism is less than one month (Spika *et al.* 1989). The spectrum of clinical symptoms is wide. The main clinical features include constipation, listlessness, lethargy, difficulty in sucking and swallowing, weak cry, general muscle weakness, and loss of head control (Midura 1996). In about half of the cases, respiratory failure is noted due to upper airway flaccidity and obstruction, aspiration or respiratory paralysis (Schechter 1999). The first sign is often constipation, which is defined as three or more days without defecation (Arnon and Chin 1979), and, as a consequence, one non-specific clinical feature is ileus (Kothare and Kassner 1995). However, the absence of constipation does not exclude infant botulism in an infant presenting with hypotonia (Rick *et al.* 1999, Mitchell and Tseng-Ong 2005). Infant botulism has even been diagnosed simultaneously with *Clostridium difficile*-associated diarrhoea (CDAD) (Schechter 1999, Fencia *et al.* 2002), and as discussed by Schechter (1999), the colonic stasis caused by *C. botulinum* multiplication could increase an infant's susceptibility to, as well as the severity of, CDAD. The role of constipation in the course of illness has also been questioned; does it result from the illness or should the slow intestinal motility be considered a risk factor for the disease (Rick *et al.* 1999).

According to Arnon (1992), sepsis is the most common admission diagnosis for patients with infant botulism. Another differential diagnosis is disorders causing hypotonia, such as Guillain-Barré syndrome (GBS), which is the most frequent cause of acute flaccid paralysis (Jones 2000). Brain stem encephalitis, meningitis, pneumonia, hypothyroidism, myasthenia gravis, failure to thrive, hypotonia of unknown aetiology, poliomyelitis and disorders in amino acid metabolism are other diagnoses applied to patients whose illness is later established to be infant botulism (Johnson *et al.* 1979, Midura 1996).

Botulinum toxin is more commonly found in the faeces than in the serum of infant botulism patients (Midura 1979). The first report of detection of botulinum toxin in serum dates back to 1978 (Alexander *et al.* 1978). The toxin type most frequently detected in serum seems to be type A. In a survey of infant botulism in Argentina, type A botulinum toxin was detected in sera from 29 of 46 patients (63%) (Fernández *et al.* 1999), and, as reported by Hatheway and McCroskey (1987), type A toxin was detected in 8 of 22 (36%) and type B toxin in only 1 of 43 (2.3%) serum samples in infant botulism cases; however, twice as many infants with type B were tested than infants with type A. In an infant botulism case in Germany, type A toxin was detected in serum (Müller-Bunke *et al.* 2000). The reported levels of toxin detected in faeces are approximately 60 000 MLD<sub>50</sub>, for both type A and type B (Paton *et al.* 1982, 1983).

The most probable site of infection is the large intestine, and therefore, detection of *C. botulinum* in faeces is often possible in infant botulism cases (Mills and Arnon 1987). *C. botulinum* may multiply in the intestinal lumen, reaching levels as high as  $1.3 \times 10^6$  to  $6.0 \times 10^8$  colony-forming units in faeces (Wilcke *et al.* 1980, Paton *et al.* 1982, 1983, Takahashi *et al.* 1988). *C. botulinum* type B has been detected in an infant's faecal samples collected within one week of the patient being transferred to hospital, using nested-PCR. The method, applied without an enrichment culture, was able to detect the toxin gene on the seventh day of the hospital stay but not later (Kakinuma *et al.* 1997). ELISA has also been used to detect type A and B toxins in faecal specimens of infant botulism patients. However, a significant cross-reactivity between type A and B antitoxins has been noted (Dezfulian *et al.* 1984).

Electrodiagnostic tests have been utilized in the diagnostics of infant botulism, and the typical electrodiagnostic triad is composed of low-amplitude compound muscle action potentials, tetanic or post-tetanic facilitation and absence of post-tetanic exhaustion (Gutierrez *et al.* 1994). However, false-negative findings have also been reported due to the special problems posed during the neonatal period and infancy, e.g. the difficulty in evaluating the morphology of the motor unit potentials (Graf *et al.* 1992, Sheth *et al.* 1999).

#### 2.5.4 Treatment and prevention

The treatment of infant botulism is supportive, including comprehensive respiratory and nutritional support until spontaneous recovery occurs. When intensive care facilities are available, the prognosis is usually excellent (Schreiner *et al.* 1991). Although rare, recurrence of infant botulism is sometimes observed (Schreiner *et al.* 1991, Ravid *et al.* 2000). The clinical condition of the patient may be markedly improved even before the level of toxin reaches the maximum (Paton *et al.* 1982). Due to the sepsis-like symptoms, infants are often treated with antibiotics, including aminoglycosides, which may further potentiate the neurotoxic effect of the botulinum toxin (Krishna and Puri 2001). In addition to the potentiating effect of aminoglycoside antibiotics, antibiotic treatment can enhance the quantity of toxin released in the intestinal lumen upon vegetative cell death and lysis. Antibiotic therapy is thus not part of the treatment protocol for uncomplicated infant botulism (Midura 1996).

Human botulism immunoglobulin treatment of hospitalized patients has been shown to significantly shorten the hospital stay, and is now accepted for use in infant botulism cases (Arnon *et al.* 2003, Thompson *et al.* 2005). Prior to the development of human immunoglobulin, no suitable antitoxin treatment was available, as the equine-derived immunoglobulin was not recommended for treating infant botulism due to possible hypersensitivity reactions (Black and Gunn 1980, Fox *et al.* 2005).

To prevent of infant botulism, immunization of the mother by vaccination might be an alternative. However, this should be done before pregnancy (Brent 2003).

#### 2.5.5 Occurrence

The first cases of infant botulism were reported in the United States (Midura and Arnon 1976, Pickett *et al.* 1976) and this is the most common form of botulism in the US today (CDC 2004), the annual incidence being two cases per 100 000 live births (Reddy *et al.* 2003). In 2003, infant botulism represented 81 of the 130 reported cases of botulism intoxications (CDC 2004). Infant botulism has also been described on four other continents: Asia, Australia, Europe and South America.

The first case of infant botulism outside the US took place in 1978 in England (Turner *et al.* 1978), and the first case in the European continent was reported in 1981, in former Czechoslovakia (Neubauer and Miláček 1981). The first European case of infant botulism demonstrated to be associated with honey was reported in Italy (Fenicia *et al.* 1993), and it was caused by *C. botulinum* type B. In Germany, one to two cases of infant botulism are reported every year (Böhnel *et al.* 2001). In the Netherlands, the first infant

botulism case was reported as late as 2000, but since then, two more cases have emerged (Wolters 2001, Thomasse *et al.* 2005).

In the Nordic countries, the first case of infant botulism was reported by Jansson *et al.* (1985) in Sweden, following reports from Denmark (Balslev *et al.* 1997, Jung and Ottoson 2001) and Norway (Tølløfsrud *et al.* 1998, Anonymous 1999, Hasseltvedt and Hoel 1999). In most of these cases, a history of honey consumption was reported. No infant botulism cases have been reported in Finland.

In South America, infant botulism has only been described in Argentina, where a total of 146 cases of infant botulism were diagnosed between 1982 and 1997, all caused by *C. botulinum* type A (Fernández *et al.* 1999). In Australia, the first case of infant botulism was reported in 1978 (Shield *et al.* 1978), and in the 10-year period from 1980 to 1989, six cases of infant botulism occurred in South Australia, all of the patients being male. Four of the cases were caused by *C. botulinum* type B and two by type A (Thomas 1993). In Asia, the first laboratory-confirmed case of infant botulism took place in 1986, in Japan (Noda *et al.* 1988). It was caused by type A, which was also isolated from the honey fed to the infant (Noda *et al.* 1988, Takahashi *et al.* 1988). According to a review by Ying and Shuyan (1986), one case of infant botulism may have occurred before 1984 in China, but no additional information is given for this case. During the following 10-year period a total of 14 cases were reported in Japan (Kozaki *et al.* 1998).

Despite its normally sporadic incidence, infant botulism has also been reported to exist as clusters of more than one infant being affected in a restricted area during a short period (Istre *et al.* 1986, Reddy *et al.* 2003).

The low incidence of infant botulism in many countries may be underestimated. Due to the numerous differential diagnoses suitable for the variable symptoms of infant botulism, such as constipation, failure to thrive and even sudden death of an infant, cases of infant botulism may have been overlooked. The lack of easily performed diagnostic tests for infant botulism may add to the number of misdiagnosed cases.

