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Incidence of *Clostridium botulinum* Spores in Honey and Infant Food Samples Collected from Vietnam and Germany

Dissertation
to obtain the Ph.D. degree in the Faculty of Agricultural Sciences,
Georg-August-University Göttingen

submitted by

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Göttingen, November 2006

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Oral examination: 02 November 2006

Dedication

to ***my family*** with love

CONTENTS

ABBREVIATIONS

Ab	antibody
AOAC	Association of Official Agricultural Chemists
BA	blood agar
BoNT	botulinum neurotoxin
CDC	Centers for Disease Control and Prevention
dATP	2’-deoxyadenosine 5’-triphosphate
dCTP	2’-deoxycytidine 5’-triphosphate
dGTP	2’-deoxyguanosine 5’-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EYA	egg yolk agar
FAAB	fastidious anaerobe agar with blood
FAAE	fastidious anaerobe agar with egg yolk
FAB	fastidious anaerobe broth
FDA	U.S. Food and Drug Administration
GPB	gelatine phosphate buffer
HPLC	high performance liquid chromatography
i.p.	intraperitoneal injection
i.v.	intravenous injection
LD	lethal dose
MAB	modified Anellis Broth

MBA	mouse bioassay
MLD	median lethal dose
MLD_{50}	mouse 50% lethal dose
NAPs	neurotoxin associated proteins
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT-PCR	reverse transcription PCR
SE	standard error
SeM	Segner Medium modified
SIDS	sudden infant death syndrome
SM	sporulation medium
SNAP-25	synaptosomal associated protein-25
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SSS	salt solution for sporulation
TM	trypticase medium
TPGYT	trypticase-peptone-glucose-yeast extract broth with trypsin
VAMP	vesicle associated membrane protein

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

Botulism is caused by the ingestion of preformed botulinal toxin produced by *Clostridium botulinum* (*C. botulinum*), which can grow in improperly preserved foods. It remains the most frequent form in humans worldwide. Recently, infant botulism has been recognised as a different type of toxico-infectious botulism in infants less than 1 year of age (Pickett et al., 1976; Arnon et al., 1979). Epidemiologic and laboratory studies have shown that infants may develop botulism after ingesting *C. botulinum* organisms or their viable spores that subsequently germinate, proliferate in the infant's intestine, and produce botulinal toxin (Midura and Arnon, 1976; Arnon et al., 1977; Arnon et al., 1979; Arnon, 1980; Sakaguchi, 1988). The infection occurs in early infancy because of the immaturity of the microflora of the intestine (Shapiro et al., 1998). Since the first case recognised in 1976, there have been over 1,500 infant botulism cases reported in more than 15 countries worldwide. The youngest baby was just 56 hours of age.

According to statistical data from the Robert-Koch Institute, there is about one case of infant botulism reported each year in Germany. This is probably an underestimated figure due to an inadequate registration of infant botulism cases (Behrens, 1998; Böhnel, 1999). In his personal report, Arnon noted that more than 650 cases were reported from 1976 to 1986 in the United States, and also in other countries throughout the world. In the United States, about 75% of all botulism cases were infant botulism cases (Shapiro et al., 1998). Between 1982 and 1997, 146 cases of infant botulism were reported in Argentina (Fernández et al., 1999).

C. botulinum is an anaerobic spore-forming organism producing an extremely potent neurotoxin in foods (Lund and Peck, 2000). There are seven types of botulism toxin designated by the letters A through G, which are serologically distinct. Types A, B, and E have been reported as the most common causes in humans, while type F is involved in rare cases. The first case of infant botulism due to *C. botulinum* type C was reported in Japan. Most reported cases of infant botulism were caused by type A or B (Arnon, 1980). A few infant botulism cases were associated with *C. butyricum* and *C. baratii*, which produce type E and F

neurotoxins respectively (Hall et al., 1985; Aureli et al., 1986; McCroskey et al., 1986; Paisley et al., 1995; Fenicia et al., 2002; Barash et al., 2005; Keet et al., 2005).

C. botulinum spores are widespread in soil, dust inside or outside houses, marine sediments, intestinal tracts of animals and fish, animal manure, vegetables, fruits, and honey. Therefore, they are potentially present in a wide range of raw materials used in the food industry, as well as in the environment of food processing factories.

To date, honey has been regarded as the only foodstuff being a significant risk factor for infant botulism (Arnon et al., 1979), although scientific studies have recognised the great medical value of honey. *C. botulinum* spores have been detected in honey samples in the United States (up to 10% of the tested samples), Japan (8.5%), Brazil (7.5%), and Italy (6.5%). The contamination levels were estimated at between 5 and 80 *C. botulinum* spores per gram. About 60% of infant botulism cases in Europe had a history of honey consumption (Aureli et al., 2002). *C. botulinum* organisms isolated from five affected patients were of the same toxin type as that found in the honey they were fed (Fenicia et al., 1993; Balslev et al., 1997; Tollofsrud et al., 1998; Monsalve and Alcolea, 1999; Jung and Ottosson, 2001). In a short preliminary study**,** 20 out of 50 infants of a paediatric clinic in Göttingen, Germany, were infected. However, clinical signs were not recorded (Böhnel, personal communication).

Spore production under laboratory conditions is not always successful. Moreover, spore production of *C. botulinum* faces the difficulty of selecting suitable media to achieve the highest sporulation rates, as well as retaining the toxigenicity of the spores. This is usually required in various fields of research. There are no media that can support sporulation of all strains of this bacterium.

Most Probable Number method (MPN), which involves enrichment in a liquid medium and a mouse bioassay (MBA), is frequently used to enumerate *C. botulinum* spores in foods. This method is time consuming and requires experimental animals. The use of the MPN method followed by PCR has been widely investigated with the aim of reducing time consumption and avoiding ethical concerns.

For detection and identification of botulinal neurotoxins, the only universally recognised method is the mouse toxicity and toxin neutralization bioassay. The use of Polymerase Chain Reaction (PCR) in screening for the presence of botulinal spores in honey samples may shorten detection time, lower the costs, and avoid ethical concerns associated with animal testing.

Detection of the presence and the number of *C. botulinum* spores in honey is necessary. It will give information about the *C. botulinum* spore contamination level of honey, which will decide whether honey is suitable for human consumption. Although it has been reported that the *C. botulinum* spore contamination in honey has no effect on children and adults, the risk of infection is relatively high to infants, to HIV patients, to patients who had taken antibiotics for a long time, or to patients with a history of gastrointestinal surgery. Moreover, the presence of *C. botulinum* spores in honey reflects the *C. botulinum* contamination of the environment because honey is considered an environmental indicator of the *C. botulinum* load.

Although there have been a great number of food poisoning cases in Vietnam recorded annually by its Ministry of Health and Department of Preventive Medicine, there is a lack of information about the incidence of pathogenic bacteria and their toxins in food. With respect to infant botulism, there have been no investigations carried out to survey the presence of *C. botulinum* and its spores in honey and infant foods. Therefore, information related to this disease is insufficient in this country.

2 REVIEW OF LITERATURE

2.1 *C. botulinum*

Belonging to the family *Bacillaceae*, *C. botulinum* is an anaerobic, Gram positive, spore-forming soil-borne pathogen that produces a potent biological toxin. *C. botulinum* comprises a group of culturally distinct bacteria. They are straight to slightly curved, motile rods, 0.5 to 2.0 µm in width and 1.6 to 22 µm in length, with peritrichous flagella (Cato et al., 1986; Rocke, 1993; ICMSF, 1996). They are natural inhabitants of the soil, marine, and fresh water sediments. They are also found in the intestines of mammalian species such as horses, chickens, companion animals, and humans. The vegetative bacteria are inactivated by chlorine compounds for about 20 min, sunlight for 1-3 h, and heat for 5 min at 85 ^{o}C.

2.1.1 Taxonomy

C. botulinum strains produce seven neurotoxins that differ antigenically and therefore are differentiated into seven types: A, B, C, D, E, F, or G, named according to their chronological discovery (Sperber, 1982; Kriek and Odendaal, 1994). Based on their physiological characteristics, *C. botulinum* strains were differentiated into four groups and later confirmed by their phylogenetic relationship (Eklund and Dowell, 1987; Sugiyama, 1988). Group I consists of proteolytic strains of type A and proteolytic strains of type B and F. Proteolytic is defined as the ability of bacteria to digest casein in milk, coagulated serum, egg white, and cooked meat. Group II is the non-proteolytic group, including non-proteolytic strains of types B and F, and all strains of type E. Group III includes toxigenic strains of types C, D, as well as *C. novyi* type A strains that are culturally quite similar. Group IV consists of type G strains and *C. subterminale* strains with their similar cultural characteristics. *C. argentinense* and *C. hastiforme* are also members of this group (Suen et al., 1988). The relationship of the various clostridial strains was explored by Lawson et al. (1993) using 16S rRNA sequencing. A dendogram showing the genealogical interrelationships of

the strains was established, which demonstrated marked genetic heterogeneity within the genus *Clostridium*.

Although many *C. botulinum* strains produce a single type of botulinum neurotoxin (BoNT), some strains produce two types of toxin, designated Af (indicating the major and minor toxin produced, respectively), Ab, Ba, and Bf (Hauschild, 1990; Franciosa et al., 1994; Cordoba, 1995). Some type C strains produce C_2 toxin alone, which is not a neurotoxin, or combinations of dominant C_1 and C_2 (Eklund et al., 1987). Table 1 shows a summary of the *C. botulinum* strains and their neurotoxins.

Table 1: Strains versus neurotoxins of *C. botulinum*

Strain	Neurotoxin[1,2]
A	A, Ab, Af
B	B, Ba, Bf
C	C_1, (D)
D	D, (C_1)
E	E
F	F
G	G

[1], Capital letter indicates the type of toxin produced in greater amount, while lower case letter indicates the type of toxin produced in lesser amount in some cases.

[2], In the parentheses, the toxin type produced in minor amount.

