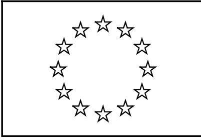

EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
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OPINION OF THE

SCIENTIFIC COMMITTEE ON VETERINARY MEASURES RELATING TO PUBLIC HEALTH

ON

HONEY AND MICROBIOLOGICAL HAZARDS

(adopted on 19-20 June 2002)

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1. BACKGROUND

Honey is used in the EU by the food industry and is also consumed directly by the population. It is known that honey may occasionally contain *Clostridium botulinum* spores, probably picked up and carried by bees (passive vector). Thus, some health authorities have discouraged its consumption by infants less than one year old, because infants of that age have not yet developed a competitive micro-flora in the gut.

Some Member States would like to take appropriate measures, such as labelling, at the Community level.

Chapter 2 of Annex II to Directive 92/118/EEC establishes that the health conditions applicable to trade and import of honey intended for human consumption shall be laid down by the Commission in accordance with the Standing Veterinary Committee procedures.

2. TERMS OF REFERENCE

The Scientific Committee on Veterinary Measures relating to Public Health is requested to assess the risk particularly for infants acquiring botulism from the consumption of honey, as honey seems to be epidemiologically linked with infant botulism. Furthermore, to advise on the possibility of setting criteria or possible preventive measures.

The Committee is requested to consider other microbiological hazards, related to honey, which could be relevant for Public Health, and to consider possible preventive measures or the potential need for setting criteria.

3. GENERAL INTRODUCTION ON HONEY

3.1. Origin of Honey (definition)

Honey, as laid down in Council Directive 2001/110/EC, is the natural sweet substance produced by *Apis mellifera* 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 honeycombs to ripen and mature. The two types of honey are, according to their origin:

- blossom honey or nectar honey: honey obtained from the nectar of plants;
- honeydew honey: honey obtained mainly from excretions of plant sucking insects (*Hemiptera*) on the living part of plants or secretions of living parts of plants.

3.2. Composition of Honey

The chemical composition of honey is highly dependent on its floral origin. However, the composition is broadly described by the following data (Bogdanov *et al.*, 1999).

<u>Carbohydrates</u>

Carbohydrates, ranging from 73 to 83%, constitute the main component of honey. The sum of the "reducing sugars" (fructose + glucose) is lower in honeydew honeys than in blossom honeys.

Constituent carbohydrates always present in honey can be listed as follows with appropriate ranges in their percentage levels:

Fructose 30.9 – 44.3%

Glucose 22.9 – 40.8%

Maltose 0.5 – 2.8%

Sucrose 0.8 – 10%

Isomaltose 0.5 – 1.5%

Turanose 0.5 – 1.5%

Nigerose 0.2 – 1.0%

The following carbohydrates are occasionally present in honey and at the percentage levels indicated:

Trehalose <2.5%[*]

Melibiose <0.5%

Palatinose <0.3%

Melezitose <5%[*]

Raffinose <1%[*]

Erlose <3.5%[**]

<u>Water</u>

The water content of honey is usually between 14.5 and 18.5%. Higher values may induce fermentation. However, some unifloral honeys

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* Trehalose, melezitose and raffinose are characteristic of honeydew honeys.
** Erlose is characteristic of some unifloral honeys, like honeydew, lavender, rosemary and acacia honeys.

normally have higher water contents (chestnut 17-19%, heather up to 21%).

<u>Other constituents</u>

Minor constituents (less than 1.5% of the whole honey):

- Organic acids (0.6%): glucoronic acid (primarily), acetic, butyric, citric, formic (also present in bee venom), lactic, malic, pyroglutamic and succinic acids. They confer an acidic pH to honey (range: 3.4 – 6.1). Honeydew honeys have a higher pH value than blossom honeys. The total acidity of honey lies between 8 and 40 meq/kg.

- Nitrogenous compounds (0.4%): proteins (0.3%), amino acids 0.05 – 0.1% (primarily proline), enzymes (amylase, α-glucosidase etc.).

- Minerals (0.1%): mainly potassium (0.05%), phosphorous (0.005%), calcium (0.0048%), sodium (0.0029%), magnesium (0.002%) have been found.

- Vitamins, lipids and aromatic substances: honey is not a good source of these compounds.

3.3. Production and Processing Practices

3.3.1. Collecting

A foraging bee goes on between twenty and fifty flights per day, each of them lasting about 15 min, gathering the nectar, which is the sweet liquid (containing about 90% water) excreted by plants.

The transformation of the nectar into honey begins during the flight in the crop where it has been stored.

After returning to the hive, the foraging bee regurgitates its load, which is taken by a worker bee. The thickened drop (now containing about 50% water) is then carried from the worker bee to another, and into a cell.

The transformed nectar stored in the cells is then evaporated by the temperature of the beehive (about 36°C) and, especially, by a continuous powerful air stream produced by the wing-flapping of specialised bees.

When the nectar has been changed into honey (about 20% water), wax-making bees cap the cells.

3.3.2. Harvesting

After the bees have been smoked out, the honeycombs are taken from the upper part of the hive. The capping of the cells is removed with a scraper or a knife and the combs are introduced into a stainless steel centrifugal extractor. During the rotation, the honey is drained from the

uncapped cells to the bottom of the centrifuge from where it is recovered (Alphandery, 1992).

3.3.3. Purification

The honey is purified by straining or decantation.

Straining

The honey is heated to 30°-35°C and then filtered through a strainer (mesh size 0.8 to 1 mm) or a tubular sieve (0.4 to 0.5 mm) and put on the honey ripener. Wax particles and foreign matter (e.g. bee fragments, small pieces of propolis, wood splinters etc.) are removed.

Decantation

The honey is put directly into the honey ripeners, maintained at 25°C, so that the air bubbles and the waxy and other impurities (except the pollen grains) come up to the surface.

3.3.4. Ripening

The liquid honey is held for two weeks at 15°C.

3.3.5. Packaging

The honey is drawn off, generally by pumping, and distributed into containers.

3.3.6. Storage

Honey stored in sealed containers can remain stable for a very long time. For practical purposes, a shelf life of two years is often stated. Processed honey should be stored between 18°–24°C (White, 1978). Honey can be exposed to higher temperature for brief periods. However, heat damage is cumulative, so exposure to heat should be limited. When honey is overheated, hexoses like fructose or glucose lose three molecules of water to form 5-hydroxy-2-furaldehyde also named hydroxy-methyl-furfural (HMF). The maximum permitted content of HMF in honey is 40 mg/kg, as laid down in Council Directive 2001/110/EC, to confirm that the honey is neither too old, overheated, nor adulterated, i.e. as a measure of quality.