### **2.5.6 Association with sudden infant death syndrome**

Infant botulism has been suggested to be one cause of sudden infant death syndrome (SIDS), according to a survey of specimens from 280 dead infants in 1978 (Arnon *et al.* 1978). In addition to detecting the *C. botulinum* organism in the samples of 4.3% of the 211 cases defined as SIDS, a highly similar age distribution was noted between infant botulism patients and SIDS patients (Arnon *et al.* 1978). Sonnabend *et al.* (1985) detected botulinum toxin of different types or both toxin and organisms from autopsy specimens of 9 out of 59 infants confirmed as SIDS. The toxin types detected were A, B, C, F and G. In four of the cases, the toxin was also found in the serum. Böhnelt *et al.* (2001) highlighted the



importance of infant botulism as a cause of SIDS, reporting an incidence as high as 20% of *C. botulinum* organisms or toxins in the specimens of 75 SIDS children. By contrast, Byard *et al.* (1992) suggested that routine postmortem culture of *C. botulinum* in the cases of SIDS would be of limited value, as no infant botulism cases were detected among 248 SIDS infants during a 10-year prospective study.

The age-related peak incidence of both SIDS and infant botulism correlates with the introduction of solid foods to the diet (Pottgen and Hillegass 1977). Arnon *et al.* (1981) have estimated that only 2600 vegetative cells of *C. botulinum* would contain a lethal amount of toxin to the infant, but as it is unlikely that the toxin from all of the cells is absorbed, the number of *C. botulinum* vegetative cells to produce a lethal amount of toxin for a 7-kg infant is likely to range from  $2.6 \times 10^6$  to  $2.6 \times 10^9$ .

The influence of nutrition on infant botulism presenting SIDS has been discussed. According to a survey by Arnon *et al.* (1982), a strong association exists between breast feeding and hospitalization for infant botulism; however, 8 of 10 cases of SIDS linked to *C. botulinum* infection had been entirely formula-fed. The remaining two cases had not been given human milk in ten or more weeks preceding death. It thus seems that nursing may moderate the severity at the onset of infant botulism (Arnon *et al.* 1982). The protection is also supposed to depend in part on the presence, the specificity, and the titre of antibodies in human milk (Arnon 1984).

## 2.6 *Clostridium botulinum* and honey

### 2.6.1 Honey production

"Honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature" (Codex Alimentarius 2001).

For thousands of years, bees have been kept to get honey, beeswax and propolis. Today, beekeeping is as much a hobby as a form of industry. Bees (*Apis mellifera*) collect nectar, with a water content of approximately 55%, from flowering plants, which, after being stored in honey combs to diminish the water content to 17-18%, will turn into a ripened honey. When the honey is ripe, the bees cap the cells with beeswax.

To make honey production more effective, bees are given wax foundations, imprinted with the shapes of hexagonal cells. Using the foundation as the basis, bees build wax cells in which honey and pollen are stored and eggs are laid. A typical artificial beehive is constructed of several wooden or styrofoam hive bodies, in which there are ten

wooden frames containing one foundation apiece. Hive bodies are placed one on top of another to form the complete beehive.

Honey is collected from the combs by uncapping the honey cells using a specific tool or device, and placing the uncapped frame of combs in a centrifugal extractor, which spins the honey from the combs into the extractor. Extracted honey is drained through a spigot into a storage tank. Before entering the tank, honey goes through a strainer that filters out large debris such as dead bees and pieces of wax. By letting honey stand in the storage tank, small debris is allowed to rise to the top of the tank, where it is removed before placing the honey into jars. The emptied frames with the ready-built combs are then either put back in the hive for the next season or melted down to produce new foundations.

## 2.6.2 Microbiological characteristics of honey and propolis

Honey is a pure natural substance, mostly composed of different sugars, the monosaccharides fructose and glucose being the main components, with average proportions of 38% and 32%, respectively (White 1978, Qiu *et al.* 1999). In addition to fructose, glucose and 17-18% moisture, honey contains a selection of over 20 different sugars, with their total proportion being ca. 9%. The rest of the honey, about 3%, is made up of small amounts of other substances such as proteins, vitamins and minerals (White 1978). However, the composition of honey varies greatly according to the foraging areas of the bees (Qiu *et al.* 1999, Serrano *et al.* 2004). The moisture content is recommended to be no more than 20%, and the level of hydroxymethylfurfural (HMF), which is related to the ageing of honey, to not exceed 40 mg/kg, excluding honey produced in tropical areas, for which the highest level of HMF allowed is 80 mg/kg (Codex Alimentarius 2001). Due to the high sugar content, an average pH of 3.9 and water activity between 0.5 and 0.6 (White 1978), microorganisms are not able to grow in ripe honey, and the only microorganisms present are spores of certain bacteria and yeasts. If the moisture content is increased to more than 20%, the yeast spores may germinate and cause spoilage by fermentation. Spores of the genus *Bacillus* are regularly found in honey, and organisms of the genus *Clostridium* may also be present (Snowdon and Cliver 1996). However, once introduced into the honey, several vegetative pathogenic bacteria have also been able to survive at low temperature storage conditions for as long as 8-34 days (Tysset and Durand 1973).

Inhibitory activity of honey against different bacteria has been reported in several studies (Molan *et al.* 1988, Willix *et al.* 1992, Taormina *et al.* 2001, Mundo *et al.* 2004, Alnaqdy *et al.* 2005, Lusby *et al.* 2005). In addition to its slight acidity and osmolarity, the antimicrobial effect of honey is mainly due to the generation of hydrogen peroxidase via glucose oxidase (Dustmann 1979). The factors contributing to the bacterial inhibition of honey are presented in Table 3. However, the sensitivity of bacteria to the inhibitory effect

of honey seems to vary depending on the composition of each batch of honey (Molan *et al.* 1988, Willix *et al.* 1992, Mundo *et al.* 2004, Lusby *et al.* 2005). Due to its antibacterial activity, honey has been used to heal infected wounds for thousands of years, but it has also more recently gained attention for its applicability in wound management (Lusby *et al.* 2005). While no honey-related cases of wound botulism have been reported, the possibility exists of honey-derived spores being a source of wound botulism. The antibacterial activity of honey is heat-labile, but according to Molan and Allen (1996), honey sterilized with gamma-irradiation does not lose its antibacterial effectiveness, and could thus be used in alternative wound management.

Besides honey and beeswax, bees produce propolis, colloquially referred to as “bee glue”. It is a resinous substance collected from various plant sources and used to seal holes in honeycombs and to coat parts of the hive (Burdock 1998). Although the chemical composition of propolis may vary depending on plant origin, geographic origin or breed of the bees (Kujumgiev 1999, Bankova 2005, Silici and Kutluca 2005), the antibacterial activity of propolis, especially against Gram-positive bacteria and yeasts, has consistently been shown (Grange and Davey 1990, Dobrowolski *et al.* 1991, Marcucci 1995, Kujumgiev 1999, Popova *et al.* 2005, Silici and Kutluca 2005, Uzel *et al.* 2005), and it is higher than that of honey (Miorin *et al.* 2003).

Table 3. Factors contributing to the inhibitory activity of honey.

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High osmotic pressure as a consequence of high sugar content
Low water activity
Low protein content
Low pH value
Low redox potential due to the high number of reducing sugars
High viscosity
The glucose oxidase system forming hydrogen peroxide
Other antimicrobial agents such as flavonoids, lysozyme, phenolic acids and terpenes

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### 2.6.3 Detection of *Clostridium botulinum* in honey

Honey is a complex material for microbiological investigation. High sugar content, low pH and the existence of different oxidases inhibit microorganisms from growing in honey, but also make the detection of these organisms more difficult. In addition, the high viscosity of honey complicates its use as a sample material. Several methods, including dilution, centrifugation, dialysis and membrane filtration, have been developed to overcome these difficulties.

The first method described for honey analysis was dialysis (Sugiyama *et al.* 1978). A 25-g honey sample was diluted in distilled water before performing a 24-h dialysis. After the dialysis, the samples were transferred to TPGY broth containing cooked meat, kept in an 80°C water bath for 25 min and incubated at 37°C for 4 days. For routine analysis of honey samples, dialysis is a time-consuming and a laborious method.

Midura *et al.* (1979) described two methods, dilution and centrifugation, for honey samples. In the first method, the supernatant gained from centrifugation was applied for toxin detection, while in the second method the supernatant was discarded. Sediments were inoculated in enrichment broths, and some of the broths were also heated in a 70°C water bath for 15 min. Incubation was performed at 30°C for 10 days. A few years later, Huhtanen *et al.* (1981) noticed that the supernatant, not used for culturing the organism, may also contain spores of *C. botulinum*, thus recommending that the supernatant be included in the analysis. The incubation temperature used in this method does not favour group I organisms, known to be the most frequent causes of infant botulism. To evaluate the possible risk for infant botulism, the incubation of honey samples should also be performed also at the optimal growth temperature of group I strains, i.e. 35-40°C.

Membrane filtration has been applied to detect spores from honey diluted in distilled water containing 1% Tween 80 solution (Hauschild and Hilsheimer 1983). The diluted samples were kept in a water bath at 65°C for 30 min before the filtration, and after filtration the filters were transferred to TPGY broth for 7 days of incubation at 35°C.