'Non-neurotoxins' C_2 and C_3 are produced by some strains of *C. botulinum* types (Gunnison and Meyer, 1929; Aktories et al., 1986a; Aktories et al., 1986b). While C_2 reacts as cytotoxic enterotoxin (Ohishi and Odagiri, 1984), virulence factors of C_3, which is described as an 'exoenzyme', are unclear. Ribosylation effect of C_3 on alfalfa plant cells was reported by Minic et al. (1999). A general review of toxic actions on the cellular level was given by Böhnel and Gessler (2005).

2.1.2 Growth requirements and cultural characteristics

In addition to appropriate nutrients, factors that affect the growth of *C. botulinum* in foods are temperature, pH, water activity (a_w), redox potential (Eh), food preservatives, and competing microorganisms (CDC, 1998). The nutritional requirements for *C. botulinum* are complex and include several amino acids, growth factors, and mineral salts. The optimal temperature for growth of group I, II, III and IV is 35-40, 30, 40, and 37 ^{o}C, respectively. However, in case more than one type is detected while only one temperature can be offered, incubation at 30 ^{o}C for 5-7 d is recommended (Smith and Sugiyama, 1988). According to the Centres for Disease Control and Prevention (CDC, 1998), the growth of *C. botulinum* is inhibited by refrigeration below 4 ^{o}C, pH below 4.5, and low water activity. However, holding temperatures below 4 ^{o}C may still allow nonproteolytic strains to grow and produce toxin (Schmidt et al., 1961; Eklund et al., 1967a; Eklund et al., 1967b). Optimal pH for growth of *C. botulinum* is from 7.0-7.2 (Kriek and Odendaal, 1994). Although the lowest pH for growth of *C. botulinum* is usually accepted at 4.5, growth under this pH value was observed by Smelt et al. (1982). A value of a_w above 0.93 is required to support growth and toxin production of *C. botulinum*. However, lower a_w reduces *C. botulinum* metabolic activity. While the optimal Eh for growth of *C. botulinum* is about -350 mV, toxin production was observed at Eh of +250 mV (CDC, 1998). Food preservatives such as nitrites, sorbic acids**,** sorbates, ascorbates, phenolic antioxidants, and polyphosphates inhibit growth and toxin production of *C. botulinum*. The growth of *C. botulinum* may be promoted by microbial flora in certain ecological habitats through molecular oxygen consumption and oxidation-reduction potential lowering (Dezfulian, 1999). Lactic acid producing bacteria such as *Lactobacillus*, *Pediococcus*, and *Lactococcus* may inhibit the growth of *C. botulinum* (Kim and Foegeding, 1993).

On blood agar, *C. botulinum* strains of various groups form colonies of different shapes, sizes, and characteristics. However, they are usually surrounded by a narrow margin of complete haemolysis, except colonies of group IV strains (Smith and Sugiyama, 1988). Colonies of *C. botulinum* strains of all groups except group IV on egg-yolk agar are covered and surrounded by a thin, pearl layer due to lipase production (Smith and Sugiyama, 1988; Kriek and Odendaal,

1994). Lecithinase is also formed by some strains of group III (Segner et al., 1971; Smith and Sugiyama, 1988). The distinctive cultural and physiological properties of *C. botulinum* strains of the four groups were described in detail by Hatheway (1993).

C. botulinum can grow and produce toxin in foods under the following conditions: 1) natural or nonprocessed food is contaminated with spores or vegetative cells; 2) processing treatment is inadequate to inactivate *C. botulinum* spores**,** or the product is re-contaminated after processing (Eklund, 1993); and 3) the food is particularly conductive to create anaerobic conditions that allow *C. botulinum* spores to germinate and outgrow or vegetative cells to grow and produce toxin.

2.1.3 Properties of *C. botulinum* spores

Spores of *C. botulinum* strains of all groups are sub-terminal; those of group I strains swell the vegetative sporangium markedly compared to those of groups II and III strains. *C. botulinum* spores have the same structure as that of other *Clostridium* spp. and of *Bacillus* spp*.* (Smith and Sugiyama, 1988). They are resistant to heat, light, drying, and radiation. They are inactivated by heating at 121 ^{o}C under 1.0-1.3 KPe for at least 20 min (ICMSF, 1996). They are readily killed by chlorine, formaldehyde, strong acids, strong bases, and ethylene and propylene oxides. The spore coating with high sulphur content may contribute to the radiation resistance of the spores. The spores have resistance to ultraviolet light, alcohols, phenolic compounds, and organic mercurial compounds. Sporulation temperature, water activity (a_w), and pH of spore or food suspension obviously affect heat resistance of *C. botulinum* spores (Smith and Sugiyama, 1988). *C. botulinum* spores are widely distributed in soil, in dust, on fresh fruits and vegetables, or in various meats and fish (Ferrari and Weisse, 1995).

The transformation of spores into multiplying vegetative cells consists of three stages: activation, germination, and outgrowth. Heat is often used to activate spores, and recovery media should contain lysozyme (Smith and Sugiyama, 1988). Germination requires neutral pH, temperatures between 15-45 ^{o}C, and

the presence of amino acids and vitamins (Ferrari and Weisse, 1995). Kriek and Odendaal (1994) reported that an addition of 0.1% of starch promoted germination, because starch binds long chain fatty acids capable of inhibiting spore germination. Outgrowth of spores requires many nutrients and growth conditions that are necessary for vegetative multiplication (Smith and Sugiyama, 1988).

2.1.4 Bacteriophages, bacteriocins, and plasmids

Bacteriophage, a large phage with an octahedral head in electron photomicrographs of *C. botulinum* type C cells, was first described by Vinet and his colleagues in 1968. Bacteriophages of types A to F were then examined with an electron microscope by Inoue and Iida (1968). It was reported that they were involved in the toxicity of types C (Inoue and Iida, 1970; Eklund et al., 1971) and D (Eklund et al., 1972). Toxigenic isolates were recovered from non-toxigenic cultures incubated in broth containing filtrates of the toxigenic strains. Eklund and Poysky (1981) found that toxigenic cultures often lost their toxigenicity when sporulation occurred or when culture medium contained antiserum against the toxigenic phages. The biochemical properties of types C and D did not change when toxigenic bacteriophages and toxigenicity were lost in media containing antiserum (Oguma et al., 1986). Bacteriophages may convert one type of *C. botulinum* to another and *C. botulinum* type C to culturally similar *C. novyi* type A (Eklund et al., 1974).

C. botulinum is susceptible to bacteriocins that were divided into three groups. The first group, designated as “boticin P”, has a molecular mass of $4x10^3$ kDa and acts on non-proteolytic types B and F and all strains of type E. It is produced by a non-toxigenic strain of *C. botulinum* type E, PM-15 (Lau et al., 1974). It does not inhibit germination and outgrowth of *C. botulinum* spores. The second group, called “boticin E”, is produced by some non-toxigenic clostridia whose biochemical properties and morphological characteristics are similar to *C. botulinum* type E. This group includes two distinct boticins: a small form with a molecular mass < 30 kDa and a large form with a molecular mass > 40 kDa (Ellison and Kautter, 1970). These boticins are bacteriolytic for vegetative cells

and bacteriostatic for spores of *C. botulinum* type E strains (Kautter et al., 1966). The third group is the antibiotic-like bacteriocin produced by a strain of *Moraxella* spp. (Kwan and Lee, 1974). It affects the outgrowth of type E spores, but does not affect their germination.

Plasmids, or free cyclic DNA molecules that are capable of autonomous replication, were found in a toxigenic type A, a toxigenic type E, and several non-toxigenic strains (Scott and Duncan, 1978). Strom et al. (1984) indicated that plasmid carriage is widespread in *C. botulinum* and related species. The plasmids were not responsible for any special characteristics of the bacteria carrying them, except for a plasmid carried by type G strains that is responsible for toxin production (Eklund et al., 1988).

2.1.5 Botulinum neurotoxins

Botulinum neurotoxin (BoNT) is the most potent lethal toxin known (Lamanna, 1959; Gill, 1982). Compared to sarin, a potent organophosphate nerve agent, it is 15,000 to 100,000 times more toxic. The lethal dose (LD) of the toxin for man is estimated at 1 ng kg^{-1} (Bonventre, 1979). Approximately for a 70-kg man, lethal dose of crystalline type A would be only 70 µg orally, 0.09-0.15 µg intravenously, and 0.7-0.9 µg inhalationally (Schantz and Johnson, 1992). A single gram of crystalline toxin that is evenly dispersed and inhaled would kill more than 1 million people, although technical factors would make such dissemination difficult (Arnon et al., 2001). The median lethal dose (MLD) per gram of body mass of the toxin type A, B, C_1, D, E, and F for mice is 1.2 ng i.p. (intraperitoneal injection), 0.5-2 ng i.p., 1.1 ng i.v. (intravenous injection), 0.4 ng i.p., 1.1 ng i.p., and 2.5 ng i.v., respectively (Gill, 1982). The MLD of botulinum toxin type C for cattle, according to Moeller and David (2003), is 0.25-2.5 ng per kilogram of body mass.

BoNTs of all types, designated A through G, have similar pharmacologic actions. However, they are serologically distinct and are distinguished by neutralisation of biological activity with serologically type-specific antibodies. Exceptionally, some toxins have the same antigen characteristics: C_1 and D toxins (Oguma et al., 1984) and E and F toxins (Kozaki et al., 1986), which may

result in a cross reaction in serologic tests. Several subserotypes that are immunologically distinguishable within a serotype have been reported. Two subtypes of serotype A were identified by restriction mapping and DNA sequencing, designated A1 and A2 (Cordoba et al., 1995). These two subtypes exhibit amino acid identity of 89% (Willems et al., 1993).