3.3.7. Optional operations

Melting

Most honeys are supersaturated with respect to glucose, which may cause glucose to crystallise spontaneously at room temperature in the form of glucose monohydrate. The rate at which crystallisation occurs depends on the composition of the honey. A honey with a glucose/water ratio >2.1 usually crystallises quickly (Doner, 1977).

Spontaneous crystallisation of honey with a high water content (>18.5%) causes the product to become cloudy and less appealing to the consumer. This results in separation into two phases: a liquid phase at the top of the container, and a more solid phase at the bottom. In such cases, it is necessary to reverse the crystallisation by heating the honey, which melts the crystals, because the higher moisture content of the liquid phase could allow any osmophilic yeasts present to multiply and ferment the honey.

Melting is a common operation in honey production.

Controlled crystallisation

It is possible to induce and control crystallisation to produce creamed honey. This process yields very fine crystals and a smooth product with a texture resembling peanut butter.

The process consists of mixing a totally liquid honey (90%) with a fine crystallised honey (10%) at 25°–27°C. After a few hours decantation at the same temperature, the honey is put into the containers and stored at 14°C. The complete crystallisation occurs within 4–5 days (Assil *et al.*, 1991).

Pasteurisation

Honey can be heat-treated to prevent unwanted fermentation by osmophilic yeasts and/or to delay crystallisation (up to 9-10 months). One common heat treatment is 77°C for 2 min. followed by rapid cooling to 54°C (e.g. with plate heat exchangers).

Other treatments include heating honey to 60°C for 30 min. or 71°C for 1 min., or an equivalent heat treatment between those two temperatures.

Many honeys are not pasteurised.

Filtration

The honey is first heated to about 60°C at which it is totally liquefied. It is then filtered through ceramic or diatomaceous filters, the mesh of which is less than 50 μ. The result of this operation is the removal of almost all of the extraneous solids and pollen grains. The disadvantage of this process is that it becomes impossible to determine the floral origin, and consequently the geographical origin, of such filtered honey without the pollen grains. Another risk is that the HMF level of the filtered honey may exceed the upper limit of 40 mg/kg fixed by Council Directive 2001/110/EC. Consequently, most of the main honey-producing countries do not produce filtered honey.

3.4. Production and Trade data

3.4.1. World honey production

The annual world honey production is estimated by the FAO at 1,200,000 tonnes. The Annex (see Tables 4 to 7) shows the distribution of honey between the world's continents (Anon., 2001).

3.4.2. EU production

Within the EU there are approximately 50,000 professional beekeepers (some 10,000 of whom have beekeeping as their only source of income) and 400,000 amateur beekeepers (Anon., 2001).

The annual honey production in the EU is estimated at 130,000 tonnes, mainly produced in the South of Europe (France 31,000; Spain 30,000; Greece 15,000; Portugal 11,000 and Italy 10,000). (See Annex).

In the North of Europe (United Kingdom, Belgium, The Netherlands, Luxembourg, Germany and Denmark) there are generally only amateur beekeepers whose products are only sold locally.

3.4.3. International trade

International trade in honey is ca. 300,000 tonnes per annum (25% of the whole production), usually packed in 300 kg barrels.

The three main exporting countries are: China with 75,000 tonnes/year, Argentina with 60,000 tonnes/year; and Mexico with 30,000 tonnes/year). Others exporting countries include Canada, Australia, New Zealand, Cuba, Turkey, Hungary, Romania and Poland.

The main importing countries are the USA with 60,000 tonnes/year; Japan with 36,000 tonnes/year and the EU with 140,000 tonnes/year. Other importing countries include Saudi Arabia, the Maghreb (Morocco, Algeria, Tunisia) and the Middle East, but in smaller quantities, and only in jars.

An overview of the individual consumption through all the EU Member States is shown in Table 1.

Table 1: Annual honey consumption in the EU (kg/person) (Anon., 2001)

	1996	1997	1998
Austria	1.5	1.3	1.1
Belgium & Luxembourg	0.5	0.8	0.7
Denmark	0.6	0.9	0.8
Finland	0.6	0.4	0.6
France	0.6	0.6	0.6
Germany	1.4	1.0	1.1
Greece	1.5	1.5	1.6
Ireland	0.5	0.5	0.3
Italy	0.4	0.4	0.4
The Netherlands	0.4	0.4	0.4
Portugal	0.5	1.1	1.1
Spain	1.0	0.7	0.8
Sweden	0.5	0.7	0.5
United Kingdom	0.4	0.4	0.4
Average	**0.7**	**0.8**	**0.7**

The annual honey consumption in the EU totals 270,000 tonnes. Therefore the EU has to import practically half of its needs. The EU imports from 1995 to 1999 are shown in the Annex (Anon., 2001).

4. MICROORGANISMS IN HONEY

An extensive review of the literature and of epidemiological data revealed *C. botulinum* and other clostridia producing botulinum neurotoxin to be the only microbiological hazard in honey. Although *Bacillus* spp. are often detected in honey, sometimes in high numbers, there is no record of their having caused illness.

Primary sources of microbiological contamination are likely to include pollen, the digestive tracts of honeybees, dust, air, earth and nectar. Such sources make the microbial contamination impossible to control.

The same secondary (post-harvest) sources of contamination that influence any food product are also sources of contamination of honey. These include buildings, equipment, food handlers, cross-contamination and air. With the exception of air, contamination from these sources is controlled by good manufacturing practices (GMP).

The microbes of concern in honey are primarily yeasts and spore-forming bacteria (Snowdon and Cliver, 1996). Total plate counts from honey samples can vary from zero to tens of thousands per gram for no predictable or

discernible reason. Most samples of honey contain detectable numbers of yeasts. Although yeast counts in many honey samples are below 100 colony-forming units per gram, some osmophilic yeasts can grow in honey to very high numbers. This is monitored by standard industry practices, including viable counts of microbes. Bacterial spores, particularly those in the *Bacillus* genus, are regularly found in honey. Clostridial spores are also found, but less frequently. The spores of *C. botulinum* are found in a fraction of the honey samples tested, normally at low levels (Snowdon and Cliver, 1996).

No vegetative forms of pathogenic bacterial species have been found in honey, because honey has inhibitory properties that discourage the growth or persistence of many microorganisms (Snowdon and Cliver, 1996; Anon., 2000).

The factors that may contribute to the inhibitory properties of honey include (Molan, 1992a, b; Snowdon and Cliver, 1996):

- High osmotic pressure, low water activity (0.5 - 0.6)
- Low pH: 3.4-5.5 (acidic environment)
- Glucose oxidase system (forms hydrogen peroxide)
- Low protein content (high carbon to nitrogen ratio)
- Low redox potential (high content of reducing sugars)
- Viscosity (opposes convection currents and limits dissolved oxygen)
- Antioxidants (pinocembrin, pinobanksin, chrysin, galagin)
- Other chemical agents (lysozyme, phenolic acids, terpenes, benzyl alcohol etc.).