Direct inoculation of honey into a liver-cooked meat medium (Hartgen, 1980), cooked meat medium (Huhtanen *et al.* 1981, Rall *et al.* 2003), fluid thioglycollate medium (Huhtanen *et al.* 1981), Robertson's medium (Hetland 1986) or TPGY medium (Mäde *et al.* 2000, Rall *et al.* 2003) has also been reported, as has dilution of honey with a saline solution before inoculating the solution in Robertson's medium (Schocken-Iturrino *et al.* 1999).

In the aforementioned studies, the detection of *C. botulinum* was performed using the mouse bioassay, excluding the study by Rall *et al.* (2003), who conducted a microscopic analysis of the enrichment medium in addition to biochemical tests of the colonies, and Mäde *et al.* (2000), who detected *C. botulinum* after enrichment using DNA extraction and PCR. However, no comparison of pre-processing methods to detect *C. botulinum* in honey by PCR has been performed, despite PCR being known to be a sensitive method, easily inhibited by various components in foods and other sample material. Also the inhibitory effect of sucrose has been demonstrated by Rossen *et al.* (1992).

#### 2.6.4 Prevalence of *Clostridium botulinum* in honey

The prevalence of *C. botulinum* spores in honey samples has been estimated in several studies, and, according to all previous results, the mean prevalence is approximately 4% (Table 4), varying up to 20% in different countries (Table 5). Despite the relatively high number of samples studied, *C. botulinum* spores have not been detected in honey produced in Canada, France, Germany and Norway (Flemming and Stojanowic 1980, Hartgen 1980, Hetland 1986, Hauschild *et al.* 1988, Nakano *et al.* 1990, Delmas *et al.* 1994). The incubation time, 4-10 days, does not seem to correlate with the number of positive samples observed, and positive samples have been detected with all methods used (Table 4). The types most often detected are A and B, with type C being detected only by Nakano *et al.* (1990), but it was present in as many as ten samples and isolated from three of them. Type D has been observed and isolated only by Schocken-Iturrino *et al.* (1999) in Brazilian honey.

Midura *et al.* (1979) noted that *C. botulinum* spores are unevenly distributed in honey. As it is difficult to obtain a homogeneous sample from honey, examination of several samples is recommended. Uneven distribution of spores also makes it difficult to perform a reliable quantitative analysis. In the majority of honey samples studied, the number of spores is low; however, Nakano and Sakaguchi (1991) did report an unusually high quantity of type F spores (Table 4). In honey samples with a known association with infant botulism, the number of spores has been at the level of thousands of spores/kg (Table 4).

The contamination routes of *C. botulinum* spores in honey remain obscure. Huhtanen *et al.* (1981) inoculated bee colonies experimentally with spores of *C. botulinum* by feeding them sugar solution containing  $1.6 \times 10^5$  spores. A number of spores equal to the original number introduced to the bees, was shown to accumulate in the honey over a five-week period, but the number of spores was not increased in the beehive. Nakano *et al.* (1994) demonstrated that *C. botulinum* spores are able to germinate and vegetative bacteria can multiply under aerobic conditions in dead bees and pupae. However, dead bees and diseased pupae are efficiently removed from the beehive by the bees, and thus, do not pose a significant risk in healthy colonies. No beehive-derived material except dead bees has been shown to constitute a material suitable for germination and multiplication of *C. botulinum* spores.

Table 4. Prevalence and quantity of *Clostridium botulinum* in honey.

Survey	Samples purchased from	Sample preparation method <sup>a</sup>	Sample size (g)	Incubation time/temperature	No. of positive samples/ Total no. of samples (%)	Type and number of <i>C. botulinum</i> detected	Type and number of <i>C. botulinum</i> isolated	Associated with illness	Quantity of spores (per kg)
Aureli <i>et al.</i> 1983	Italy	DC	10	7 d/30°C	0/107	ND <sup>b</sup>	NI <sup>c</sup>	No	NO <sup>d</sup>
Berry <i>et al.</i> 1987	UK	DC and MF	20	NR <sup>e</sup>	0/122	ND	NI	No	NO
Centorbi <i>et al.</i> 1994	Argentina	DC	20	7 d/30°C	1/42 (2)	A(1)	NI	No	1*
Criseo <i>et al.</i> 1993	Italy	DC	20	10 d/35±1°C	2/39 (7)	B(2)	B(2)	No	3*
Delmas <i>et al.</i> 1994	France	Dialysis	25	4 d/35°C	0/116	ND	NI	No	NO
Du <i>et al.</i> 1991 <sup>f</sup>	Taiwan	Dialysis	-	-	2/152 (1)	B(2)	-	No	-
Flemming and Stojanowic 1980	Germany	Dialysis	25	4 d/37°C	0/92	ND	NI	No	NO
Guilfoyle and Yager 1983	USA	Dialysis	25	7 d/35°C	0/48	ND	NI	No	NO
Hartgen 1980	Germany	DI	1	7 d/30°C	0/210	ND	NI	No	NO
Hauschild <i>et al.</i> 1988	Canada	MF	75	7 d/35°C	0/43	ND	NI	No	<1
Hauschild <i>et al.</i> 1988	Canada	DC and MF	75	7 d/35°C	0/106	ND	NI	No	<1
Hauschild <i>et al.</i> 1988	Canada	DC and MF	75	7 d/35°C	1/1 (100)	A(1)	NI	Yes	8 × 10 <sup>3</sup>
Hetland 1986	Norway	DI and dialysis	5 or 25	4 d/37°C	0/134	ND	NI	No	NO
Huhtanen <i>et al.</i> 1981	USA	DC and DI	20	7 d/30°C	10/80 (13)	A(NR), B(NR)	NI	No	7*
Kautter <i>et al.</i> 1982	USA	Dialysis	25	4 d/37°C	2/100 (2)	A(2)	NI	No	0.8*
Midura <i>et al.</i> 1979	USA	DC	30	10 d/30°C	0/81	ND	NI	No	NO
Midura <i>et al.</i> 1979	USA	DC	30	10 d/30°C	9/9 (100)	A(2), B(7)	A(2), B(7)	Yes	5-80 × 10 <sup>3</sup>
Monetto <i>et al.</i> 1999	Argentina	DC	20	7 d/30°C	2/44 (5)	Af(1), F(1)	Af(1), F(1)	No	<1000
Monetto <i>et al.</i> 1999	Argentina	DC	20	7 d/30°C	1/1 (100)	A(1)	A(1)	Yes	15 × 10 <sup>3</sup>
Mäde <i>et al.</i> 2000	Germany	DI	25	5 d/30°C	1/52 (2)	A(1)	A(1)	No	0.8*
Nakano <i>et al.</i> 1990	Japan	DC	20	7 d/30°C	23/270 <sup>g</sup> (9)	A(11), B(2), C(10), F(1)	A(11), B(1), C(3), F(1)	No	5*
Nakano and Sakaguchi 1991	Japan	DC	20	7 d/30°C	1/36 (3)	F(1)	F(1)	No	36-60 × 10 <sup>3</sup>

Table 4. Continued.

Survey	Samples purchased from	Sample preparation method	Sample size (g)	Incubation time/temperature	No. of positive samples/ Total no. of samples (%)	Type and number of <i>C. botulinum</i> detected	Type and number of <i>C. botulinum</i> isolated	Associated with illness	Quantity of spores (per kg)
Rall <i>et al.</i> 2003	Brazil	DI	8	7 d/35°C or 28°C	3/100 (3)	NS <sup>h</sup> (3)	NI	No	NO
Schocken-Iturrino <i>et al.</i> 1999	Brazil	DI	10	5-10 d/37°C	6/85 (7)	A(2), B(1), D(3)	A(2), B(1), D(1)	No	7*
Sugiyama <i>et al.</i> 1978	USA	Dialysis	75-125	4 d/37°C	18/241 (7)	A(11), B(7)	NI	No	80-280

<sup>a</sup>Sample preparation methods: DC, dilution-centrifugation method, (Midura *et al.* 1979); MF, membrane filtration method, (Hauschild and Hilsheimer 1983); DI, direct inoculation of honey into enrichment broth.

<sup>b</sup>ND, not detected.

<sup>c</sup>NI, not isolated.

<sup>d</sup>NO, not observable.

<sup>e</sup>NR, not reported.

<sup>f</sup>Article in Chinese. The results are based on the English summary.

<sup>g</sup>One sample contained two types of *C. botulinum* spores.

<sup>h</sup>NS, not specified.

\*Number of spores is estimated by author using most probable number method for a single dilution

Table 5. Prevalence of *Clostridium botulinum* spores in honey of different countries.