BoNTs are produced intracellularly by the bacteria and released during autolysis of the bacterial cells. They are initially synthesised as an inactive form, a single-chain polypeptide with a molecular mass of about 150 kDa, which is activated by bacterial or tissue proteases (or trypsin) following bacterial lysis. The activated protein consists of a heavy (H) chain (100 kDa) and a light (L) chain (50 kDa), which are linked by a single disulfide bond (Goonetilleke and Harris, 2004).

While *C. botulinum* spores are heat resistant, the neurotoxin is heat-labile. Heating at 85 ^{o}C for at least 5 min destroys the toxin (ICMSF, 1996). It has been reported that boiling food to ensure thorough heating of the interior will destroy the toxin.

BoNTs are zinc-dependent endopeptidases targeting various specific proteins: type A and E target SNAP-25, a protein of the presynaptic membrane; type C targets SNAP-25 and syntaxin, an integral membrane protein that is involved in membrane fusion; and types B, D, F, and G target VAMP (synaptobrevin), a protein of the neurotransmitter-containing vesicles. However, the hydrolysis of the target protein, blockade of transmitter release, and the resultant flaccid paralysis are the same final result of these toxins' actions (Schiavo et al., 1992; Goonetilleke and Harris, 2004). With the hypothesis about interference in the neurological control of intestinal physiology caused by long-lasting absorption of low quantities of botulinum toxin, Böhnel and colleagues (2001b) proposed the name 'visceral botulism' to describe the chronic form of botulism in cattle. Binding sites of botulinum toxin in the central nervous system, especially in the hippocampus, cerebellum, as well as in the cerebral cortex have also been described (Black and Dolly, 1987; Simpson, 1989; Li and Singh, 1998; Böhnel and Gessler, 2005).

The toxins are absorbed from the gastrointestinal (GI) tract into the circulation. They spread to all parts of the body except the central nervous system as they cannot penetrate the blood-brain barrier. However, apparently the only sites for which they have significant affinity are peripheral cholinergic nerve endings. They interfere with the release of acetylcholine from cholinergic neurons of the neuromuscular junction and autonomic synapses. The blockade of acetylcholine activity results in a flaccid paralysis and imbalances in the autonomic nervous system (Montecucco and Schiavo, 1994; Rossetto et al., 1995; Simpson et al., 1999).

Types A, B, and E toxins are most frequently implicated in human botulism (Lamanna, 1959; Simpson, 1999), while type C_1 and D toxins mainly cause the disease in domestic animals and birds and type B toxin affects cattle and horses (Swerczek, 1980; Barsanti, 1990; Hatheway, 1995). *C. botulinum* type C outbreaks in human are extremely rare (Segner et al., 1971). The human gastrointestinal tract does not have receptors for botulinum neurotoxin for translocation into the circulation (Maksymowych and Simpson, 1998). Dogs are most often affected by type C_1 botulinum toxin (Barsanti et al., 1978; Barsanti, 1990).

2.2 Methods of detection, isolation, and quantification

Detection and isolation of *C. botulinum* are difficult. There are no selective media available for culturing both proteolytic and non-proteolytic *C. botulinum*. The detection of the microorganism in the environment and in foods and their raw materials is complicated because of the presence of abundant spores and/or indemonstrable toxins (Hyytia et al., 1999).

The isolation of *C. botulinum* in the environment and food samples is subject to various limitations due to the presence of non-toxigenic strains. These phenotypically and genotypically resemble *C. botulinum* (Lee and Riemann, 1970; Lindström et al., 2001). Moreover, no single procedure, medium, or temperature is best for the incubation of all toxin types and cultural varieties of the microorganism (Smith and Sugiyama, 1988).

2.2.1 Cultural methods for isolation

The cultivation of *C. botulinum* requires strict anaerobic techniques and incubation. Different conditions are required for the isolation of proteolytic and non-proteolytic *C. botulinum* because they are physiologically distinct. Heat treatment is frequently used to eliminate competing vegetative bacteria and activate dormant spores to germinate. While heating at 75 ^{o}C to 80 ^{o}C for 10 to 15 min is useful in culturing spores of proteolytic *C. botulinum*, a lower temperature, e.g. 60 ^{o}C for 15 to 30 min or 71 ^{o}C for 15 min, is used in isolation of non-proteolytic strains and group III strains, respectively (Segner et al., 1971). Alternatively, ethanol treatment is used for isolation of spores of non-proteolytic *C. botulinum* to eliminate non-spore formers that can compete with the target microorganism and adversely affect its growth (CDC, 1998).

However, using heat and alcohol treatment may fail to isolate vegetative bacteria (Dezfulian et al., 1981) and toxigenic strains may become non-toxigenic (Eklund and Poysky, 1972). For isolation of vegetative cells or spores that are not fully heat resistant, the heat treatment is not required.

Enrichment of *C. botulinum* must be carried out under anaerobic conditions (Hatheway, 1988; Kautter et al., 1992; Solomon and Lilly, 2001). Cooked Meat Medium (CMM) incubated at 35 ^{o}C should be used to enrich proteolytic *C. botulinum*, while for non-proteolytic *C. botulinum* CMM fortified with glucose, chopped meat glucose starch medium, or trypticase-peptone-glucose-yeast extract broth containing trypsin (TPGYT) may be used and incubated at 26 ^{o}C (Hauschild, 1989). Lysozyme (5 μg mL^{-1}) was recommended to add to enrichment medium to stimulate germination of *C. botulinum* spores (Smith and Sugiyama, 1988). An addition of 0.1% starch may promote the germination (Kriek and Odendaal, 1994). It was recommended to add trypsin to an enrichment medium, except for medium containing cooked meat particles, to inactivate bacteriocins inhibitory to *C. botulinum*. Cooked meat particles reduce rapidly the proteolytic activity of the trypsin (Smith and Sugiyama, 1988). It was reported that enrichment cultures should be incubated for 5-7 d, or even longer, to allow growth, botulinum toxin formation and sporulation (Smith and Sugiyama, 1988; Broussolle et al., 2002).

After the enrichment, the culture can be used for detecting neurotoxins by mice injection and plating on solid media. Egg-yolk agar is a suitable non-selective medium because on this agar, strains of both group I and group II have colony appearance associated with their lipase activity (Smith and Sugiyama, 1988; Lund and Peck, 2000). *C. botulinum* Isolation (CBI) agar (Dezfulian et al., 1981) and Botulinum Selective Medium (BSM) (Mills et al., 1985) have been used for selective detection of proteolytic strains. Non-proteolytic strains are inhibited by trimethoprin in these media. Colonies that are suspected to be *C. botulinum* are used for further analyses and identification.

2.2.2 Quantification of *C. botulinum*

Enumeration of *C. botulinum* was conventionally performed by using a pour-plate procedure (Hauschild and Hilsheimer, 1977; Glasby and Hatheway, 1985) or the most probable number method (MPN) combined with MBA or PCR (Hielm et al., 1996). Recently, real-time PCR, which is based on the quantification of specific amplified DNA, has been applied for the quantitative detection of *C. botulinum* type A (Yoon et al., 2005) and type E (Kimura et al., 2001) in food and of *Clostridium sp.* in faeces of autistic children (Song et al., 2004). Competitive reverse transcription-PCR (RT-PCR) was developed for enumerating non-proteolytic type E, which accurately measures the level of toxin-encoding mRNA in *C. botulinum* cells (McGrath et al., 2000).

2.2.3 Cellular fatty acid analysis

Based on fatty acids produced by different types of *C. botulinum*, cellular fatty acid analysis by gas liquid chromatography (GLC) has been applied to differentiate *C. botulinum* types (Reiner and Bayer, 1978; Gutteridge et al., 1980; Ghanem et al., 1991). By means of gas chromatography (GC), Rieke (1981) could differentiate clostridia strains based on analysis of acetic acids, propanoic acids, iso-butyric acids, and iso-valeric acids. Ghanem et al. (1991) found that several toxin types of *C. botulinum* and their non-toxic counterparts could be differentiated so well by their cellular fatty acid compositions,

especially in view of the phenotypic and genetic similarities (at the species level). One limitation of this technique is that toxin types of bacteria in the same group could not be separated from each other or from those of related species (Heitefuß, 1991).

2.2.4 Detection of neurotoxin by mouse bioassay

Intraperitoneal injection into mice called mouse bioassay (MBA) has been used as the standard method for detection and identification of botulinum toxins (Schantz and Kautter, 1978; Smith and Sugiyama, 1988; CDC, 1998). Trypsin treatment is required for neurotoxin detection of types B, C, D, E, and F if the culture medium does not contain trypsin (Smith and Sugiyama, 1988). Trypsinisation activates the toxins by converting the single chain toxin to the more toxic dichain form (Lund and Peck, 2000). It is recommended to use two mice for a test (Smith and Sugiyama, 1988; CDC, 1998). However, due to ethical reasons one mouse can be used for a test (Sandler et al., 1993).

Typical symptoms of botulism in mice begin with ruffling of the fur, followed by laboured abdominal breathing, wasp-like narrowed waist, weakness of limbs, paralysis, and respiratory failure resulting in death (Smith and Sugiyama, 1988; CDC, 1998). The specificity of MBA is based on the use of specific antisera and the observation of the typical symptoms.

Toxin neutralisation test, also called mouse protection test, has been used for identifying presence of toxic botulism bacteria. The toxin-antitoxin mixtures are injected i.p. into mice that will be observed for four days. The antitoxin type that protects the mice reflects the toxin type in tested samples (Smith and Sugiyama, 1988; CDC, 1998). Monovalent types or polyvalent of neurotoxins can be used in neutralisation tests (CDC, 1998). However, false-positive results due to cross reactions between botulinum and tetanus toxins were reported by Dolimbek et al. (2002) and Saeed (2005).