Having reviewed the scientific literature, *C. botulinum* appears to be the main microorganism in honey of concern to human health.

5. HAZARD IDENTIFICATION : *CLOSTRIDIUM BOTULINUM*

Clostridium botulinum was defined by Prévot (1953) as the species designation for all organisms producing botulinum neurotoxin, currently comprising seven serologically distinct toxin types (A-G). A toxin type may not be restricted to one phenotypic group of organisms. The most important groups in foodborne botulism in man are Group I (proteolytic, mesophilic) and Group II (non-proteolytic / saccharolytic, psychrotrophic). The main distinguishing biochemical features differentiating Groups I and II, and those of clostridia producing botulinum neurotoxin are shown in the Annex (Tables 8 and 9). The taxonomic status of *C. botulinum*, based on phenotype, was reviewed by Hatheway (1993). The relationship of the various types of *C. botulinum* and other clostridia capable of producing botulinum neurotoxin, based on 16S rRNA sequencing, is explored by Lawson *et al*. (1993).

Four different forms of botulism are recognized. If *C. botulinum* multiplies in a food, and that food is subsequently ingested without heating, the person consuming it suffers botulism via **intoxication** from preformed toxin. There are reports that a form of botulism occurs when *C. botulinum* multiplies in the gut, producing neurotoxin and causing a **toxico-infection** (Matveev *et al*., 1967; Chia *et al*., 1986; McCroskey and Hatheway, 1988). In **wound botulism**, *C. botulinum* establishes itself and multiplies in damaged tissues, and in doing so produces neurotoxin, which is absorbed, causing botulism (Merson and Dowell, 1973). The fourth form is **infant botulism** when multiplication and toxin production occurs in the infant gut (Chin *et al*., 1979; Arnon, 1998; www.infantbot.org).

Clostridium botulinum has the capacity to affect essentially all vertebrates (Table 2).

Table 2. Types of *Clostridium botulinum* and species affected by botulism

Type	Species affected	Vehicle
A	Man (also wound & infant)	Home - canned vegetables
A	Chicken ("limberneck")	Fruit, meat, fish
B	Man (also wound & infant)	Prepared meats (esp. pork), cattle, horses
Cα	Aquatic wild birds ("Western duck sickness")	Toxin-bearing invertebrates Carrion, forage
Cβ	Cattle ("Midland cattle disease"), Horses, ("forage poisoning") Mink	Feed or grazing contaminated with decomposing poultry waste; accidental ingestion of parts of putrid mice or cats Feed is raw minced (ground) offal and abattoir waste; unless temperature is reduced rapidly and controlled below 10°C, type C is able to grow and produce toxin
D	Cattle ("lamziekte")	Carrion
E	Man	Uncooked products of fish and marine mammals
F	Man	Home-made liver paste "deer-jerky"
G ***C. argentinense***	Unknown (man ?)	?

Man is most commonly affected by types A, B and E, with a few reports of type F. Types C and D have been suspected of causing human botulism and reported in the literature, but by the criteria applied today, types C and D botulism have not been confirmed in man (discussed in Roberts and Gibson, 1979; Hatheway, 1993). While surveying soil in Argentina, Gimenez and Ciccarelli (1970) isolated an organism that produced a toxin that killed mice with symptoms indistinguishable from those of botulism, but not neutralised by any monovalent or polyvalent *C. botulinum* antitoxins. This was named *C. botulinum* type G, but has since been reclassified as *C. argentinense* (Suen *et al*., 1988). Although type G was isolated retrospectively after sudden death in adults (Sonnabend *et*

al., 1981) and infants (Sonnabend *et al*., 1985) doubt remains whether it was the cause of the deaths (Hatheway, 1993). Type G has never been demonstrated in, or isolated from, foods.

Clostridium botulinum can be isolated from soil, mud and aquatic sediments, the frequency of isolation and the type isolated varying with the geographical region. For example, soil in the eastern states of the USA mainly carries type B, and that in western states type A. The Great Lakes region of the USA and the Baltic countries and associated seas carry mainly types E. The occurrence of *C. botulinum* in the environment is comprehensively reviewed by Dodds (1993a). It is also relatively common in some raw foods e.g. Baltic herring (Johannsen, 1963), Pacific salmon, trout farmed in ponds with earth bottoms (Bach and Muller Prasuhn, 1971; Huss *et al*., 1974a, b), pork (Roberts and Smart, 1976, 1977; Tompkin, 1980), mushrooms (Hauschild *et al*., 1975). It has also been isolated from fruit, vegetables, onion skins and garlic cloves (Solomon and Kautter, 1986, 1988), cabbage, spinach (Insalata *et al*., 1970), honey (Sugiyama *et al*, 1978; Midura *et al*, 1979), but not corn syrup (Lilly *et al*., 1991).

No means are known to prevent, or control, the frequency of contamination, so food processors assume spores of *C. botulinum* may be present and adjust processing to destroy spores, or preservation to prevent toxin production.

6. HAZARD IDENTIFICATION OF INFANT BOTULISM

6.1. Characteristics of the pathogen and its ability to cause disease in the host.

Epidemiological information worldwide indicates that *C. botulinum* type A and type B are most frequently involved in infant botulism, while *C. botulinum* type E and type F have more rarely caused the disease (Arnon, 1998). *Clostridium botulinum* concomitantly producing 2 different neurotoxins (type B and type F) has been isolated from infant botulism (Hatheway and McCroskey, 1987). Unusual strains of *C. butyricum* and *C. baratii* that produce botulinum toxins type E and F, respectively, have also caused infant botulism (Aureli *et al.*, 1986; Hall *et al.*, 1985; Trethon *et al.*, 1995). Strains producing type C and G botulinum toxins have supposedly been associated with the disease (Oguma *et al.*, 1990; Sonnabend *et al.*, 1985).

The different serotypes found in human cases seem to reflect the environmental distribution, rather than differences in the ability to cause disease in the host. Proteolytic strains of *C. botulinum* are more frequently related to infant botulism than non-proteolytic strains (Arnon, 1998).