Country	No. of positive samples/ Total no. of samples (%)	Type and number of <i>C. botulinum</i> detected	Type and number of <i>C. botulinum</i> isolated	References
Argentina	8/103 (8)	A(4), Af(1), D(1), F(2)	A(2), F(2)	Nakano <i>et al.</i> 1990, Nakano and Sakaguchi 1991, Centorbi <i>et al.</i> 1994, Monetto <i>et al.</i> 1999
Australia	0/6	ND <sup>a</sup>	NI <sup>b</sup>	Nakano <i>et al.</i> 1990, Delmas <i>et al.</i> 1994
Brazil	8/108 (7)	A(2), B(1), D(3), NS <sup>c</sup> (2)	A(2), B(1), D(1)	Schocken-Iturrino <i>et al.</i> 1999, Rall <i>et al.</i> 2003
Canada	0/156	ND	NI	Hartgen 1980, Hauschild <i>et al.</i> 1988, Nakano <i>et al.</i> 1990, Delmas <i>et al.</i> 1994
China	9/79 (11)	A(3), B(2), C(4)	A(3), B(1)	Nakano <i>et al.</i> 1990, Delmas <i>et al.</i> 1994
former CSSR	0/1	ND	NI	Hartgen 1980
France	0/92	ND	NI	Nakano <i>et al.</i> 1990, Delmas <i>et al.</i> 1994
Germany	0/282	ND	NI	Flemming and Stojanowic 1980, Hartgen 1980
Hungary	1/18 (6)	A(1), C(1) <sup>d</sup>	A(1), C(1)	Hartgen 1980, Nakano <i>et al.</i> 1990, Delmas <i>et al.</i> 1994
Italy	2/146 (1)	B(2)	B(2)	Aureli <i>et al.</i> 1983, Criseo <i>et al.</i> 1993
Japan	6/65 (9)	A(3), C(3)	A(3), C(1)	Hartgen 1980, Nakano <i>et al.</i> 1990, Nakano and Sakaguchi 1991
Mexico	1/5 (20)	C(1)	NI	Hartgen 1980, Nakano <i>et al.</i> 1990, Delmas <i>et al.</i> 1994
New Zealand	0/1	ND	NI	Hartgen 1980
Norway	0/134	ND	NI	Hetland, 1986
Romania	0/1	ND	NI	Delmas <i>et al.</i> 1994
Russia	0/1	ND	NI	Delmas <i>et al.</i> 1994
Spain	1/7 (14)	A(1)	A(1)	Nakano <i>et al.</i> 1990, Delmas <i>et al.</i> 1994
Turkey	0/1	ND	NI	Delmas <i>et al.</i> 1994
UK	0/1	ND	NI	Nakano <i>et al.</i> 1990
USA	32/404 (8)	A(13), B(14), NS(5)	A(2), B(7)	Sugiyama <i>et al.</i> 1978, Midura <i>et al.</i> 1979, Hartgen 1980, Huhtanen <i>et al.</i> 1981, Nakano <i>et al.</i> 1990
former USSR	0/4	ND	NI	Nakano <i>et al.</i> 1990
Vietnam	0/1	ND	NI	Delmas <i>et al.</i> 1994

<sup>a</sup>ND, not detected.

<sup>b</sup>NI, not isolated.

<sup>c</sup>NS, not specified.

<sup>d</sup>One sample contained *C. botulinum* spores of types A and C.

## 2.6.5 Honey and infant botulism

In early epidemiological studies, approximately 30% of infant botulism cases in California were associated with the ingestion of honey containing botulinum spores (Arnon *et al.* 1979a, Chin *et al.* 1979). After public notification, only 12 of the 160 patients (7.5%) diagnosed with infant botulism between 1993 and 1997 had received honey (Schechter 1999). In Europe, a history of honey consumption has been reported for more than half of the infant botulism cases (Aureli *et al.* 2002). The minimum infective dose of *C. botulinum*



spores for an infant is not known, but from exposure to honey containing botulinal spores it has been estimated to be as low as 10-100 spores (Arnon *et al.* 1979a). Paradoxically, honey is a traditional remedy to calm crying babies. This has been given some scientific basis by Ramenghi *et al.* (2001), who showed that oral administration of honey significantly reduces the cry response to painful experiences in newborn babies, as compared with the effect of placebo.

### 3. AIMS OF THE STUDY

This thesis is based on the previously recognized association between honey and infant botulism. The studies were conducted in order to:

1. examine the involvement of *Clostridium botulinum* in a case of sudden infant death syndrome (I),
2. develop a method for efficient and sensitive detection of *C. botulinum* spores in honey using polymerase chain reaction (II),
3. optimize a pulsed-field gel electrophoresis (PFGE) method for the analysis of *C. botulinum* group I, and evaluate the genetic diversity of *C. botulinum* group I using PFGE (III-V),
4. determine the prevalence and quantity of *C. botulinum* spores in honey sold in Finland, and study the prevalence of *C. botulinum* spores in honey produced in the Nordic countries (II, IV),
5. evaluate the contamination routes of *C. botulinum* in the honey production environment, and develop prevention measures to decrease the contamination level (V).

## 4. MATERIALS AND METHODS

### 4.1 Sampling in a case of a sudden infant death syndrome (I)

Autopsy specimens of the serum, faeces, intestine and spleen of a suddenly deceased, 11-week-old infant were collected by a pathologist. In addition, the opened package of the infant's last meal of corn gruel was examined. Family members of the deceased child were interviewed, and household samples, including soil from flowerpots and a vacuum cleaner dust bag, were collected. The interview covered questions about the previous health of the infant, feeding and nursing habits, nearby environment, possible pets and travelling. A permission to examine the tissue samples was obtained from the National Authority for Medicolegal Affairs (decision number 3380/32/200/01).

### 4.2 Bacterial strains (II, III)

To compare three different methods for detecting botulinal spores from honey using PCR, a spore mixture containing equal concentrations of three strains of *C. botulinum* type A (62A, ATCC 25763A, NCTC 7272A) and a mixture containing equal concentrations of four strains of *C. botulinum* type B (proteolytic strains 133-4803B, 213B, 126B and non-proteolytic strain 2B) were used (II).

In Study III, the suitability of different enzymes for a molecular epidemiological analysis of group I *C. botulinum* by PFGE was tested using five strains of proteolytic *C. botulinum*: two of type A (ATCC 3502, ATCC 19397), two of type B (ATCC 2743, FT 243) and one of type F (ATCC 25764). To evaluate the diversity of group I *C. botulinum*, a total of 55 strains from culture collections of the Department of Food and Environmental Hygiene, University of Helsinki, and the Institute of Food Research, Norwich, UK, were studied (III). Of these strains, 19 were of type A, 28 of type B and three of type F, and five strains contained the toxin gene for both type A and type B. The strains included both European and American isolates. Each of the strains; ATCC 3502, ATCC 19397, ATCC 7948, NCTC 2012, ATCC 25763, ATCC 25764 and ATCC 35415, were initially obtained from more than one laboratory.

### 4.3 Development of honey preparation method prior to PCR (II)

Three honey sample preparation methods; dilution, centrifugation and supernatant filtration (SF), were evaluated for their efficacy to detect *C. botulinum* from 25-g honey samples inoculated with 0, 0.1, 1, 10 and 100 spores/g honey. In the dilution method, the inoculated

honey samples were suspended in 25 ml of distilled water (1:1 w/v) and incubated in a water bath at 65°C for 30 min. Each suspension was then poured into a one-litre bottle containing 450 ml of trypticase-peptone-glucose-yeast extract (TPGY) broth (Lilly *et al.* 1971), corresponding to a honey dilution ratio of 1:20. In the centrifugation method, all samples were diluted in 225 ml of 1% Tween 80 (Merck KGaA, Darmstadt, Germany) solution in a 65°C water bath for 30 min and centrifuged for 30 min at 8700-9000 × g. The precipitate was then transferred into 9 ml of TPGY broth. In the SF method, membrane filtration was combined with centrifugation so that the supernatant was used instead of the precipitate. Spores were captured from the supernatant by filtering the supernatant through a 0.45-µm filter with a diameter of 47 mm (Millipore, Bedford, MA, USA). The filter was then inoculated into 9 ml of TPGY broth.

After incubating the TPGY broths under anaerobic conditions for five days at 30°C, 1 ml from each tube and bottle was transferred into a fresh 9-ml TPGY broth for a further incubation of 16 h under the same conditions. Cells from 1 ml of this overnight culture were then washed with TE (Tris-HCl 10 mM [pH 7.5], EDTA 1 mM [pH 8.0]) buffer and made into a template for PCR.