Although the biological tests are sensitive and may detect previously undiscovered botulinum neurotoxins, atypical toxins, and antigenic variants, they are time consuming, expensive, complicated, and are not suitable for

examining samples containing other lethal substances or very low levels of toxin. Such tests using animals are increasingly restricted due to ethical concerns (Dezfulian and Bartlett, 1985a). Cross reactions were reported between *C. botulinum* types C and D (Jansen, 1971; Fach et al., 1996) and between types E and F (Yang and Sugiyama, 1975). Clinical cases of botulism failed to be detected or inconclusive results were reported in some studies in which MBA was applied (Thomas, 1991; Trueman et al., 1992; Szabo et al., 1994b; Böhnel, 1999). Diagnostic methods of *C. botulinum* and its toxin are still insufficient and poorly developed (Gessler and Böhnel, 2003; Robinson and Nahata, 2003).

Mouse diaphragm assay (MDA) is currently used to detect antibodies against BoNT (BoNT-Ab). It measures BoNT-Ab induced blockade of the paralysing effect of BoNT on a mouse hemidiaphragm preparation (Dressler and Dirnberger, 2001). Dressler et al. (2005) applied this assay to detect antibodies against botulinum toxin type B (BoNT-B-Ab). The detectable concentration of BoNT-B-Ab was 0.4 mU mL^{-1}.

2.2.5 Immunological assays

Immunological methods like enzyme-linked immunosorbent assays (ELISAs) and other techniques have been used widely to detect toxins (Notermans et al., 1982b; Ferreira et al., 1990; Carlin and Peck, 1996) and to identify colonies of *C. botulinum* (Dezfulian and Bartlett, 1985b; Goodnough et al., 1993). These methods also have been used to detect *C. botulinum* neurotoxins in clinical and food specimens (Shone et al., 1985; Potter et al., 1993; Rocke et al., 1998; Poli et al., 2002; Ferreira, 2003; Ferreira et al., 2004).

Low sensitivity or specificity of immunological methods was noted in assays such as passive haemagglutination assay (Evancho et al., 1973), radioimmunoassay (Boroff and Shu-Chen, 1973), and immunodiffussion assay (Ferreira et al., 1981). ELISAs have been modified to improve their sensitivity and specificity, for instance, amplified immunoassay ELISA-ELCA (Roman et al., 1994), colorimetric capture ELISAs (Szilagyi et al., 2000; Poli et al., 2002), magnetic-bead ELISA (Kourilov and Steinitz, 2002; Gessler and Böhnel, 2006),

immunoaffinity column assays (Gessler et al., 2005), and amplified enzyme-linked immunosorbent assay (Ferreira, 2003).

To achieve a higher sensitivity with immunological methods used for detection of *C. botulinum*, immuno-PCR methods have been developed in which a specific DNA molecule is used as label (Sano et al., 1992; Wu et al., 2001). The sensitivity of the antigen detection by immuno-PCR was enhanced from 100-fold (Joerger et al., 1995; Wu et al., 2001) to 100,000-fold (Sano et al., 1992) in comparison to that of conventional ELISA.

The sensitivity and specificity of some of the ELISAs are similar to that of the mouse bioassay**,** (Doellgast et al., 1993; Doellgast et al., 1994; Ferreira, 2003) or even more sensitive (Roman et al., 1994; Hanna et al., 1999). These techniques are recommended for use as an alternative to mouse bioassay to save time and avoid using animals *in vivo* toxicity tests.

However, applying ELISAs have limitations. Many ELISAs require complex and expensive applications (Shone et al., 1985; Doellgast et al., 1993). Some may react with biological inactive toxin (Huhtanen et al., 1992) and cross react with other clostridia (Fernandez and Peck, 1997). Depending on the type examined and the culture media used, false positive results from 1.5 to 28.6% of samples due to the cross reaction with other toxin types were reported by Gessler and Böhnel (2003). Cross reactions were observed between types A and B by Dezfulian et al. (1984), between types C_1 and D by Notermans and his co-workers (1982a), and between types C and D and *C. novyi* type A (Thomas, 1991). Commercial ELISAs are limited to pathogenic botulinum neurotoxin types A, B, and E for humans (Gessler and Böhnel, 2003).

Immunochromatographic assays are a logical extension of the technology used in latex agglutination tests. The first assays were developed in 1956 by Singer and Plotz. These assays utilise antigen-antibody reaction on a nitrocellulose membrane where a colour band from attached gold beads indicates the antigen-antibody reaction. This principle is identical to the widely accepted “Pregnancy test” technology.

Chiao and colleagues (2004) developed an immunochromatographic assay, the colloidal gold-based immunochromatographic assay. This essay was designed

to detect botulinum neurotoxin type B based on the sandwich format using polyclonal antibodies. In less than 10 min, botulinum neurotoxin type B was detected at concentrations of 50 ng mL^{-1}. The treatment of test strips with silver enhancer can increase the assay sensitivity to 50 pg mL^{-1}. No cross reaction to type A and E neurotoxins occurred.

The lateral-flow assays developed by the Naval Medical Research Centre (Silver Spring, MD) and Alexeter Technologies (Gaithersburg, MD) for detection of botulinum neurotoxin types A, B, and E in liquid, solid, and high-fat-content foods were evaluated by Shamar et al. (2005). The detectable concentration was 10 ng mL^{-1} for types A and B, and 20 ng mL^{-1} for type E. Although these assays were found less sensitive in comparison to other *in vitro* detection methods, and the result assessment is strictly qualitative, they are rapid, user friendly, long-term stable, and relatively inexpensive.

The biowarefare agent detection devices (BADD) were developed by Osborn Scientific Group. They are easy-to-use test sets performing an *in vitro* immunochromatographic assay capable of qualitatively detecting the presence of specific biological warfare agents. The botulinum toxin test set (BOT-E500-4, USA) is used to examine swab samples from contaminated surfaces or liquid materials. This test set can detect botulinum neurotoxin types A to G in concentrations of 50 ng or greater on a hard, smooth surface, or 100 ng mL^{-1} in solution within 15 min. However, the test set cannot specify which types of neurotoxin are present in the test samples. Moreover, the test results cannot be observed 15 min after the test has been done because colour signals will be no longer observable.

The flow through and lateral flow immunochromatographic assays for detection of botulium neurotoxin type D were developed by Klewitz (2005). The formation of a “mobile sandwich complex” was optimised with an effective sample pre-treatment. An enzymatic staining reaction was used in the flow through immunochromatographic assay for the detection of the immobilised sandwich complex within 6 h. It had a test sensitivity of 50 pg mL^{-1}. Other flow through assays where gold conjugated antibodies were used decreased the test sensitivity, i.e. 50 ng mL^{-1}. In the lateral flow assay, colloidal gold particles were used as a labelling reagent. The minimum detectable concentration of

botulinum neurotoxin type D was 50 pg mL^{-1} within less than 5 h.

According to manufacturers, theses assays are recommended for initial screening of environmental and food samples.

2.2.6 Endopeptidase activity assays

Another approach to the botulinum toxin detection is based on its endopeptidase activity. Highly specific antibody-enzyme reagents are used to monitor and detect the cleavage of immobilised peptide substrates by botulinum toxin (Schmidt and Bostian, 1995; Ekong et al., 1997a; Ekong et al., 1997b; Wictome et al., 1999). These methods have several advantages over ELISA procedures (Lund and Peck, 2000). These *in vitro* assays may directly measure the biological activity of the light chain because the cleavage reaction is highly specific. Therefore, false negatives are rare.

There are several types of endopeptidase activity assays for measurement of botulinum neurotoxin types A and B: the high pressure liquid chromatography (HPLC) based methods (Schmidt and Bostian, 1995; Ekong et al., 1997b) and the methods using a combination of endopeptidase activity and immunological reactions (Hallis, 1996; Ekong et al., 1997a). The HPLC methods have disadvantages because they are less sensitive than the MBA and are not suitable for the rapid detection of toxin in large numbers of samples. The latter methods were type specific and did not cross react with other botulinum neurotoxins (Hallis, 1996) and were more sensitive than MBA (Ekong et al., 1997b; Wictome et al., 1999). Another assay, a rapid, mass spectrometry-based method was developed by Barr et al. (2005). Botulinum neurotoxin types A, B, E, and F at concentrations of 0.62 MLD_{50} mL^{-1} were detected by matrix-assisted laser-desorption ionisation time-of-light mass spectrometry (MALDI-TOF-MS). All seven neurotoxin types in a multiplexed assay format were detected by MALDI-TOF-MS with no cross-reactions and the detection limits from 0.039 to 0.625 MLD_{50} mL^{-1} (Boyer et al., 2005).

2.2.7 Biosensors

Recently, biosensors have been developed and widely applied to examine a variety of potentially threatening biological and chemical substances. This approach is also seen in botulinum toxin research.

Fibre-optical biosensors were developed for the quantitative detection of botulinum toxin. Type A toxin at concentrations of as low as 5 ng mL^{-1} is detected by using evanescent wave technology within a minute. Rhodamine-labelled polyclonal antitoxin A immunoglobin G (IgG) antibodies were used as a sandwich immunoassay for generation of the specific fluorescent signal. The technique was found to be highly specific, and no response was observed against tetanus toxin (Ogert et al., 1992). Kumar et al. (1994) also used an evanescent wave immunosensor for a quantitative analysis of bound botulinum neurotoxin and quartz fibre-immobilised antibody (FiAb) by Dot-Blot analysis using avidin-horseradish peroxidase (HRP) conjugate.

The array biosensor developed by Ligler et al. (2003) was capable of rapidly detecting the simultaneous toxic compounds of staphylococcal enterotoxin B, ricin, cholera toxin, botulinum toxoids A and B, trinitrotoluene, and the mycotoxin fumonisin at levels as low as 0.5 ng mL^{-1}. Sandwich and competitive fluoroimmunoassays were developed to detect high and low molecular mass toxins in environmental and food samples and clinical fluids, with minimal sample preparation. A mechanosensor was developed by Liu et al. (2003). This technique relied on the detection of an agarose bead detachment from the tip of a micromachined cantilever. The cantilever action is dependent on botulinum neurotoxin type B acting on its synaptic protein. This substratum called synaptobrevin 2 was attached to the beads. Within 15 minutes, type B neurotoxin at concentrations of 8 nM could be detected.