6.2. Host susceptibility

Age is the only recognized predisposing factor for infant botulism: indeed, most of the reported cases have occurred in patients < 6 months old and all in infants less than 1 year old. Healthy adults and older children normally ingest *C. botulinum* spores in contaminated fresh foods, without developing illness. The reason is related to the host

intestinal microflora, which is quantitatively and qualitatively simpler in young infants, and does not efficiently prevent colonisation of the intestine by exogenous spores of neurotoxigenic clostridia, as demonstrated in animal models (Sugiyama and Mills, 1978). Although the diet of infants (breast feeding, formula feeding, consumption of solid foods) influences the composition of the intestinal flora, a clear cause-effect relationship between the type of diet and illness cannot be drawn. In fact, while most hospitalised patients are breast-fed, age at onset of botulism is younger in formula-fed infants, possibly as a consequence of their weaker, or unbalanced, immune status or the gut microflora. Comparative studies of the immune status of infants are revealing roles for the environment and nutrition (Björksten *et al*., 2001; Bottcher *et al*., 2000; Julge *et al*., 2001; Kalliomäki *et al*., 2001; Van Ogtrop *et al*., 1991).

Additional predisposing factors may be of a physiological nature, such as reduced intestinal motility, that can favour colonisation by clostridial spores. It has recently been observed that patients suffering from infant botulism due to type E neurotoxigenic *C. butyricum* were affected with Meckel's diverticulum, a vestigial form of the umbilicus channel, which might constitute a favourable niche for microbial persistence and colonisation (Fenicia *et al.*, 1999).

6.3. Food matrix: infant botulism

Although the environment (soil, dust) is thought to play a critical role as a source of infecting clostridia for infants, honey is the only dietary vehicle so far definitely linked to infant botulism (Arnon *et al.*, 1979), with the outstanding exception of a formula milk powder provisionally implicated in a case of infant botulism in the UK (O'Brien, 2001).

Both epidemiological and laboratory data contributed to identifying honey as a risk factor for infant botulism. In Europe, a majority of infant botulism patients (30 out of 49 cases) had a history of having been fed honey (see Table 3); *C. botulinum* organisms of the same toxin type as isolated from affected patients were found in honey fed to 5 cases (Balslev *et al.*, 1997; Jung and Ottoson, 2001; Fenicia *et al.*, 1993; Greve *et al.*, 1993). A few cases have been traced to contamination of the infant gut from the environment, presumably transmitted orally or nasally.

7. HAZARD CHARACTERISATION : INFANT BOTULISM

7.1. Epidemiology

A case definition used by CDC (1997) for Infant Botulism is: A clinically compatible case that is laboratory-confirmed, occurring in children under 1 year of age.

In Europe, since the first report in 1978 (Turner *et al.*, 1978), forty-nine cases of infant botulism have been described (in the Czech Republic, Denmark, France, Germany, Hungary, Italy, Netherlands, Norway,

Spain, Sweden, Switzerland, and UK). All affected babies were less than 1 year old, with 93% being younger than 6 months; the mean age at onset was 13 weeks. Males and females were affected in approximately equal proportions, with a slightly higher prevalence in males. The majority of patients had been breast-fed, while most had a history of having been fed honey. Most cases were due to *C. botulinum* type B (18 cases) and type A (11 cases), reflecting the distribution of toxin types in the environment. Exceptional cases, due to *C. butyricum* producing type E botulinum neurotoxin have been reported in Italy (Aureli *et al.*, 1986) and *C. baratii* producing type F botulinum neurotoxin in New Mexico (Hall *et al.*, 1985) and Hungary (Trethon *et al.,* 1995).

Cases of infant botulism in Europe are summarised in Table 3.

To date, more than 1,000 cases of infant botulism have been reported in all continents, except Africa; 90% of them have occurred in the US.

Table 3 Infant botulism in Europe[1]

Country	Strain Type	Age at onset (weeks)	Milk/Feeding	History of honey consumption	References
Czech Republic	*C. botulinum* type B	4	breast fed	No	Neubauer and Milaceck, 1981
Denmark	*C. botulinum* n.t.	5	n.s.	Yes*	Jung and Ottosson, 2001
	C. botulinum types A, E	12	breast fed	Yes*	Balslev *et al.*, 1997
France	*C. botulinum* type B	44	n.s.	No	Paty *et al.*, 1987
Germany	*C. botulinum* n.t.	8	n.s.	No	Greve *et al.*, 1993
	C. botulinum n.t.	6	n.s.	Yes	#
	C. botulinum n.t.	10	breast fed	n.s.	#
	C. botulinum type A	14	breast fed	Yes	Mueller-Bunke *et al.*, 2000
Hungary	*C. barattii* type F	n.s.	breast fed	Yes	Trethon *et al.*, 1995
	C. botulinum type A	12	n.s.	n.s.	#
Italy	*C. butyricum* type E	16	formula milk	Yes	Aureli *et al.*, 1986
	C. butyricum type E	16	breast fed	Yes	Aureli *et al.*, 1986
	C. botulinum type B	6	breast fed	Yes	Aureli *et al.*, 1989
	C. botulinum type A	12	breast fed	Yes	Fenicia *et al.*, 1989
	C. botulinum type B	8	breast fed	Yes	#
	C. botulinum type B	9	breast fed	Yes*	Fenicia *et al.*, 1993
	C. botulinum type B	12	breast fed	Yes	Calvani *et al.*, 1997
	C. botulinum type B	8	breast fed	n.s.	#
	C. botulinum type B	28	breast fed	No	#
	C. butyricum type E	20	breast fed	Yes	Franciosa *et al.*, 1998
	C. botulinum type B	4	breast fed	No	#
	C. botulinum type A	8	breast fed	Yes	#
	C. butyricum type E	28	breast fed	No	#
	C. botulinum type B	20	breast fed	No	#
	C. botulinum type B	8	breast fed	Yes	#
	C. botulinum type B	8	breast fed	No	#
	C. botulinum type B	10	breast fed	No	Li Moli *et al.*, 1996

[1] Modified from Aureli *et al.*, 2002.