#### 4.4 PFGE analysis (I, III-V)

##### 4.4.1 DNA preparations (I, III-V)

After being incubated anaerobically at 37°C for 2 days, single colonies of pure cultures were picked from blood agar plates and inoculated into 10-ml TPGY broths and incubated for 16 h at 37°C. DNA isolation was performed according to a previously described method (I) (Hielm *et al.* 1998) or with slight modifications (III-V). Briefly, a 4-ml volume of the 16-h culture was chilled on ice, and cells were harvested by centrifugation (1100 × g) at 4°C and suspended in PIV buffer (10 mM Tris [pH 7.5], 1 M NaCl) supplemented with 10% (v/v) formaldehyde solution. The cell suspensions were kept on ice for 1 h with gently shaking every 15 min. The cells were then washed twice with PIV buffer and resuspended in 1 ml of double-strength lysis solution (12 mM Tris [pH 7.5], 2 M NaCl, 200 mM EDTA [pH 8.0], 1% Brij 58, 0.4% deoxycholate, 1% sodium lauroyl sarcosine, 40 µl of RNase/ml, 2 mg of lysozyme/ml, 40 U of mutanolysin/ml). A volume of 500 µl of each cell suspension was mixed with an equal amount of 2% (wt/vol) low-melting-point agarose (InCert agarose, Cambrex Bio Science, Rockland, ME, USA) and cast in GelSyringe dispensers (New England Biolabs, Beverly, MA, USA). The gel plugs formed were then incubated in lysis solution (6 mM Tris [pH 7.5], 1 M NaCl, 100 mM EDTA [pH 8.0], 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 20 µl of RNase/ml, 1 mg of lysozyme/ml, 20 U of mutanolysin/ml) with gentle shaking at 37°C overnight. The next day, the plugs were

rinsed with TE buffer, and the DNA isolation was completed by washing the plugs twice with ESP (0.5 M EDTA [pH 8.0], 10% sodium lauroyl sarcosine, 100 µg of proteinase K/ml) at 50°C for 3 h. The inactivation of proteinase K was performed with Pefablock SC (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C overnight. On the third day, the plugs were washed with TE buffer twice at 37°C for 2 h with gentle shaking.

#### **4.4.2 Restriction enzyme digestions (I, III-V)**

In Study III, nine rare-cutting restriction enzymes; *ApaI*, *AscI*, *MluI*, *NruI*, *PmeI*, *RsrII*, *SacII*, *SmaI* and *XhoI* (New England Biolabs), were chosen to test the cleavage of DNA of proteolytic *C. botulinum*. Samples were electrophoresed at 8°C through a 1% (wt/vol) agarose gel (Seakem Gold agarose, BMA) in 0.5-x TBE buffer (Amresco, Solon, OH, USA) at 200 V for 20-22 h with a Gene Navigator system (Pharmacia, Uppsala, Sweden) equipped with a hexagonal electrode. Different pulse-time ramps were tested when necessary to determine the optimal running conditions. Low Range PFG marker (New England Biolabs) was used for fragment size evaluation.

In Studies IV and V, the DNA was digested using rare-cutting restriction enzyme *SacII* (New England Biolabs), and a running protocol with a pulse-time ramp of 1-26 s for 22 h at 200 V was applied.

In Study I, the DNA was digested using two rare-cutting restriction enzymes, *SmaI* and *XhoI* (New England Biolabs). With *SmaI*, the running time was 22 h using a pulse-time ramp of 1-26 s, and with *XhoI* 18 h using a pulse-time ramp of 1-15 s.

#### **4.4.3 Imaging the PFGE results (I, III-V)**

After electrophoresis, the gel was stained for 30 min in running buffer or in distilled water containing 0.5 µg/ml of ethidium bromide. The gel was destained in pure running buffer or in distilled water for at least 2 h. DNA fragment sizes were estimated by comparing their running lengths with those of molecular weight markers. The gels were photographed using the AlphaImager 2000 documentation system (Alpha Innotech, San Leandro, CA, USA).

#### **4.4.4 PFGE profile analysis (I, III-IV)**

Fingerprint profiles of the isolates were analysed with BioNumerics software version 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity between all pairs was expressed by the Dice coefficient correlation, and Unweighted Pair-Grouping Method of Averages (UPGMA) clustering was used to construct the dendrogram. The position tolerance was set

at 2.0% (III, IV) or at 3.0% (V), with no increase towards the end of the profile, and the optimization value was set at 0.5%.

#### 4.5 Sampling of honey and materials related to honey production (II, IV, V)

Altogether 448 extracted and 256 comb honey samples were collected in Studies II, IV and V. In Study I, a total of 114 extracted honey samples were obtained from Finnish beekeepers and 76 samples from honey imported to Finland. In Study III, 294 samples from extracted and comb honey produced in Denmark, Norway and Sweden were collected via national beekeepers' associations.

In a follow-up survey (V), a total of 1168 samples of honey and other materials related to honey production were collected from a total of 100 apiaries from 37 beekeepers during a three-year period (2001-2003). Samples included bees, beebread, feeding sugar, honey, honeycomb, pollen grains and soil. The sampling was divided into two periods each year.

#### 4.6 Sample preparation (II, IV, V)

All extracted honey (II, IV, V), comb honey (IV, V) and feeding sugar (V) samples were prepared according to the SF method before PCR. The other samples in Study V (bees, beebread, pollen grains and soil) were examined by performing direct one-gram inoculations into 10-ml TPGY broths, producing 2-20 repetitions, the number of repetitions depending on the amount of each sample material available. Beebread, which is composed of pollen packed in beeswax cells, was divided before weighing into two portions: pollen and beeswax. All tubes were kept in a water bath at 65°C for 30-45 min to diminish the number of vegetative organisms and induce the germination of botulinal spores.

In Study I, the tissue samples were inoculated in several parallel TPGY broths. The household samples were also inoculated in TPGY medium, each subsample weighing 1.0 g or 0.5 g for soil and dust, respectively.

All TPGY broths were incubated in anaerobic conditions at 30°C and parallel tubes at 37°C to enable the germination and growth of non-proteolytic and proteolytic strains of *C. botulinum*, respectively (II-V), excluding the broths in Study II, which were incubated at 30°C.

#### 4.7 PCR analysis (I-V)

A volume of 1 ml of the TPGY cultures was further transferred to fresh TPGY medium for overnight culturing. Cells from 1 ml of this culture were then washed with TE buffer, and 1 µl of the cell suspension was used as a template for PCR.

In Study II and with certain strains in Study III, previously described primer sets for the *botA* and *botB* genes were used in PCR analysis (Franciosa *et al.* 1994) at concentrations of 0.4 µM. For amplification of gene fragments, the following thermal profile was used: 10 min of denaturation at 98°C before adding the DNA polymerase (DynaZyme, Finnzymes, Espoo, Finland), after which there were 28 cycles with 30 s of denaturation at 94°C, 30 s of annealing at 50°C and 80 s of extension at 72°C, ending with an extension of 2 min at 72°C.

In Studies I and III-V, a previously described multiplex-PCR method targeted to *botA*, *botB*, *botE* and *botF* genes (Lindström *et al.* 2001), with slight modifications, was applied to detect *C. botulinum* types A, B, E and F. The 50-µl volume of the reaction mixture contained 1 µl of cell suspension as template, 0.26 µM of each primer (Sigma-Genosys Ltd., Cambridgeshire, UK), 190 nM of each deoxynucleotide triphosphate (dNTP Mix, Finnzymes), 28 mM Tris-HCl, 69 mM KCl, 4.1 mM MgCl and 2 U of DNA polymerase (DynaZyme, Finnzymes). The polymerase enzyme was added after an initial denaturation of 10 min at 98°C. Each PCR cycle consisted of denaturation at 95°C for 30 s, annealing at 60°C for 25 s, and extension at 72°C for 85 s. The cycles were repeated 27 times and followed by a final extension at 72°C for 3 min. The PCR products were visualized in agarose gel electrophoresis using 2% agarose gels (I.D.N.A. agarose, BioWhittaker Molecular Applications, Rockland, ME, USA). All PCR reactions were reproduced.

#### 4.8 Isolation of *Clostridium botulinum* (I, II, IV, V)

*C. botulinum* was isolated from PCR-positive samples by plating 0.1 ml of the enrichment media (TPGY) on egg yolk agar (EYA) plates and growing them anaerobically for 2 days at 30°C or 37°C. Blood agar was applied to purify the isolates. Proteolytic activity was tested by plating the isolates on reinforced clostridium medium (RCM) containing 5% (v/w) skimmed milk (Peck *et al.* 1992).

#### 4.9 RAPD analysis (I)

RAPD analysis was performed as previously described (Hyytiä *et al.* 1999b), with Ready-To-Go RAPD analysis beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), using universal primers OPJ-06 and OPJ-13. The thermal profile consisted of denaturation at 95°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min.

#### 4.10 Most probable number method (I, II, V)

Most probable number method (MPN) was applied to estimate the number of *C. botulinum* spores in positive samples. The quantity was approximated from MPN tables or calculated using Thomas's approximation (Thomas 1942), according to the number of PCR-positive tubes in the series. In Study II, the series constituted subsamples of 5 x 0.1 g, 5 x 1 g and 5 x 10 g from each PCR-positive honey sample, prepared by the SF method and studied with PCR. If the result was below the detection limit of the MPN series (<18 spores/kg), a quantity of 9 *C. botulinum* spores/kg was assigned for estimating the mean and the median spore concentrations. In Studies I and V, the series consisted of several repetitions containing equal amounts of the sample, and the estimate was not calculated when the number of subsamples was less than five.