Another approach to detecting botulinum neurotoxin is by using a biosensor based on gangliosides, i.e., natural cellular receptors for the toxin. This occurs when they are incorporated onto a liposome surface. Liposomes containing gangliosides mimic cells that are invaded by bacterial toxins and can be used as sensitive probes for detecting these toxins. The fluorescent liposomes, containing both a marker (rhodamine) and a receptor (GT1b or GM1) in a

bilayer, were used in sandwich fluoroimmunoassays for tetanus, botulinum, and cholera toxins at levels as low as 1 nM of each toxin (Singh et al., 2000). A similar receptor immunoassay for botulinum toxin using ganglioside-incorporated liposomes was applied by Ahn-Yoon et al. (2004). In this assay, GT1b gangliosides were incorporated into the surface of dye encapsulated liposomes. Botulinum toxin bound to the GT1b-liposomes was captured by anti-botulinum toxin antibodies immobilised in a band on a nitro-cellulose membrane strip. The intensity of the coloured band was visually estimated, or measured by densitometry using computer software. The sandwich assay using GT1b-liposomes for detection of botulinum toxin within less than 20 min was found to be rapid and very sensitive. The threshold of toxin detection was determined to be as low as 15 pg mL^{-1}. It was suggested that the assay could be used for detecting botulinum toxin in field screening, simply and reliably, without the need for complex instrumentation.

2.2.8 Detection of neurotoxin genes by polymerase chain reaction (PCR) and gene probes

With their high sensitivity and specificity, molecular biological techniques have been developed for the non-specific detection of genes for all types of botulinum neurotoxins (Campbell et al., 1993) and for the specific detection of genes for each neurotoxin type (Szabo et al., 1993; Franciosa et al., 1994; Fach et al., 1993; Fach et al., 1995). Several PCR-based detection methods have been widely utilised (Szabo et al., 1994a; Szabo et al., 1994b; Takeshi et al., 1996; Branconnier et al., 2001).

The use of a cultural enrichment followed by a PCR procedure has been recommended (Hielm et al., 1996; Dahlenborg et al., 2001) because it provides higher number of target bacteria and reduces problems due to the presence of extracellular DNA, bacterial death, or to interference by food components. Quantitative detection of *C. botulinum* using a MPN series of dilutions, with cultural enrichment, followed by PCR tests has been used to quantitatively detect *C. botulinum* type E in inoculated fish. It has also been used to investigate the presence of these bacteria in aquatic sediments and in fish

(Aranda et al., 1997; Hielm et al., 1996; Hielm et al., 1998). *In situ* (without prior enrichment) detection of *C. botulinum* type C_1 neurotoxin by using a nested PCR assay was reported by Williamson et al. (1999). In the reverse transcription-PCR developed by McGrath et al. (2000), gene expression is detected rather than the gene itself. It may distinguish between viable and dead bacteria.

Compared to MBA, PCR has been found to be more sensitive, rapid, and does not require experimental animals (Fach et al., 1993; Szabo et al., 1993; Szabo et al., 1994b). A sensitivity of 10-12.5 fg of DNA, that is approximately equal to 3-5 cells in a reaction volume was reported by Fach et al. (1993) and Szabo et al. (1993). An amount of 0.3 ng of DNA was also detected by Craven et al. (2002).

The technique developed by Fach et al. (2002) based on identification of the most highly conserved region of botulinal neurotoxin genes can be automated and readily applied for simultaneous detection of *C. botulinum* types A, B, E, and F on a large scale. Multiplex-PCR developed by Lindström et al. (2001), which was then slightly modified by Nevas et al. (2005a) was used to detect *C. botulinum* types A, B, E, and F in food, faecal materials, and honey.

The PCR-based assays have demonstrated their advantages for rapid and accurate identification of pathogenic bacteria. However, the limitations of these assays are that they are primarily qualitative techniques and are not appropriate for accurate quantification of a target sequence. Numbers of quantitative PCR approaches to the detection and quantification of target gene numbers have been developed, such as most probable number PCR (MPN-PCR) (Hielm et al., 1996), competitive PCR (Lee et al., 1996; Janse et al., 1998; McGrath et al., 2000), and PCR-ELISA (Gutierrez et al., 1997; Gonzalez et al., 1999). Most recently, the TaqMan real-time PCR assays have been developed based on sequence-specific hybridization probes; i.e., the 5'-3' exonuclease activity of Taq polymerase. These assays have been applied for quantification of various pathogenic bacteria such as *Mycobacterium tuberculosis* (Desjardin et al., 1998), *Vibrio parahaemolyticus* (Kaufman et al., 2004), *Yersinia pestis* (Higgins et al., 1998), and *Salmonella* (Nogva and Lillehaug, 1999; Mercanoglu and Griffiths, 2005).

2.3 Botulism in man

Botulism was first recognised as “sausage poisoning” in the 18th and 19th centuries. The causative agent was first described by van Ermengem in 1897 from a large outbreak in Ellezelles, Belgium. Human botulism is mainly caused by *C. botulinum* strains that produce toxin types A, B, and E. Neurotoxigenic strains of *C. butyricum* (Aureli et al., 1986) and *C. baratii* (Hall et al., 1985; McCroskey et al., 1991), which produce types E and F toxin, respectively, have also been recently reported in human botulism. In humans there have been only two reported outbreaks of poisoning with type C, and one with type D toxin (Lamanna, 1959). Type C_α reported by K.F. Meyer (cited by Prevot et al., 1955) as type C botulism in the USA, type C_β in a foodborne outbreak in France (Prevot et al., 1955), and type D in an outbreak of botulism in humans in Chad (Demarchi et al., 1958) were reported. Humans are very sensitive to botulism. There is no person-to-person transmission.

There are now five clinical forms of human botulism (Cherington, 1998; 2004). Classic (also called food-borne) and wound botulism, which were the only two forms of botulism known until the last quarter of the 20th century, were recognised in 1897 and 1943, respectively. The other forms have been described over the past decades, except for botulinal intestinal infections reported in 1928 by Meyer. Infant botulism, first described in 1976, is the most frequently reported form in the USA. The hidden form, described in 1977, is the adult variation of infant botulism. Inadvertent botulism, the most recent form to be described (in 1997), is an unintended consequence of treatment with injections of botulinum toxins for therapeutic or cosmetic use.

The neurological signs and symptoms are the same for all epidemiological categories and may include respiratory paralysis or neuronal failure (Dressler, 2002). However, the pathogenesis varies among the different forms. While classic or food-borne botulism is an intoxication that results from the ingestion of preformed toxin, infant, hidden, and wound botulism are infectious forms (Cherington, 1998; 2004).

2.3.1 Food-borne botulism

Food-borne botulism occurs after the ingestion of foods containing preformed toxin. The contamination occurs in susceptible foods stored at ambient temperatures for prolonged periods. The sources of food-borne botulism are mainly fish, meat, dairy products, and vegetables. Home canned foods made from fish, vegetables, or potatoes were often implicated in food-borne botulism outbreaks (Hauschild, 1989; Dodds, 1993b). It has been shown that in home canned foods *C. botulinum* spores survive inadequate cooking and canning processes, germinate, reproduce, and produce toxin. Types A, B, and E are implicated in most human cases. Although the clinical presentations of these three types are similar, type A cases may be more severe and longer lasting with a higher fatality rate than those caused by type B (Woodruff, 1992). Type E was reported the prevalent type associated with botulism outbreaks in Alaska, Canada, Denmark, Norway, the former Soviet Union, Iran, and Japan (Hauschild, 1993). The number of reported cases associated with type F toxin was low (Midura, 1972; Hauschild, 1993).

Mortality was reported to be high in the USA from 1899-1949 with the approximate case-fatality ratio at 60% (CDC, 1998) because treatment was unsatisfactory (Meyer, 1964). Due to improvements in supportive and respiratory intensive care and to the prompt administration of antitoxin, the case-fatality ratio has decreased over years for all toxin types. In the USA, it was 15.5% in the period from 1950 to 1996 (CDC, 1998).

2.3.2 Infant botulism

In reference to the present study, infant botulism is focused on and reviewed in more detail.

2.3.2.1 Epidemiology

Infant botulism is defined as: “a clinically compatible case that is laboratory-confirmed, occurring in children under 1 year of age” (CDC, 1997). This form

occurs when *C. botulinum* spores are ingested, germinate, colonise, and produce neurotoxin in the gastrointestinal tract. Infants are susceptible to gut colonisation due to immaturity of the host gut microflora.

Infant botulism was first recognised and described in the USA in 1976 (Midura and Arnon, 1976). Since the first case of infant botulism was reported in England in 1978 (Turner et al., 1978), 49 cases have been described in Europe (Aureli et al., 2002). To date, the CDC has documented more than 1,400 cases in all continents, except Africa. Approximately, 90% of the cases have been diagnosed in the USA (Shapiro et al., 1998). In Germany, as many as 100-200 cases of so called “sudden infant botulism” per year were estimated (Böhnel et al., 2001a).

Most infant botulism cases are due to *C. botulinum* types A and B. Proteolytic *C. botulinum* strains are more frequently related to infant botulism than non-proteolytic strains (Arnon, 1998). The first case of infant botulism caused by type C was reported in Japan (Oguma et al., 1990). In addition to cases caused by *C. botulinum*, there have been cases due to *Clostridium butyricum* and *Clostridium baratii* producing types E and F toxin respectively (Hall et al., 1985; Aureli et al., 1986; Gimenez and Sugiyama, 1988). It was postulated by Sonnabend et al. (1985) that the strain producing type G botulinum toxin was also associated with infant botulism.

While 99% of affected babies were less than one year old, 94% of cases occurred in infants less than six months old with a median of ten weeks (Brown, 1984; Spika et al., 1989). Two cases of infant botulism at six and eight days of life were reported by Thilo and Townsend (1993). Recently, a case of botulism in a 54-hour-old infant has been recognised the youngest case of infant botulism. It is the third reported case caused by *C. botulinum* type F (Keet et al., 2005). A slightly higher prevalence in male infants was reported.