Country	Strain type	Age at onset (weeks)	Milk/feeding	History of honey consumption	References
Netherlands	*C. botulinum* n.t.	10	breast and formula milk fed	Yes	Wolters, 2000
Norway	*C. botulinum* type A	12	breast fed	Yes*	Tollofsrud *et al.*, 1998
	C. botulinum type A	14	n.s.	Yes*	Yndestad (2002) personal communication
	C. botulinum type A	10	n.s.	Yes*	Yndestad (2002) personal communication
	C. botulinum type A	5	n.s.	Yes*	Yndestad (2002) personal communication
Spain	*C. botulinum* type A	20	n.s.	Yes	#
	C. botulinum n.t.	8	n.s.	Yes	#
	C. botulinum type B	12	n.s.	Yes	#
	C. botulinum n.t.	12	n.s.	Yes	#
	C. botulinum n.t.	16	n.s.	No	#
	C. botulinum n.t.	12	n.s.	Yes	#
	C. botulinum type A	20	breast fed	Yes	Torres Tortosa *et al.*, 1986
	C. botulinum n.t.	20	breast fed	Yes	Lizarraga Azparren *et al.*, 1996
	C. botulinum type B	8	breast fed	Yes*	Castell Monsalve and Nieto Sandoval Alcolea, 1999
Sweden	*C. botulinum* type A	7	breast fed	Yes	Jansson *et al.*, 1985
Switzerland	*C. botulinum* type A	12	breast fed	No	Gautier *et al.*, 1989
United Kingdom	*C. botulinum* type A	24	breast fed	n.s.	Turner *et al.*, 1978
	C. botulinum type B	20	breast fed	n.s.	Anon., 1987
	C. botulinum type BF	16	breast fed	No	Smith *et al.*, 1989
	C. botulinum type B	16	n.s.	n.s.	Gilbert and Brett., 1993
	C. botulinum type A	16	breast fed	Yes	Gilbert and Brett., 1994
	C. botulinum type B	20	n.s.	No**	CDSC, 2001

*honey containing *C. botulinum* organisms of the same type as isolated from affected infants

** patient fed with a formula milk powder contaminated with *C. botulinum* spores of the same toxin type as those isolated from faecal specimen

n.s. = not specified

n.t. = not typed by monovalent antitoxins, but confirmed by polyvalent antitoxins.

Data concerning cases for which a bibliographic reference is not available but were provided as personal communication by Gervelmeyer, A. (Germany); Zsuzsa, B. (Hungary); Aureli, P. (Italy); Tello Anchuela, O. (Spain).

7.2. Infant botulism disease: pathogenesis and clinical spectrum

Infant botulism, first recognised in 1976, is an intestinal toxaemia caused by botulinum toxin-producing clostridia, which affects babies under 1 year of age. Ingested spores of neurotoxigenic clostridia can survive the acidity of the stomach and reach the intestine. Lack of competition by the immature host gut microflora permits clostridial multiplication and colonisation within the intestine, with subsequent botulinum toxin production and its release into the gut lumen (Arnon, 1995). The neurotoxin is then absorbed into the bloodstream and carried to the peripheral nerve endings, where it enzymatically cleaves specific protein targets involved in the process of neuroexocytosis (Montecucco and Schiavo, 1993). Their cleavage impairs the release of the acetylcholine neurotransmitter at the nerve endings, progressively blocks the nerve pulse transmission, and ultimately produces the symmetric and descending flaccid paralysis characteristic of botulism. The high potency of the botulinum toxin (estimated lethal dose for humans: 1 ng/kg body weight) is due both to the high specificity of the molecular targets and to the protease activity.

The severity of the paralysis is related to the number of affected nerves. The clinical spectrum of the disease ranges from mild to fatal; early symptoms including constipation, lethargy, poor feeding and weak crying. These symptoms may be followed by more serious hypotonia, floppiness, loss of head control, and progression to coma and respiratory arrest (Arnon, 1998). It has been speculated that the course of the illness might either be affected by differential absorption of the botulinum toxin, due to individual variation among infants, or depend on the site of the intestine where the toxin is absorbed (Arnon, 1980). Treatment with penicillin, results in lysis of the bacterial cells and the release of neurotoxin previously bound inside the cell.

While sub-clinical infections may be under-estimated because they do not need hospitalisation, severe outcomes of infant botulism may result in sudden unexpected death, which led to the hypothesis that infant botulism might be a cause of the Sudden Infant Death Syndrome (SIDS) (Arnon *et al.*, 1981). However, investigations of 248 SIDS cases in Australia failed to detect *C. botulinum* in samples from the small and large intestines and led to the conclusion that infant botulism was not a significant factor in the cause of sudden death (Byard *et al.*, 1992). Similar conclusions were reached in a separate survey of over 200 cases of SIDS in the UK (Berry *et al.*, 1987; Urquhart and Grist, 1976).

Duration of the illness varies from a few days to weeks or months, and the recovery also takes weeks to months, as new motor axon end-plates are formed.

Possible complications in infant botulism include adult respiratory distress syndrome, hypoxia, concomitant aspiration and obstruction, hyponatremia; secondary infections, such as urinary tract infections,

pneumonia, acute otitis and *C. difficile* colitis with manifestations of necrotising enterocolitis have also been reported.

Nevertheless, the mortality rate in "classic" infant botulism cases (i.e., excluding cases of SIDS) is extremely low in Europe, because all of the affected babies survived after appropriate treatment, which normally includes nasoenteric intubation as a preventive and/or supportive measure, depending on the severity of the symptoms.

7.3. Diagnosis

The clinical diagnosis of infant botulism relies on the careful examination of neurological symptoms; absence of fever and alert senses are also distinctive of infant botulism. However, despite the fact that the disease has been known for more than 25 years, it is still frequently misdiagnosed with other diseases, most often Guillain-Barré syndrome and myasthenia gravis (Arnon, 1998). Therefore, definitive confirmation of the clinical diagnosis requires demonstration of botulinum toxin and/or botulinum toxin-producing clostridia in the faecal specimen from the patient. Moreover, the persistence of botulinum toxin and/or the toxigenic microorganism in the faeces of affected infants is consistent with intestinal colonisation and toxaemia, typical of infant botulism (Wilcke *et al.*, 1980).

7.4. Dose-response relationship

Intra-gastric dose-response studies have been conducted on animal models. For infant mice, a 50% infective dose (ID_{50}) of 700 *C. botulinum* spores has been reported (Sugiyama and Mills, 1978), not significantly different from the ID_{50} = 1,500 observed in infant rats (Moberg and Sugiyama, 1980).

However, uncertainties may arise from several sources of variability, e.g. differences between different botulinum toxin-producing clostridial strains and variation in host susceptibility. This variability might influence the number of organisms required to produce illness, and possibly the severity of illness.

The minimum infective dose of *C. botulinum* spores for human infants is not known, but from exposure to spore-containing honey, it has been estimated to be as low as 10 to 100 spores (Arnon *et al.,* 1978). In one experiment the infective spore dose for infant mice was as low as 10 spores.

8. Exposure Assessment

8.1. Behaviour of *C. botulinum* in honey during processing

The only food so far implicated in infant botulism is honey. Primary sources of spore contamination of honey may include pollen, the digestive tracts of honey bees, dust, air, earth and nectar. In addition the

sweeteners destined as food for bees may be a source of spores in honey (Nakano *et al.,* 1992).

Secondary sources are contamination through air, food handlers, cross-contamination, equipment etc.. The primary sources of spores of *C. botulinum* are impossible to control.