#### 4.11 Pollen analysis (II)

Pollen analysis was applied to confirm the geographical origin of honey samples. The honey samples were prepared according to standard methods of palynology (Louveaux *et al.* 1978). Samples of 10 g were dissolved in 20 ml of distilled water and centrifuged for 10 min at 2500 rpm. After removing the supernatant, the procedure was repeated and the sediment was transferred to a slide and spread out over an area of about 15 x 15 mm. After drying, the sediment was sealed with a cover slide using glycerine gelatin. All pollen grains were recorded by evaluating the whole sediment by microscopic examination (×400 magnification).

#### 4.12 Mouse bioassay (I, II)

The toxicity of isolates obtained from the patient's intestine and from the vacuum cleaner dust was confirmed using a mouse bioassay (I). Also the toxin production of the strains isolated from honey in Study II was confirmed by a mouse bioassay. Animal testing, performed according to national guidelines, was approved by the Committee for Animal



Experimentation, University of Helsinki, and the County Administrative Board (decision numbers STU 525 A, ESLH-2000-04531/Ym-23, and STU 512 A, ESLH-2000-04527/Ym-23).

#### 4.13 Questionnaire and observation study (V)

A questionnaire and observation study was performed in relation to each apiary as well as concerning the production methods and the extraction facilities of the beekeeper. The questionnaire and observation of the apiaries covered bee breed, bee diseases and the environmental characteristics of the apiary, including information about the distance to the nearest animal farm, pasture or water source, and the type of soil surface in the apiary. The questionnaire and observation of the extraction facilities included the cleaning and cleanliness of the extraction room and the extractor, the area of the extraction facilities and the capacity of the extractor, the straining method used, the level of lighting in the extraction room, the availability of proper hand-washing facilities and protective clothing and the usage of the same footwear outside and inside the extraction room. Also enquired about was whether the extraction room was used for purposes other than honey production.

#### 4.14 Statistical methods (IV,V)

The significance of the difference in the prevalence of *C. botulinum* between different sample materials was estimated using the chi-square test (IV, V).

In Study V, all individual results were recorded using MS Excel 2002 software (Microsoft Corporation, Redmond, WA, USA), and the statistical analysis was performed with the Statistical Package for Social Sciences version 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Answers from the questionnaire concerning environmental factors at the apiary were examined in relation to results of the presence of *C. botulinum* in various sites and items at the same apiary. The proportions of samples positive for *C. botulinum* in various sampling sites and items during different sampling years, among different bee breeds, and at different distances from the nearest animal farm as well as 95% confidence intervals (95% CIs) for proportions were calculated. A non-parametric Spearman's rank order correlation coefficient with a two-tailed *P*-value was calculated for bivariate correlations between positive *C. botulinum* samples in various sites or items and various environmental factors. An odds ratio (OR) with 95% CI was calculated for each sample, being positive for *C. botulinum* according to the outcome of various environmental parameters. *C. botulinum* absent in an apiary was taken as a dependent variable in the logistic multivariable regression model. In this model, various environmental and hygienic parameters were considered independent variables, and their predictive values for the

dependent variable were analysed by computing the coefficient estimates (B-values), *P*-values for the B-values, and ORs with 95% CIs from the B-values.

## 5. RESULTS

### 5.1 A case of sudden infant death syndrome (I)

The *C. botulinum* type B toxin gene was detected in two subsamples of the infant's intestine, incubated at 37°C, and *C. botulinum* type B was isolated from one of the subsamples. *C. botulinum* type B was also detected and isolated from the vacuum cleaner dust. All other autopsy samples were negative for *C. botulinum*, and serum did not contain botulinum toxin. The corn gruel was also negative for *C. botulinum*.

According to the interview, the infant had been healthy since birth except for a two-day period of constipation at the age of four weeks, but no signs of constipation preceded the death. He had been breast-fed until six weeks of age, after which the breast milk had gradually been replaced with commercial formulas: milk and corn gruel. The death had occurred a few hours after the child had been fed, the final feeding being slower than normal. Honey was not part of the infant's diet. No environmental predisposing factors to infant botulism were noted.

The type B isolates from the infant's intestine and from the vacuum cleaner dust were confirmed to be proteolytic. When analysed with PFGE, using restriction enzymes *SmaI* and *XhoI* and with RAPD using primers OPJ-06 and OPJ-13, the fingerprint profiles were shown to be identical.

### 5.2 Development of honey preparation method prior to PCR (II)

The supernatant filtration (SF) method in pre-processing honey prior to PCR was shown to provide the highest sensitivity in detecting *C. botulinum* spores in honey samples. All samples inoculated with spores of *C. botulinum* were detected by the SF method. By using the dilution or centrifugation method in preparing the honey samples, some false-negative results were obtained.

### 5.3 Development of PFGE analysis suitable for *Clostridium botulinum* group I (III)

Of the nine enzymes tested, *SacII*, *SmaI* and *XhoI* produced a suitable fingerprint profile for molecular epidemiological analysis, the number of fragments ranging from 10 to 22, from 9 to 21 and from 14 to 25, respectively. With *SacII* and *SmaI*, the preferable pulse-time ramp was 1-26 s and the run was completed in 22 h. With *XhoI*, the optimal pulse-time ramp was 1-15 s in 18 h. The median number of fragments generated was 17, 13 and 19 with *SacII*,

*SmaI* and *XhoI*, respectively. With *SacII*, a total of 33 different PFGE profiles were produced. With *SmaI* and *XhoI*, the number of profiles was 29 and 32, respectively. By combining *SacII* with *SmaI*, or with *XhoI*, an increase was noted in the discriminatory index compared with the discriminatory index of *SacII*, and the number of different PFGE types produced was increased to 38 in both cases. When all three enzymes were combined, the number of PFGE types was decreased.

Of the other enzymes, *AscI*, *MluI* and *RsrI* created only a few fragments, and the majority of the fragments generated by *ApaI* and *PmeI* were too small (<100 kb) to allow reliable profile analysis. *NruI* occasionally produced an appropriate number of fragments, but with certain strains the digestions of the genome were very frequent, producing several fragments too poorly separated to be reliably analysed.

## 5.4 Prevalence and quantity of *Clostridium botulinum* in samples related to honey production (II, IV, V)

### 5.4.1 Honey (II, IV, V)

The prevalence of *C. botulinum* was 12% in the 448 extracted honey samples studied (II, IV, V) (Table 6). Spores of types A, B and E were detected by PCR in 17, 40 and 5 extracted honey samples, respectively, and on nine occasions, types A and B were detected in the same sample. In Europe, the prevalence of *C. botulinum* in extracted honey was 11%. Of the 214 Finnish samples (II, V), 18 (8%) were found to be positive for *C. botulinum*. The highest numbers of samples of extracted honey were collected from Finland (n=214), Norway (n=100), Sweden (n=61) and Denmark (n=58). The prevalence of *C. botulinum* was significantly higher in Danish than in Finnish ( $\chi^2=11.0$ ,  $P<0.01$ ), Norwegian ( $\chi^2=10.4$ ,  $P<0.01$ ) or Swedish ( $\chi^2=16.6$ ,  $P<0.001$ ) honey samples. The estimation of quantity in all extracted honey samples studied was 3 spores/kg, as calculated by MPN for a single dilution.

In comb honey samples (n=256), the overall prevalence was 24% (IV, V). In Finnish honey, the prevalence of botulinum spores was significantly higher in comb honey than in extracted honey samples ( $\chi^2=17.8$ ,  $P<0.001$ ). In Danish or Norwegian samples, no significant difference was noted between these two sample materials.

### 5.4.2 Other samples related to honey production (V)

The highest frequency of *C. botulinum* spores in samples taken from the beehives was observed in samples associated with beeswax; comb honey, pollen from beebread and the beeswax itself. Of all the samples, the highest prevalence was detected in soil. Prevalence

in different materials and the number of samples studied are shown in Table 6. No significant annual variation was noted in the prevalence of *C. botulinum* in any apiary- or soil-derived material.

### 5.4.3 Isolation of *Clostridium botulinum* (I, II, IV, V)

A total of 242 *C. botulinum* isolates were obtained from the PCR-positive samples, of which 15 were type A and 227 were type B isolates. All type B isolates were confirmed to be group I strains. The origin and serotype of the isolates as well as the types of *C. botulinum* detected are presented in Table 6.

Table 6. Numbers and types of *Clostridium botulinum* detected and isolated from different sample materials in Studies II, IV and V.