In approximately 85% of infant botulism cases the source of spore ingestion is unknown and in up to 15% of cases honey is suspected to be the source of the ingestion (Spika et al., 1989; Arnon, 1998).

While the minimum infective dose of *C. botulinum* spores for infants is not known, it has been estimated to be as low as 10 to 100 spores (Arnon, 1998). A

dose of 10 spores was found to be infective for experimental infant mice (Sugiyama and Mills, 1978).

2.3.2.2 Clinical spectrum

Infant botulism is difficult to recognise because of its insidious onset. The disease has a broad clinical spectrum ranging from a mild infection to fulminating, even fatal illness. The initial clinical sign is usually constipation. This symptom is followed by poor feeding, lethargy, weak cry, poor suck, and generalised decrease in muscle tone characterised by loss of head control. The baby often appears “floppy” (Arnon et al., 1977; Arnon, 1998). Respiratory difficulties occur as a late sign of the disease and quickly lead to respiratory arrest (Schmidt and Schmidt, 1992). A mortality rate of 5% has been reported (Kothare and Kassner, 1995). The illness may last from a few days to weeks or months, and recovery may also take weeks or months.

2.3.2.3 Diagnosis

Electromyogram (EMG) studies are usually the quickest way to diagnose botulism. EMG also may help to distinguish botulism from myasthenia gravis and Guillain-Barré syndrome, which are still frequently misdiagnosed as botulism (Arnon, 1998).

A definitive diagnosis includes the detection of botulinum toxin and the isolation of *C. botulinum* from stool specimens. A serum sample is necessary for a toxin assay. Additionally, other potential samples, such as honey, corn, infant formula foods, dust, and other materials in the surrounding environment of affected babies, should be collected for diagnosis (Arnon, 1998; CDC, 1998; Cox and Hinkle, 2002; Nevas et al., 2005b).

Clinical diagnosis is based on a careful examination of neurological symptoms. In addition to the neurological symptoms, absence of fever despite alertness of senses is considered distinctive for infant botulism.

It has been noted that infant botulism goes undetected in many areas (Fox et al., 2005). The differential diagnosis of infant botulism includes other neuromuscular disorders associated with hypotonia. These include myopathies, Guillain-Barré syndrome, familial infantile myasthenia gravis, spinal muscular atrophy, and poliomyelitis (Cherington, 1998; Cox and Hinkle, 2002). Böhnel et al. (2001a) proposed to use “sudden infant botulism” for cases of unexpected infant death with post-mortem findings of *C. botulinum* bacteria/toxins to differentiate from clinical floppy infant syndrome.

2.3.2.4 Treatment

The treatment for infant botulism has been based on supportive treatment including respiratory and nutritional care. Prompt clinical diagnosis and treatment with Botulism Immune Globulin (BIG) may reduce the recovery time. BIG is obtained from pooled plasma of adults immunised with pentavalent botulinum toxoid and selected for high titres of neutralizing antibodies against types A and B toxin (Frankovich and Arnon, 1991). Since BIG was approved by the Food and Drug Administration in 2003 for use in infant botulism in the USA, therapy with BIG significantly reduces hospital stay and hospital costs and diminishes the potential complications of the disorder (Arnon, 1998; Thompson et al., 2005). It is recommended in the USA that BIG treatment should be requested without waiting for laboratory confirmation. Antibiotics are not used in uncomplicated infant botulism cases because the toxin may be released into the intestine following death and lysis of vegetative cells.

2.3.2.5 Prevention

Currently, the only known way to prevent the acquisition of infant botulism is to avoid feeding honey to infants less than one year old. This recommendation has been endorsed by paediatric and public health agencies. Moreover, breast-feeding has been recommended to moderate the rapidity of onset and the severity of the disease (Arnon et al., 1982; Spika et al., 1989).

Botulism presents a public health emergency. It is obligated to report to departments of health services in case of a botulism case with 1) a clinical epidemiological confirmation; 2) a probable clinically confirmed case of botulism after laboratory detection confirmation; 3) an asymptomatic infection which is laboratory detected; 4) or an infection with only laboratory detection confirmation (Robert Koch Institute, 2000). If a case is clinically or laboratory confirmed or if a bio-terrorism event is suspected, all concerned agencies such as state and local infectious disease and epidemiology departments, must be notified.

2.3.2.6 Link to sudden infant death syndrome (SIDS)

Sudden Infant Death Syndrome (SIDS) is the "sudden death of an infant under one year of age which remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and review of the clinical history" (Willinger et al., 1991).

A link between infant botulism and SIDS was noted because of a similar sudden respiratory arrest found in cases of infant botulism and SIDS. Sudden unexpected death in cases of infant botulism led to the hypothesis that infant botulism might be a cause of SIDS (Arnon et al., 1981). The age distribution of SIDS is identical to that of infant botulism. *C. botulinum* and toxin were found in 10 of 212 tests of necropsy specimens (Arnon et al., 1978; Arnon et al., 1981). However, in a 10-year study conducted in Australia by Byard and his co-workers (1992), none of the small and large intestine specimens from 248 SIDS cases was positive for *C. botulinum* by culture. Similar conclusions were made by Urquhart and Grist (1976) and Berry et al. (1987). While infant botulism was not associated with SIDS in Southern Australia, *C. botulinum* and the toxin were found in cases in North America and Europe (Hauschild et al., 1983; Hauschild and Gauvreau, 1985; Midura, 1996; Böhnel et al., 2001a).

2.3.2.7 Risk factors for infant botulism

Through laboratory and epidemiological evidence, it is conclusive that consumption of honey is associated with infant botulism. Therefore, consumption of honey should be considered as the most important risk factor associated with infant botulism.

In a two-year prospective case-control study conducted by Spika et al. (1989), risk factors for infant botulism were defined. In addition to honey consumption, lack of breast-feeding and decreased frequency of bowel movement, namely less than one per day for at least two months, were also associated with the disease in infants less than two months of age. Another significant risk factor is living in a rural area or on a farm (Hoffman et al., 1988).

2.3.3 Wound botulism

Until recently, wound botulism has been considered a rare form of botulism. From 1943 to 1990, there were 47 cases of wound botulism reported (Weber, 1993). Wounds contaminated with *C. botulinum* spores result in wound botulism. The rarity of this form is due to the failure of the spores to germinate readily in tissue. Since 1991, there has been a dramatic increase in the numbers of wound botulism reported in drug addicts injecting black tar heroin subcutaneously (Maselli, 1997; Passaro et al., 1998; Werne et al., 2000; CDC, 2003). The fatality rate for wound botulism is approximately 15% (Hatheway, 1995).

2.3.4 Hidden botulism

This form refers to adult patients who accommodate toxin-producing clostridia bacteria in their intestinal tracts. This occurs while there is no known contaminated food, no wound, and no history of drug abuse. Toxin production is similar to that of infant botulism. The hidden botulism patients often have a history of gastrointestinal tract abnormalities, abdominal surgery, or recent antibiotic treatment (Dowell, 1977; Chia, 1986; Griffin, 1997).

2.3.5 Inadvertent botulism

Inadvertent botulism is the most recently recognised form. This form of botulism occurs in patients treated dystonic and other movement disorders with botulinum toxin (Cherington, 1998; Munchau and Bhatia, 2000). Botulinum neurotoxin types A, B, C, and F are used now in these treatments (Eleopra et al., 2004). Clinical weakness and electrophysiologic abnormalities were observed in inadvertent botulism cases (Bakheit et al., 1997).

3 OBJECTIVES

Objectives of this study were:

1. Development of a procedure to produce spore suspensions of different strains of *C. botulinum* A, B, C, D, E, and F by applying different media.
2. Development of a MPN-PCR method to enumerate spores of these strains in honey samples, targeting the neurotoxin encoding genes.
3. Detection for the presence of viable *C. botulinum* spores in honey samples and infant foods purchased from retail stores and supermarkets in Ho Chi Minh City, Vietnam. In addition, some honey and infant food samples that were purchased from retail supermarkets in Germany were also examined. The purpose of this survey was to determine if honey and infant foods in these market areas would present a potential threat to infants in Vietnam and Germany.

4 MATERIALS AND METHODS

4.1 Bacterial strains and culture conditions

Bacterial strains used were *C. botulinum* types A, C, D, E and proteolytic types B and F. Strains were obtained from the culture collection at the Institute of Agronomy and Animal Production in the Tropics and Subtropics, Georg-August-University Göttingen. Table 2 shows all strains with their origins.

Table 2: Origin of the *C. botulinum* strains

Institute's number	Type	Original number	Source
2298	A	62 A	Prof. Kozaki, Osaka Prefecture University, Japan
2299	B	Okra	Prof. Kozaki, Osaka Prefecture University, Japan
2300	C	003-9	Prof. Kozaki, Osaka Prefecture University, Japan
2145	C	REB 1455	National Reference Centre for Clostridia, Erfurt, Germany
2294	C	5295	New Zealand Reference Culture Collection, New Zealand
2256	Cß	AO28	IDLO, Lelystad, Netherlands
2257	Cα	468 C	IDLO, Lelystad, Netherlands
1969	C	NC 03180	NCTC*
2279	Cα	CCUG7970	Culture Collection, University of Göteborg, Schweden
2301	D	CB-16	Prof. Kozaki, Osaka Prefecture University, Japan
2302	E	35396	Prof. Kozaki, Osaka Prefecture University, Japan
2271	E	CECT 4611	Spanish Collection of Culture Type, Valencia, Spain
2625	E	CB-S 21E	Miia Lindström, Helsinki
2303	F	Langeland	Prof. Kozaki, Osaka Prefecture University, Japan

(*): NCTC, National Collection of Type Cultures, United Kingdom

The stock cultures of each strain were maintained frozen at - 80 ^{o}C in 2 mL of RCM (Reinforced Clostridial Medium) cultures with 10% glycerine added. These

were inoculated into 5 mL of RCM and incubated at 37 ^{o}C under anaerobic conditions. The incubation conditions were a combination of 90% N_2, 5% CO_2, and 5% of H_2 (ANOXOMAT® SYSTEM, MART, Lichtenvoorde, the Netherlands). These were applied for all stages of the study. After two days of incubation, growth and gas production of the cultures were controlled. These cultures were then inoculated into 5 mL RCM tubes and checked for any contamination by further BA (Blood Agar with RCM basis) culture with two days of incubation. The pure cultures that did not have any contamination, i.e. having given apparently only one kind of colony on BA, were further inoculated into 20 mL of RCM tubes and incubated under anaerobic conditions for 2-4 d. Then the growing cultures were frozen in 2 mL tubes at - 80 ^{o}C. These cultures were used for subsequent studies.