Honey prepared for direct consumption may be unheated or pasteurised. Some producers also filter honey. None of these processes remove or inactivate spores of *C. botulinum*. While these processes may eliminate or reduce the numbers of many microbes, spores of *C. botulinum* types A or B of the proteolytic Group I, which are most frequently found in honey, will survive because of their heat resistance. Generally, the spores of Group I have a $D_{121°C}$ between 0.1 and 0.2 min and are, therefore, very heat resistant. Spores of Group II are less heat resistant but readily survive heating at 70°-77°C (ACMSF, 1992; ICMSF, 1996; Fernandez and Peck, 1997).

Heating to over 100°C (even 121°C) would be required to kill *C. botulinum*: however, heating honey to over 100°C would caramelise it and lead to exceeding the limit of HMF, the level of which must not exceed 40 mg/kg: also the diastase activity, which must not be lower than 8 (Schade scale), would be affected.

During maturation certain impurities rise to the surface and are removed (scraped off) but spores of *C. botulinum* remain in the honey.

Centrifugation to remove spores is not feasible because it would also remove pollen, which is an essential component for honey identification and labelling.

Honey used as an ingredient is subjected to several different processes depending on the food to which it is added. Some of these processes may inactivate any vegetative microbes and spores present, usually due to heat treatment (Snowdon and Cliver, 1996).

Regarding infant botulism, honey for direct consumption is most relevant and in the subsequent discussion we are referring to honey for direct consumption unless other types of honey are specified.

8.2. General growth and survival characteristics of *C. botulinum* in honey

In addition to *C. botulinum* type A and B, two cases of infant botulism due to a strain of *C. baratii* that produced a type F-like botulinum toxin in New Mexico in 1979 and more recently in Hungary, and a *C. butyricum* strain that produced a type E-like botulinum neurotoxin in Rome in 1986 have been described (Aureli *et al.*, 1986; Hall *et al.*, 1985; Hoffman *et al.*, 1982; Trethon *et al.*, 1995). From Japan one case of infant botulism caused by a strain of *C. botulinum* type C has been reported (Oguma *et al.*, 1990). With the exception of these cases, all cases of infant botulism have been caused by *C. botulinum* strains of the proteolytic Group I.

Although there are few experimental data regarding the fate of spores in honey during long-term storage, some laboratories have the experience that the spores have survived in honey for at least two years or more. Our general knowledge of survival of spores in nature and food also indicates that it is realistic to consider that spores of *C. botulinum* will survive for years in honey.

However, the duration of survival of spores in honey may depend on the storage temperature. According to Nakano *et al.*, (1989) the *C. botulinum* spore population in honey did not change over a year when stored at 4°C. At 25°C, however, the number of spores began to decrease after 100 days and no spores were detected after 5 days of storage at 65°C (Nakano *et al.*, 1989). However, these observations have not been confirmed by other researchers.

Clostridium botulinum will not multiply in honey even if the storage temperature is optimum for germination. This is due to honey's low water activity and its inhibitory properties.

Ingested spores of *C. botulinum* germinate and colonise the susceptible infant colon and produce botulinum neurotoxin. In turn the toxin is absorbed, binds to peripheral cholinergic synapses, and causes flaccid paralysis.

8.3. Prevalence of *C. botulinum* in honey

Spores of *C. botulinum* are widespread in nature. There is also strong evidence that the greatest exposure of infants to spores is from the general environment. In the USA it is well documented that the asymmetrical distribution of case toxin types parallels the distribution of *C. botulinum* spores in soil (Smith, 1978). Although some geographical regions of the world can be associated with a particular type of *C. botulinum* in the soil, it is not possible to identify countries as the origin of honey with a greater risk of containing *C. botulinum.*

Baseline surveys of honey samples have revealed that approximately 2 to 7% of the samples contain *C. botulinum* spores (Kautter *et al.*, 1982; Schocken-Iturrino *et al.*, 1999), but there is great variation between different geographical areas. Of 2,033 samples tested in different parts of the world, 5.1% contained detectable levels of *C. botulinum* spores (Snowdon and Cliver, 1996). Many studies have failed to detect spores of *C. botulinum* in honey.

Honey samples collected at retail level were found to be contaminated with *C. botulinum* spores in the USA (10% of the analysed samples), Japan (8.5%), Brazil (7.5%) and Italy (6.5%); with contamination levels between 5 and 80 spores/g of product (Nakano and Sakaguchi, 1991).

Nakano *et al.* (1989) found 23% positive samples of honey collected from apiaries, 18% from drums, and 5% in samples from retail packages. This corresponds well with results from another survey that reported higher incidence from samples taken from apiaries than from retail supplies (Sugiyama *et al.*, 1978). Nakano *et al.* (1989) observed a

decrease in viable spores after storage of the honey for a year at 25° C and concluded that some processes during purification and prolonged storage contributed to the lower incidence in marketing honey than in honey at apiaries.

Dodds (1993b), reported spore levels of *C. botulinum* in contaminated honey of between 1 and 10 spores per kg. However, the level was higher in honey samples associated with infant botulism, approximately 10^4 spores per kg (Dodds, 1993c; Austin, 1996). Snowdon and Cliver (1996), in their review, note that *C. botulinum* spores, if present, are typically found at levels of <1 cfu/g, but are infrequently found at levels as high as 60 cfu/g. The reason why samples of honey occasionally contain higher than usual levels of spores is unclear.

In the most recent survey (Nevas *et al.*, 2002) spores of *C. botulinum* were detected in 8 (7%) of the 114 Finnish honey samples and in 12 (16%) of the 76 imported honey samples, numbers in PCR-positive samples ranging from less than 18 to 140 spores/kg. Neurotoxin gene sequences corresponding to *C. botulinum* type A were detected in 17 samples and proteolytic type B in 12 samples by PCR analysis. Both types A and B were detected in nine samples. Of the 20 PCR-positive samples, strains of *C. botulinum* type A were isolated from 14 and type B from 2. This is the first report of type A spores of *C. botulinum* being detected and isolated in Fennoscandia.

Due to the low prevalence of *C. botulinum* spores in honey, a statistically-based sampling plan would require huge numbers of tests to be made, with a very low chance of successful detection.

Furthermore, no other microorganism is known to be a useful indicator of contamination of honey by *C. botulinum*.

8.4. Consumption data

The annual world honey production is estimated by the FAO to 1,200,000 tonnes.