Sample material	Number of samples	Number of positive samples (%)	Type and number of <i>C. botulinum</i> detected	Type and number of <i>C. botulinum</i> isolates <sup>b</sup>
Bees	156	14 (9)	B(10), E(2), F(2)	B(3)
Beeswax	176	40 (23)	A(2), B(34), E(3), F(1)	B(43)
Comb honey	256	61 (24)	A(3), B(54), E(3), F(1)	A(1), B(52)
Extracted honey	448	53 (12)	A(17), B(40), E(5)	A(14) B(27)
Feeding sugar	87	6 (7)	B(6)	B(6)
Pollen grains	61	4 (7)	B(3), E(1)	B(1)
Pollen from beebread	173	26 (15)	B(13), E(3)	B(33)
Soil <sup>b</sup>	235	73 (31)	A(7), B(64), E(6), F(1)	B(62)
Total	1592	277 (17)	A(29), B(224), E(23), F(4)	A(15), B(227)

<sup>a</sup>Different types of *C. botulinum* were detected in one sample.

<sup>b</sup>On several occasions, more than one strain was isolated from the subsamples of a single sample.

### 5.5 Diversity of *Clostridium botulinum* group I (III-V)

All *C. botulinum* strains were typeable with PFGE. In Study III, within the 55 strains of group I types A, B and F that were investigated using restriction enzyme *SacII*, 33 different fingerprint profiles emerged, and, according to the dendrogram, a high heterogeneity was present in the group I. However, 15 of the analysed 28 type B strains (54%) were grouped into two clusters at a similarity level of 95%, and the type F strains were shown to be highly similar. The same strains of type A and type F, originating from different laboratories, were shown to be similar with all three enzymes.

In Studies IV and V, distinct clusters composed of highly similar group I type B strains were observed. The clusters included fingerprint patterns obtained from isolates of different countries and from different sample material. In Study IV, eight different PFGE profiles were formed within the 24 analysed strains, and 20 of the strains (83%) were included in the three largest clusters. In Study V, two main PFGE profiles covered 96% of the 202 group I type B isolates, but the two profiles shared only 62% similarity with each other.

## 5.6 Factors affecting contamination of *Clostridium botulinum* in the honey production environment (V)

Based on the most significant bivariate correlations, four variables; size of extractor, usage of the same footwear outdoors and in the extraction room, presence of hand-washing facilities in the extraction room, and presence of *C. botulinum* in soil samples, were selected for use in a multivariate logistic regression model. The presence of hand-washing facilities had a significant ( $P < 0.05$ ) negative predictive value (OR 19.465, 95% CI 1.580-239.746) for the extracted honey being not negative for *C. botulinum* (V, Table 7). *C. botulinum* contamination in bees was shown to be influenced by the soil surface in the apiary, with gravel and moss enhancing the possibility of bees carrying spores.

No significant ( $P > 0.05$ ) non-parametric bivariate correlations were observed between various bee diseases and occurrence of *C. botulinum* in bee, beehive or honey samples. There were no significant ( $P > 0.05$ ) differences between any type of samples positive for *C. botulinum* and different bee breeds. Significant ( $P > 0.05$ ) non-parametric bivariate correlations were not observed between extracted honey samples positive for *C. botulinum* and bee, beehive, or pollen grain samples positive for *C. botulinum*. There was also no correlation between the different types of straining methods used and the existence of botulinal spores in honey.

## 6. DISCUSSION

### 6.1 *Clostridium botulinum* as a cause of sudden infant death (I)

The defined case can be considered infant botulism based on the case definition used in the United States (CDC 1997), which describes infant botulism as an illness, consistent with the known paralysing action of botulinum toxin, in which *C. botulinum* toxin and/or organisms are identified in serum or faecal specimens. In practice, botulinum toxin is rarely found in the serum of infant botulism patients with fecally diagnosed illness (Hatheway and McCroskey 1987). *C. botulinum* has previously been shown not to be part of the normal human infant intestinal microflora (Stark and Lee 1982a, 1982b, Hatheway and McCroskey 1987). Being able to isolate the organism from the intestinal contents suggests that either a relatively high number of spores was initially ingested or the multiplication in the intestinal lumen has been significant, or both.

The similarity between *C. botulinum* isolates from an infected infant and from household dust was demonstrated by both PFGE and RAPD analyses. However, according to the results of the genetic diversity of *C. botulinum* group I type B strains (IV, V), straightforward conclusions cannot be made based merely on the observed similarity between isolates from the patient and those from the environment. Nevertheless, the assumption about the route of infection is not founded only on data of the genetic similarity of the two strains. The infant's food proved to be negative for *C. botulinum*, and no predisposing factors were noted in the interview of family members. An exposure to dust-related spores thus appears to be the cause of the illness, resulting in a form of the illness resembling SIDS; the route of dust-containing spores from the surroundings to the infant's alimentary canal could not be determined. The diagnosis of infant botulism should not be overlooked in sudden infant death cases in countries with a low reported incidence of infant botulism.

### 6.2 Development of honey preparation method prior to PCR (II)

A PCR method to overcome the difficulties associated with *C. botulinum* detection in honey was developed. Supernatant filtration in pre-processing of the samples provided a sensitivity sufficiently high to detect 0.1 spore of *C. botulinum* in 1 g of honey. This may be considered adequate in determining whether honey is a potential cause of infant botulism, as the number of spores in honey samples associated with infant botulism has been reported to vary from 5 to 80 spores/g (Midura *et al.* 1979). Although Hauschild and Hilsheimer (1983) were the first to describe the combination of centrifugation and membrane filtration,

they inoculated both supernatant and precipitate in the same enrichment broth. According to our results, the most sensitive outcome was obtained using the pure supernatant, which suggests that most spores are suspended in the supernatant and/or the precipitate contains inhibitory substances. A direct inoculation of honey into the enrichment broth, as performed by Mäde *et al.* (2000), may diminish the sensitivity of PCR detection. By using supernatant filtration, spores of *C. botulinum* were separated from the honey and the amount of inhibitory substances affecting bacterial growth and PCR analysis was effectively minimized.

### 6.3 Diversity of *Clostridium botulinum* group I by PFGE (III-V)

In developing a PFGE method to analyse group I *C. botulinum* (III), certain modifications were applied to a method previously described for *C. botulinum* group II (Hielm *et al.* 1998a). When a volume of 8 ml of overnight culture was used to isolate of DNA, the amount of DNA was too high to produce clear fragments. To decrease the DNA yield, cells were washed from 4 ml of TPGY broth rather than 8 ml. In addition, a twofold volume of double-strength lysis solution was used when casting the plugs compared with the earlier protocol. The gels were stained in a solution prepared in distilled water, as presented elsewhere (Maslow *et al.* 1993), which produced a good resolution of the small fragments.

According to the results of Study III, the restriction enzymes *SacII*, *SmaI* and *XhoI* are recommended for the differentiation of proteolytic *C. botulinum* strains. Based on the analysis of 55 strains, *SacII* yielded the highest number of different fingerprint profiles, and thus seems to be the best choice for genomic analysis of proteolytic *C. botulinum* strains as a single enzyme. If further discrimination of strains or confirmation of strain similarity is needed, a combination of *SacII* and *XhoI* should be applied. With any of the enzymes tested, no specific fragments associated with different types of strains were noted that could be used in determining the toxin type.

Compared with earlier results on the diversity of group I *C. botulinum* using RAPD and rep-PCR (Hyytiä *et al.* 1999a), PFGE revealed a higher heterogeneity between the isolates. According to Hyytiä *et al.* (1999a), five type A strains (ATCC 3502, ATCC 19397, ATCC 25763, ATCC 7948, SL-3) and two type B strains (McClung 133-4803 and ATCC 7949) were confirmed to be similar at the toxin type level by RAPD, but as demonstrated here, the strains were clearly discriminated from each other by PFGE.

Despite the ability of PFGE to differentiate certain strains efficiently, relatively large clusters were also observed, particularly among type B strains. In Study III, two highly similar clusters of group I type B strains were observed, including strains isolated from different materials, from different continents and over an extended period of time. A high similarity was also noted between a few Finnish isolates. The formation of clusters



composed of highly similar PFGE profiles of type B group I isolates was further emphasized when analysing isolates from Danish and Norwegian honey samples (IV). Since all isolates were obtained from honey, the similarity could be speculated to be related to the honey production environment. The analysis of Finnish type B group I isolates in Study V showed that strains isolated from apiary-related samples and from soil produced identical fingerprint profiles. The similarity thus does not appear to strictly correlate with the origin of the isolates. Based on the PFGE results in Study V, no conclusions about the contamination routes in honey production environment could be drawn due to the similarity of fingerprint profiles. There was, however, a clear difference between the main profiles observed in the dendrogram of PFGE profiles (IV Fig. 1, V Fig. 3), suggesting the existence of different genetic lineages within group I type B isolates.