4.2 Media

For spore production, various media were applied (Table 3), whereas for spore enumeration, Fastidious Anaerobe Broth (FAB) and Cooked Meat Medium (CMM - DIFCO, Detroit, USA) were used. (Detailed components see Appendix).

Table 3: Media applied for spore production of *C. botulinum*

Procedure	Media	Authors/Manufacturers
Spore production	Blood Agar (BA)	see Appendix
	Egg Yolk Agar (EYA)	see Appendix
	Fastidious Anaerobe Agar with Blood (FAAB)	Saeed, 2005
	Fastidious Anaerobe Agar with Egg Yolk (FAAE)	Saeed, 2005
	Duncan-Strong Medium (DSM)	Duncan and Strong, 1968
	Modified Anellis Broth (MAB)	Gaze and Brown, 1988
	Reinforced Clostridial Medium (RCM)	see Appendix
	RCM-hay *	see Appendix
	Segner Medium (SeM)	Segner et al., 1971
	Sporulation Medium (SM)	Medium 531 - German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany
	SM-hay	see Appendix
	Trypticase medium (TM)	Durban et al., 1970
Spore enumeration	Fastidious Anaerobe Broth (FAB)	see Appendix
	Cooked Meat Medium (CMM)	DIFCO, Detroit

*, addition of hay to medium (Mitscherlich - personal communication to Böhnel, 1985).

4.3 Honey and infant food samples

Honey and infant food samples were purchased from retail stores and supermarkets in Ho Chi Minh City, Vietnam. The infant food samples had instant cereal as the basic component and another component like pork, chicken, shrimp, or fish meat, milk, fruit, vegetables, rice, or soya bean. These samples were divided into five groups based on the other components. Some

other samples were purchased from retail supermarkets in Germany. The numbers and types of samples tested are listed in Tables 4 and 5.

Table 4: Types of samples collected from Ho Chi Minh City, Vietnam

Types of samples		Number of samples
Honey		99
Infant Foods	Pork/Chicken	50
	Milk	35
	Fruit/Vegetables	35
	Shrimp/Fish	30
	Rice/Soya bean	29
Total		**278**

Table 5: Types of samples purchased in Germany

Types of samples	Number of samples
Honey	13
Infant cereal formula	9
Infant milk powder	9
Potato purée	8
Vegetable formula	7
Total	**46**

4.4 Spore production

Spores of the *C. botulinum* A to F strains were produced separately by growing in various media (Table 6). 0.1 mL of bacterial cultures of *C. botulinum* A to F strains were inoculated into 5 mL of FAB, incubated anaerobically at 30-37 ^{o}C for 2 d. Two millilitres of this culture were then inoculated into 20 mL FAB and incubated under the same condition for 2-4 d. The whole 20 mL of the FAB culture were then transferred to media for spore production. The sporulation level was checked daily by microscopic examination after Gram or spore staining. If spores were produced on FAAE or BA, anaerobic incubation for 4 d at 30-37 ^{o}C was carried out. Spores on agar plates were washed off with cold sterile PBS. If there were more than approximately 60% of spores compared

with rod forms observed, spores were collected by centrifugation at 5,000 x *g* for 15 min and resuspended in spore salt solution (SSS, see Appendix - Böhnel, personal communication). This was stored at 4 ^{o}C for further 4-7 d for further transformation into the spore form from the vegetative cells. Finally, spores were harvested by centrifugation at 5,000 x *g* for 15 min and washed with cold sterile water five times. Sonication in an ultrasonic bath (SONOREX DK 514 BP, BANDELIN, Berlin, Germany) with washing was carried out five times to avoid clumping of spores (Peck et al., 1992). The washed spores were then resuspended in Phosphate Buffered Saline solution (PBS) pH 7.2, and stored at 4 ^{o}C. The degree of sporulation was estimated by examining ten microscopic fields (1,000 x magnification). It was classified as poor (lower than 30%), fair (30-70%), and good (from 70-100%).

Table 6: Media applied for spore production of different *C. botulinum* strains

Strains	**Media**									
	BA	DSM	EYA	FAAB	FAAE	MAB	RCM-hay	SM/SM-hay	SeM	TM
A 2298										+
B 2299	+		+	+		+				
C 1969	+						+	+	+	
C 1974	+						+	+	+	
C 2145	+	+		+	+					
C 2256	+						+	+	+	
C 2257	+						+	+	+	
C 2279	+	+		+			+	+	+	
C 2294	+						+	+	+	
C 2300	+	+			+	+	+	+	+	
D 2301	+					+	+	+	+	
E 2302	+		+	+	+	+	+	+	+	
E 2625	+		+	+	+	+		+	+	
F 2303	+		+	+		+		+		

4.5 Enumeration procedure

The enumeration of *C. botulinum* spores in spore suspensions and in spiked honey was determined using the five-tube-MPN method (Most Probable Number) (Peeler et al., 1992; Blodgett, 2001) in CMM and FAB. This was confirmed by mouse bioassay (MBA) and PCR.

Series of 5 tubes containing 4.5 mL of CMM and FAB were inoculated with 0.5 mL of spore suspension or 0.5 g honey. This was considered as the 10^{-1} dilution. For further dilutions, spore suspension and honey samples were 10-fold serially diluted in sterile water. From each dilution, 0.5 mL was transferred to 5 tubes containing 4.5 mL of enrichment media. For spore suspension, duplicate test samples were tested in FAB; while for honey samples CMM and FAB were used in parallel to determine which medium was better for recovery of *C. botulinum* spores. All tubes were heated at 60 ^{o}C for 30 min, and then cooled immediately with cold water. The number of spores in suspension of each strain was obtained after an anaerobic incubation for 4 d at 35-37 ^{o}C. Tubes showing bacterial growth were checked with PCR and MBA. The number of *C. botulinum* spores was obtained by converting the number of positive tubes to a MPN from the reference table (Peeler, 1992).

4.6 Procedures for detection of *C. botulinum* spores in honey and infant foods

After enrichment in the liquid media, PCR was carried out targeting the botulinal neurotoxin encoding genes. The confirmation of PCR positive samples was based on the restriction enzyme analysis of PCR products and DNA sequencing. A parallel confirmation by mouse bioassay was carried out.

4.7 Preparation of honey and infant food samples

A 20-25 g portion of honey was weighed under a laminar hood and added to a 500 mL screw-capped flask (ROTH, Karlsruhe, Germany) containing 200 mL of CMM

or FAB that had been warmed up to about 40-45 ^{o}C, and mixed well. For infant food samples, FAB was used as enrichment medium. From this diluent, 0.5 mL were pipetted to each of 4.5 mL broth tubes of FBA, RCM, and CMM, and mixed well. All of the tubes and flasks were then heated in a water bath at 60 ^{o}C for 30 min and incubated at 35-37 ^{o}C for 3-7 d. The proteolytic activity was examined by observation of the ability to digest cooked meat pellets in CMM.

4.8 Preparation of spiked honey samples for enumeration of *C. botulinum* spores

Honey was purchased from supermarkets and sterilised by autoclaving it at 115 ^{o}C for 30 min. After sterilisation, the honey was streaked onto blood agar plates and also put into FAB tubes. This was incubated aerobically and anaerobically for 3-7 d for examination of sterility. Under a laminar flow hood, 99 g of honey were put into 100 mL screw-capped bottles (ROTH, Karlsruhe, Germany). The honey bottles were heated at 50 ^{o}C for 1 h. Aliquots of 1 mL of individual spore suspensions of each *C. botulinum* type A, B, C, D, E, or F were added to these bottles. To evenly distribute the inoculated spores, the honey bottles were placed on a magnetic stirrer at 50 ^{o}C for 1 h.

The amount of spores of each strain of *C. botulinum* artificially inoculated to each honey sample is shown in Table 7. In addition, other bottles were inoculated with 1 mL of a mixture of all strains containing equal numbers of spores of each strain. Triplicate samples of inoculated honey were prepared.

Table 7: Numbers of *C. botulinum* spores inoculated in honey (spores g^{-1})

Strain	Number of spores	95% confidence intervals	
		Low	High
A 2298	$7x10^4$	$2x10^4$	$2x10^5$
B 2299	$1x10^4$	$3x10^3$	$3x10^4$
C 2145	$1x10^4$	$3x10^3$	$3x10^4$
D 2301	$7x10^4$	$2x10^4$	$2x10^5$
E 2625	$2.6x10^4$	$7x10^3$	$8x10^4$
F 2303	$1.6x10^4$	$5x10^3$	$5x10^4$
Mixed (each type)	$1x10^3$		

4.9 Preparation of spiked infant food samples for detection of *C. botulinum* spores

Infant foods were first checked for the presence of *C. botulinum* and its toxins by MBA and PCR after enrichment in FAB under anaerobic conditions at 35-37 ^oC for 7 d. The negative samples in both procedures were used for the spiking.