The EU production is calculated to about 130,000 tonnes. In addition the EU imports about 140,000 tonnes yearly. The greatest exporting countries are China (75,000 tonnes/year), Argentina (60,000 tonnes/year) and Mexico (30,000 tonnes/year). The consumption in the EU is about 0.7-0.8 kg honey/person/year (see Table 1). The consumption for infants below 1 year of age is probably less than 1% of the whole consumption e.g. 7 to 8 mg per infant per year. As the honey has sometimes been shown to contain from 10,000 to 60,000 spores/kg (Dodds, 1993c; Austin, 1996) the infants would be exposed, on average, to 0.08-0.45 spores per year. However, many infants are never given honey, making the exposure for the infants in the group repeatedly exposed to honey because of traditional practices higher than indicated. Therefore it can be concluded that exposure of infants to spores of *C. botulinum* through consumption of honey is normally very low. However, for certain groups of infants exposure is higher and should not be ignored.

8.5. Risk factors for infant botulism

Laboratory and epidemiological evidence definitely associate the consumption of honey to infant botulism. Other products, like corn syrups, have also been considered, but it has been concluded that corn syrup does not constitute a source of *C. botulinum* spores or a risk factor for infant botulism. (Lilly *et al.*, 1991).

In addition to specific testing of honey and syrup, hundreds of traditional and non-traditional infant foods items, including formula milk, have been examined and found not to contain *C. botulinum* spores (Midura, 1979).

Thus consumption of honey must be considered as the most important risk factor associated with infant botulism. It should also be recognised that a slow intestinal transit time is also identified as a risk factor.

The infants are exposed to honey mainly where:

- the mother dips the nipple in honey before putting it in the mouth of the infant
- the parents smear the lips of the infant with honey
- the parents add honey to the formula fed to infants.

Applying a mouse model system for intestinal colonisation, studies have demonstrated that the intestinal flora normally prevents colonisation of the gut by *C. botulinum* (Burr and Sugiyama, 1982; Moberg and Sugiyama, 1979; Sugiyama and Mills, 1978). Infant mice, however, were susceptible to intestinal colonisation by *C. botulinum* with the highest susceptibility between days 8 and 11. This is a pattern that fits well with human infant botulism, which has a susceptibility peak between 2 and 4 months of age (Sugiyama and Mills, 1978; Arnon *et al.,* 1981). However, there seem to be considerable variation in this susceptibility (Hurst and Marsh, 1993; Hubert *et al.,* 1987).

The onset of infant botulism occurs at a significantly younger age in formula-fed infants (7.6 weeks) than in breast-fed infant (13.7 weeks) (Arnon *et al.,* 1982). The difference could be due to earlier ability in formula-fed infants to establish ecological niches in addition to absence of immune factors normally present in human milk (Arnon, 1980; Stark and Lee, 1982a, b).

There is general agreement that when children are about one year of age or older, there is no risk of infant botulism. However, there are a few exceptions. For children and adults undergoing heavy treatment with some antibacterials, or having some intestinal disturbances, there is always a risk of colonisation and toxin production by *C. botulinum*. However, very few cases have been reported for those categories worldwide and the risk remains low.

9. METHODS OF DETECTION

Microbiological monitoring for *C. botulinum* currently requires culturing for 3-5 days, and testing for toxin in a mouse bio-assay (slow, expensive, requires dedicated facilities, and in many countries a licence for animal experimentation).

Alternative molecular / genetic assays are being developed, but none available commercially have so far been validated against the mouse bio-assay.

The reference method for detection of botulinum neurotoxin remains the mouse bioassay (Schantz and Kautter, 1978) coupled with neutralisation with monovalent antisera. Production of those antisera has offered poor commercial returns, and, as a consequence, antisera are difficult to obtain.

For many years attempts to find alternative methods suffered from lack of sensitivity and specificity. In recent years many alternative methods of detection have been developed (see Annex for examples), but none are being produced commercially.

The (UK) Centre for Applied Microbiology and Research (CAMR) has developed a panel of sensitive assays for the botulinum toxin serotypes associated with human disease (types A, B, E and F). These novel *in vitro* assay systems are a modified ELISA (Hallis *et al*., 1996) in which the biological (endopeptidase) activities contained within the toxins are used to amplify the assay signal to provide tests that are of equivalent sensitivity to the mouse bioassay. The *in vitro* assay for botulinum type B neurotoxin has been assessed in a broad range of foodstuffs and shown to provide a robust method of detection with a sensitivity that exceeds that of the mouse assay (Wictome *et al*., 1999b). A major advantage of the assay format is the extremely low incidence of false-positive results, because the assay signal depends on both the recognition of the neurotoxins by specific antibodies and also the unique endopeptidase activities contained within the toxin light subunits.

Continuing research at CAMR is aimed at generating validated assays systems and reagents for all for botulinum serotypes associated with human disease, and also improving and simplifying the assay protocols to provide a reliable alternative to the mouse bioassay.

10. POSSIBLE PREVENTIVE MEASURES

In the absence of a process that would eliminate spores of *C. botulinum*, and because of the large numbers of tests that would be required to detect the low levels and sporadic occurrence of *C. botulinum*, labelling the products appears to be the most appropriate action.

A number of countries already label honey, recommending that it is not given to infants / children less than 12 months of age. Following the use of such labelling, the number of cases of infant botulism in those countries appears to have fallen. However because of the extremely low incidence of cases, no direct cause-effect relationship can be inferred.

Labelling can be supplemented with other means of conveying the message that honey should not be given to infants < 1 year of age e.g. brochures at pregnancy clinics, in languages appropriate to changing ethnicities in populations.

11. CONCLUSIONS

– Clostridial species with a capacity to produce botulinum neurotoxin (e.g. *C. botulinum*, *C. butyricum, C. baratii*) have been implicated in cases of infant botulism. The main organism implicated in cases of infant botulism is *C. botulinum.* A review of the scientific literature indicates that these clostridial species are the only microbiological hazard associated with honey. Other bacterial spores such as *Bacillus* spp. have been detected in honey, but are not known to pose a risk to human health.

– Honey has been associated generally with infant botulism in children of < 1 year of age, and mainly in infants < 6 months of age, after multiplication of clostridia and production of botulism neurotoxin in the infant gut.

– Although infant botulism is a serious illness, mortality is very low. In general, in Europe, the risk of infant botulism is extremely low. The majority of infants suffering from botulism have been given honey.

– The level and frequency of contamination of honey with spores of *C. botulinum* appear generally to be low, although limited microbiological testing of honey has been performed. The routes by which spores of *C. botulinum* contaminate honey have not been precisely identified.

– Although some geographical regions of the world can be associated with a particular type of *C. botulinum* in the soil, it is not possible to identify countries as the origin of honey with a greater risk of containing *C. botulinum.*

– *C. botulinum* can survive as spores in honey but cannot multiply or produce toxins due to the inhibitory properties of honey.

– At present there is no process that could be applied to remove or kill spores of *C. botulinum* in honey without impairing product quality.