On the other hand, the existence of large clusters, including isolates from different materials and different countries (IV, V), may indicate a relatively high genetic stability of *C. botulinum* group I type B in soil, as well as in other material unfavourable to its growth. In addition, the similarity between fingerprint profiles of the same strains obtained from different laboratories may suggest a high constancy of the genotype (III), as these formerly commercially available strains have presumably gone through cycles of sporulation and germination. Although high similarity was detected within the type F strains, due to the low number of strains analysed, no conclusions about the diversity of group I type F could be drawn.

PFGE method is a useful tool for studying the epidemiological relatedness of proteolytic *C. botulinum* isolates types A and B. However, because of the occurrence of clusters sharing a highly similar PFGE profile, especially within the type B strains, the epidemiological conclusions based merely on PFGE data should be made with caution.

#### 6.4 Prevalence and quantity of *Clostridium botulinum* in honey (II, IV, V)

The observed prevalence (11%) of *C. botulinum* in European extracted honey (II, IV, V) is high compared with earlier surveys. In Study II, the incubation was performed only at 30°C, which could have resulted in underestimating the number of positive samples, as the optimal growth temperature for the *C. botulinum* group I strains is 35-40°C. However, the prevalence of *C. botulinum* in Finnish honey samples in Study V (10%), is not significantly higher ( $\chi^2=0.62$ ,  $P>0.05$ ) compared with that observed in Study I (7%), despite of incubating the samples at both 30°C and 37°C (V). A significantly higher prevalence of *C. botulinum* was noted in all comb honey samples compared with the prevalence in all extracted honey samples (IV, V). Nakano *et al.* (1990) have previously reported that the prevalence of spores is higher in samples from apiaries than in retail packages, and the finding has been suggested to be due to the ageing of honey. In Studies IV and V, honey

samples represented the same production period as honeycomb samples, thus, the difference is more likely to be caused by dilution rather than by ageing of the product. When honey from different hives and/or apiaries is extracted and mixed in large containers to produce retail packages, the spore population is presumably diluted to some extent. On the other hand, comb honey is taken from beeswax cells, and thus, beeswax, which has been shown to frequently contain spores (V), may cause contamination.

In studies of 92 French (Nakano *et al.* 1990, Delmas *et al.* 1994), 282 German (Flemming and Stojanowic 1980, Hartgen 1980) and 134 Norwegian (Hetland 1986) honey samples, no *C. botulinum* spores were detected (Table 5). Different methods in pre-processing honey samples and in detecting *C. botulinum* from the enriched samples may contribute to the different outcome. Screening the samples with PCR may provide a more sensitive detection.

Due to the high viscosity of honey, spores of *C. botulinum*, which typically exist in low numbers in extracted honey, are unevenly distributed. This complicates both sampling and detection and may result in false-negative results. The results on prevalence and especially quantity must therefore be considered rough estimates, and honey from a producer may not be defined as free from botulinal spores based on a negative result from one sample.

## 6.5 Factors affecting contamination of honey with *Clostridium botulinum* (V)

In samples taken from apiaries and soil, the prevalence of *C. botulinum* type B was high, and among the types A, B, E and F, type B seems to predominate in all environments other than aquatic in Finland. Group I type B therefore seems to be responsible for the contamination of honey. In previous studies on the prevalence of *C. botulinum* serotypes A, B, E and F in Northern Europe, an extensive spread of type E was reported in samples from sediment and fish (Johannsen 1962, 1963, Huss 1980, Hielm *et al.* 1998b, 1998c, Hyytiä *et al.* 1998). In addition, serotype B has been found in faecal samples from Swedish pigs (Dahlenborg *et al.* 2001) and sporadically in environmental samples (Huss 1980).

The high prevalence and quantity of *C. botulinum* spores in beeswax and materials associated with wax (V) suggest a role for beeswax in contaminating honey. Contamination of the wax might occur via dust carried by bees from the soil (V). In the beehive, the soft wax of the combs provides a sticky surface for the spores to adhere. If the soil surface in the vicinity of the apiary consists of moss or gravel, the possibility of bees carrying spores of *C. botulinum* is increased. As spores seem to be captured in beeswax, the cycle of spores in honey production could be broken by the renewal of old wax frames with new foundations, produced using temperatures sufficiently high to destroy group I botulinal spores. The

straining of honey should also be performed carefully to remove all wax particles, although no significant correlation was observed between different straining techniques and the existence of *C. botulinum* in honey (V, Fig. 4). Placing the apiaries in areas covered with vegetation, other than moss, may also decrease the sporeload in the beehive. Although bees do not usually land on ground, they may land on wet or damp moss in order to gather water. Gravelly areas may release more dust to the air than vegetative areas, thus increasing the contamination of bees or the hive itself (V).

Due to the high prevalence of *C. botulinum* in Danish samples, we suggested in Study IV that the extensive pig farming in Denmark could have an impact on the contamination. This assumption is based on the report on *C. botulinum* type B being frequently detected in faecal samples of swine in Sweden (Dahlenborg *et al.* 2001) and Denmark being known for its extensive pig farming. Honeybees tend to prefer a dirty water source to a cleaner one, possibly due to its odor and salt content (Johansson and Johansson 1978), and therefore, water contaminated with manure may also be utilized (Butler 1940). In addition to being a nutritive substance, water is spread over the combs to regulate the temperature and humidity inside the beehive (Johansson and Johansson 1978). Of the Finnish apiaries participating in the survey, very few were located within 3 km from a pig farm, and no correlation between *C. botulinum* being detected in the hive or extracted honey and the vicinity of a pig farm was observed.

According to the statistical analysis, no significant correlation existed between *C. botulinum* being present in extracted honey and *C. botulinum* being present in beeswax or comb honey. The comparison between apiary-related samples and extracted honey samples may, however, have been distorted, as extracted honey typically represents the entire production of a beekeeper, not only that of the apiaries included in the survey. The statistical analysis highlighted the role of production hygiene during extraction and straining in the contamination of extracted honey. The prevalence of spores in honey could be diminished by equipping the extraction room with proper facilities for hand-washing and adequate illumination. By wearing different footwear inside and outside the extraction room, the beekeeper may avoid transporting dust and spores indoors. A spacious extraction room and a high-capacity extractor also correlate with low prevalence of spores in honey. High production hygiene standards thus seem to be as important with respect to food safety in honey production as in any other field of food production, despite honey being considered a food item not easily spoiled. Hygienic factors may also play a role in the high prevalence of *C. botulinum* in Danish honey.

By maintaining an adequate level of hygiene in the extraction facilities and by frequently renewing wax foundations, the *C. botulinum* spore level may be reduced. However, due to the observed wide existence of *C. botulinum* spores in the environment, it is not possible to produce honey totally free from spores.

## 7. CONCLUSIONS

1. *C. botulinum* was shown to have caused the unexpected death of an infant. The likely source of spores was the infant's environment; honey consumption was not associated with the case. No cases of infant botulism have previously been reported in Finland. However, the diagnosis of infant botulism may be overlooked in cases of sudden infant death, particularly in countries with a low reported incidence of infant botulism.
2. The supernatant filtration method combined with PCR enables a sensitive, rapid, and ethically acceptable means of screening honey samples for the presence of *C. botulinum* spores.
3. A PFGE protocol for *C. botulinum* group I was optimized. Restriction enzymes *SacII*, *SmaI* and *XhoI* are all suitable for strain identification. However, when using a single enzyme, *SacII* provides the most cost-efficient and yet reliable choice. When examined with PFGE, group I *C. botulinum* emerges as a heterogeneous species, with a highly consistent genotype. The clearly distinct clusters observed within the Finnish isolates and isolates from other Nordic countries suggest the existence of different genetic lineages within *C. botulinum* group I type B. The PFGE method is a useful tool for studying the epidemiological relatedness of proteolytic *C. botulinum* isolates types A and B obtained from patients and from food. However, the existence of isolates sharing highly similar PFGE profiles, especially within group I type B strains, necessitates that epidemiological conclusions based on PFGE data alone be made with caution.
4. Honey produced in Finland may also contain *C. botulinum* spores. The relatively high prevalence of spores in the honey samples analysed may be due to the sensitivity of the detection method used. The prevalence of *C. botulinum* varied considerably between Denmark, Norway and Sweden, being highest in Danish honey, and the predominating type in the Nordic countries, other than in Sweden, is group I type B.
5. The contamination of honey by spores of *C. botulinum* likely occurs either in the beehive or during the extraction phase. In both cases, the contamination seems to occur via dust, either carried into the beehive by bees and accumulating in the beeswax or carried into the extraction facilities by the beekeeper or on production equipment. Hand-washing facilities, adequate illumination and the use of different

footwear inside the extraction room appear to decrease the contamination pressure of honey with botulinal spores. The cycle of spores may be broken by replacing old wax frames with new foundations, produced using temperatures sufficiently high to destroy botulinal spores. Placement of the apiaries in areas covered with vegetation other than moss may also diminish the contamination in the hive via bees.

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