– Microbiological testing would not be an effective control option against infant botulism, due to the sporadic occurrence and low levels of *C. botulinum* in honey.

12. RECOMMENDATIONS

– Microbiological criteria for honey are not recommended because they would not contribute to preventing infant botulism.

– It is recommended that effective and targeted information regarding the risks of infant botulism from the consumption of honey should be provided, e.g. via leaflets, labelling and via advice to health-care professionals.

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14. ANNEX

Table 4: World honey production (Anon., 2001)

	Production (x 1000 tonnes)				
	1995	**1996**	**1997**	**1998**	**1999**
Africa	133	138	139	137	138
North and Central America	192	182	190	213	203
South America	105	92	104	99	119
Asia	365	365	387	382	385
Europe	319	277	293	297	296
Oceania	28	35	36	31	32
Total	**1,142**	**1,089**	**1,149**	**1,159**	**1,173**

Table 5: EU honey production (x 1000 tonnes) (Anon., 2001)

Austria	5.5
Belgium & Luxembourg	2.0
Denmark	3.0
Finland	1.0
France	31.0
Germany	15.0
Greece	15.0
Ireland	0.1
Italy	11.0
The Netherlands	0.8
Portugal	11.0
Spain	30.4
Sweden	3.3
United Kingdom	4.0
Total	**133.1**

Table 6: European Union Imports of Honey from Third Countries 1995-1999 in tonnes (Anon., 2001)

Country	1995	1996	1997	1998	1999
Argentina	45,241	20,843	17,953	29,768	40,922
Australia	2,884	4,043	4,415	4,440	3,705
Bulgaria	1,654	3,752	3,781	1,838	1,733
Canada	3,492	1,942	2,085	3,414	3,289
Chile	1,609	2,861	1,181	2,706	2,313
China	42,272	48,873	46,664	55,524	33,174
Cuba	3,911	3,282	3,453	4,126	3,914
Hungary	10,532	12,033	8,729	6,726	7,476
Mexico	17,509	17,090	16,363	18,569	17,527
Romania	1,984	4,885	8,402	4,226	5,768
Uruguay	3,726	3,335	5,396	3,630	6,264
USA	513	534	221	245	154
Others	11,647	14,227	17,190	17,548	12,546
Total	146,974	137,700	135,833	152,760	138,785

Table 7: Intra-EU Trade in Honey 1995-1999 in tonnes (Anon., 2001)

Country	1995	1996	1997	1998	1999
Austria	59	162	336	112	2,158
Belgium/ Luxembourg	3,251	2,819	1,516	3,140	2,351
Denmark	1,672	1,564	1,485	1,882	2,541
Finland	0	36	0	0	0
France	5,773	3,996	1,788	1,943	1,857
Germany	12,343	15,653	12,720	16,856	14,954
Greece	190	334	136	170	73
Ireland	184	225	335	241	114
Italy	1,003	1,507	1,527	2,055	2,217
Netherlands	338	874	544	1,208	4,345
Portugal	282	645	1,106	673	497
Spain	3,760	5,761	7,380	7,484	4,102
Sweden	5	4	27	13	29
United Kingdom	1,353	3,651	2,093	4,344	3,340
Total	30,213	38,231	30,993	40,121	38,578

Table 8: Main distinguishing features of metabolic groups of *C. botulinum* (modified from Hatheway, 1993)

	Group			
Feature	**I**	**II**	**III**	**IV**
Toxin produced	A, B, F	B, E, F	C1, C2, D	G
Proteolysis	+	-	+	+
Lipolysis	+	+	+	-
Fermentation of glucose	+	+	+	-
Fermentation of mannose	-	+	+	-
Minimum growth temperature	10°-12°C	3.3°C.	15°C	*
Inhibited by NaCl (%)	10	5	3	>3
Volatile fatty acids**	A, iB, B, iV	A, B	A, P, B	A, iB, B, iV, PP, PA

**Key to fatty acids: A = acetic; P = propionic; iB = iso-butyric; B = butyric; iV = iso-valeric; V = valeric; PA = phenylacetic; PP = phenylpropionic (hydrocinnamic).

*minimum growth temperature not determined

Table 9: Main distinguishing features of other organisms producing *C. botulinum* neurotoxin (modified from Hatheway, 1993).

	C. butyricum	*C. barati*
Toxin type	E	F
Proteolysis	-	-
Lipolysis	-	+
Fermentation of glucose	+	+
Fermentation of mannose	+	+
Minimum growth temperature	10°C	*
Volatile fatty acids**	A, B	A, B

**fatty acids: A = acetic; P = propionic; iB = iso-butyric; B = butyric; iV = iso-valeric; V = valeric; PA = phenylacetic; PP = phenylpropionic (hydrocinnamic).

*minimum growth temperature not determined

Table 10 Detection of *Clostridium botulinum* and botulinal neurotoxins – examples

Procedure	Type(s)	References
Mouse lethality	A, B, C, D, E, F, G	Schantz and Kautter, 1978
ELISA	A, B	Shone *et al*., 1985
ELISA	G	Lewis *et al* ., 1981
endopeptidase	A, B	Hallis *et al*., 1996; Wictome *et al*., 1999a
PCR + 5 gene probes	A, B, E, F G	Fach *et al*., 1995
electroimmunodiffusion	A, E	Jay, 1996
ELISA-ELCA	E	Roman *et al*., 1994
PCR + capillary electrophoresis	E	Sciacchitano and Hirschfield, 1996
colorimetric capture ELISAS	A, B	Szilagyi *et al*., 2000
PCR	E	Kimura *et al*., 2001
fluoroimmunoassays using ganglioside-earing liposomes	All*	Singh *et al*., 2000
PCR and molecular probes	A, B, E	Braconnier *et al*., 2001
immuno-PCR	A	Wu *et al*., 2001
enrichment PCR	BEF	Dahlenborg, *et al*., 2001
multiplex PCR	ABEF	Lindstrom *et al*., 2001
PCR	AB	Nevas *et al*., 2002
fibre optic-based biosensor	A	Ogert *et al*., 1992
enzyme-linked immunosorbent assay and enzyme-linked coagulation	ABE	Doellgast *et al*., 1994
evanescent wave immunosensor	All*	Kumar *et al*., 1994
PCR	A	Fach *et al*., 1993
PCR Gene probes	BEF	Campbell *et al*., 1993
enzyme-linked immunosorbent assay and signal amplification via enzyme-linked coagulation assay	ABE	Doellgast *et al*., 1993
PCR and DNA probes	ABEF	Aranda *et al*., 1997

All * = in principle

15. ACKNOWLEDGEMENTS

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The working group was chaired by

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and included the following members:

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- Dr. Christian Flamini
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