Under a laminar flow hood, an amount of 19 g of infant food samples was weighed and inoculated with one millilitre of spore suspension containing equal numbers of spores of *C. botulinum* types A, B, C, D, E, and F. The same procedure was conducted as that for spiked honey samples. The final concentrations of *C. botulinum* were 0.1, 1, and 10 spores g^{-1} of honey or infant food sample. The spiked samples were checked for the presence of *C. botulinum* spores in parallel with purchased honey and infant foods to test the sensitivity of the applied methods.

4.10 DNA isolation

For preparation of DNA templates NucleoSpin Tissue kits (MACHEREY-NAGEL, Düren, Germany) were used according to the instructions of the manufacturer. One millilitre of bacterial culture was centrifuged for 5 min at 8,000 x g and the supernatant was discarded. The pellet was lysed by 180 µL of T1 buffer and 25 µL

of proteinase K solution at 56 ^{o}C for 3 h, and then by 200 µL of B3 buffer at 70 ^{o}C for 10 min. After adjusting the DNA binding conditions with 210 µL of 96% ethanol, the sample was applied to a column and centrifuged at 11,000 x g for 1 min. This was followed by a further two step washing procedure. The first was in 500 µL of BW in a centrifuge tube (11,000 x g, 1 min), and the second was in 600 µL of B5 buffer and centrifuged again as before. After a further centrifugation at 11,000 x g for 1 min to remove any residual ethanol buffer B5 from the sample, the DNA products were eluted with 100 µL of BE. These DNA products were used as DNA templates for the PCR procedure.

4.11 PCR procedure

The multiplex PCR assay (Lindström et al., 2001) was applied to detect *C. botulinum* type A, B, E, and F toxin genes. For detection of *C. botulinum* type D toxin genes, the PCR assay developed by Takeshi et al. (1996) was used. A recently developed method was applied to detect *C. botulinum* type C toxin genes (Gessler and Böhnel, 2006). Primers used in PCR detection are shown in Table 8.

4.12 Mouse bioassay

White mice (NMRI mice, HARLAN WINKELMANN, Borchen, Germany) with 18-25 g body weight were used for testing biological activity of the culture supernatants. After incubation, cultures were centrifuged at 4,000 x *g* for 20-30 min. The supernatants were diluted 1:10 in gelatine-phosphate buffer pH 6.2 (detailed components see Appendix), and 0.5 mL of each dilution was injected intraperitoneally (i.p.) (Smith and Sugiyama, 1988; CDC, 1998). The injected mice were observed for 4 d for signs of botulism such as ruffled fur, wasp-like narrowed waist, laboured respiration, paralysis, or death. Trypsin-treated materials and untreated duplicates were conducted in parallel. For trypsinisation, trypsin solution (20 mg trypsin [SIGMA-ALDRICH, Deisenhofen, Germany] in 10 mL sterile demineralised water stored at -20 °C) was added to the culture supernatant as 1% (v/v) followed by incubation at 37 °C for 30 min. The toxicity is given in MLD mL^{-1}

corresponding to the reciprocal value of the highest dilution at which both mice showed typical signs and died.

Table 8: Primers for PCR detection of *C. botulinum* types A to F

Toxin type*	Primer name	Primer sequence (5'-3')	PCR product size (bp)	Location on gene (coding region)
A_f	CBMLA1	AGC TAC GGA GGC AGC TAT GTT	782	1788-1808
A_r	CBMLA2	CGT ATT TGG AAA GCT GAA AAG G		2569-2548
B_f	CBMLB2	CAG GAG AAG TGG AGC GAA AA	205	434-453
B_r	CBMLB2	CBMLB2 CTT GCG CCT TTG TTT TCT TG		638-619
C_f	CP3-01	CTG AAA AAG CCT TTC GCA TT	452	98-117
C_r	CP3-02	TTG TGC CGC AAA AGTATT GT		530-549
D_f	DS-11	GTG ATC CTG TTA ATG ACA ATG	497	32-52
D_r	DS-22	TCC TTG CAA TGT AAG GGA TGC		508-528
E_f	CBMLE1	CCA AGA TTT TCA TCC GCC TA	389	156-175
E_r	CBMLE2	GCT ATT GAT CCA AAA CGG TGA		544-525
F_f	CBMLF1	CGG CTT CAT TAG AGA ACG GA	543	185-194
F_r	CBMLF2	TAA CTC CCC TAG CCC CGT AT		727-708

* Subscript f, forward primer; subscript r, reverse primer.

The PCR reaction mixture consists of 25 µL of Bioron mastermix (BIORON, Ludwigshafen, Germany), 1 µL of each primer, 3 µL of DNA template, and sterile deionised water to a final volume of 50 µL. For multiplex PCR, 1.65 µL of $MgCl_2$ (0.33 mM) are added into the PCR reaction mixture. The primer sets used in the present study were adopted from the above-mentioned assays. The Bioron mastermix contains in 1.25 µL: T*aq* DNA polymerase (0.1 units μL^{-1}), antibodies to

T*aq* DNA polymerase, 130 mM TrisHCl (pH 8.8 at 25 ^{o}C), 32 mM $(NH_4)_2SO_4$, 0.02% Tween-20, 3 mM $MgCl_2$, dNTPs (0.4 mM of each of dATP, dCTP, dGTP, and dTTP).

A programmable thermocycler (TGRADIENT, BIOMETRA, Göttingen, Germany) was used for the amplification. Each multiplex PCR consists of an initial denaturation at 94 ^{o}C for 10 min; 27 cycles of denaturation (95 ^{o}C for 30 s), annealing (60 ^{o}C for 25 s), and extension (72 ^{o}C for 1 min 25 s); and a final extension at 72 ^{o}C for 3 min. For types C or D PCR, after the same initial denaturation at 94 ^{o}C for 10 min; 30 cycles of denaturation (94 ^{o}C for 1 min), annealing (55 ^{o}C for 1 min), and extension (72 ^{o}C for 1 min); and a final extension at 72 ^{o}C for 10 min followed. The amplified PCR products were visualised in 2% agarose gels (MERCK, Darmstadt, Germany) stained with ethidium bromide. Positive and negative controls were run with each PCR. The sizes of the amplification products were measured by using standard DNA fragments (DNA molecular weight marker VI, FINNZYMES, Espoo, Finland). The visualisation and photographs of the amplification products were carried out with a UV transilluminator (MWG-BIOTECH, Ebersberg, Germany).

4.13 Restriction enzyme analysis of PCR products

The PCR products were first concentrated by ethanol precipitation. A volume of 0.30 µL of PCR products were mixed well with 3 µL 3M NaOAc (pH 5.2) and 70 µL ice cold ethanol 96%, which was chilled to -20°C just prior to use. The mixture was left overnight at -20°C and then centrifuged at 10,000 x *g*. The supernatant was discarded and the precipitate was dried inside a biological hood for 30-40 min to remove the residual ethanol. The pellet was then resuspended with 10 µL of EB buffer (10 mM Tris·Cl, pH 8.5). PCR products of types A, B, E, and F were treated by DraI (QBIOGENE, Heidelberg, Germany), while PCR products of types C and D were digested by MboII (QBIOGENE, Heidelberg, Germany) (Table 9).

Table 9: Restriction enzymes used in the restriction enzyme analyses

Type	Reference strain*	Enzyme	Fragments	Fragment size
A	X52066	DraI	2	136; 646
B	M81186	DraI	3	68; 153; 472
E	X62089	DraI	2	97; 293
F	L35496 (proteolytic)	DraI	2	80; 463
F	M92906 (non-proteolytic)	DraI	2	80; 463
C	X53751	MboII	2	226; 246
D	S49407	MboII	2	145; 373

* GenBank entries offered by the NCBI

Each restriction enzyme reaction included 5 µL of ethanol-concentrated or purified PCR products, 0.01 µL BSA (Bovine Serum Albumin), 0.09 µL buffer provided by the producer, 0.9 µL distilled water, and 0.2 µL enzyme MboII (or 0.1 µL of enzyme DraI). The restriction reaction occurred at 37 °C for 1 h in a water bath and was then inactivated by heating for 10 min at 65 °C or 85 °C, for MboII or DraI respectively. The digested products were visualised and recorded using the same procedure as that used for the PCR procedure.

4.14 Purification of PCR fragments for sequencing

PCR fragments from PCR-positive samples were purified for sequencing using Montage PCR filter units (MILLIPORE, Schwalbach, Germany) following the protocol of the supplier. One hundred µL of PCR fragments and 300 µL distilled water or buffer EB (10 mM Tris·Cl, pH 8.5) were pipetted into a spin column (reservoir) that was put into a provided 1.5 mL reaction tube and spun at 1,000 x g for 15 min. The filtrate was saved until the sample was analysed. The spin column was put into another clean 1.5 mL reaction tube and 20 µL distilled water or buffer EB were pipetted carefully into the end of the reservoir without touching the

membrane surface. The reservoir was inverted into a clean eppendorf and centrifuged at 1,000 x g for 2 min. The purified PCR fragments were stored at -20 ^{o}C until sequencing.

4.15 Sequencing analysis

The DNA sequencing analysis was carried out at the Institute of Veterinary Medicine, Faculty of Agricultural Sciences, Georg-August University Göttingen, using an ABI PRISM 3100 Analyser (APPLIED BIOSYSTEMS, Foster City, USA). The primers used were similar to that used in the PCR procedures to obtain sequences of both ends from both DNA strands to obtain double-stranded sequences. A reaction mixture for sequencing contained 2 µL PCR product, 1 µL of the respective primer at a concentration of 10 pmol, 3 µL Big Dye (APPLIED BIOSYSTEMS, Foster City, USA), and distilled water to a final volume of 10 µL. For each sample, a set of mixtures of forward and reverse primers was prepared. PCR procedures for the sequencing were carried out in a T-gradient thermocycler (BIOMETRA, Göttingen, Germany). The PCR programme consisted of a denaturation at 96 ^{o}C for 30 s, followed by 30 cycles of denaturation at 96 ^{o}C for 10 s, annealing for 5 s, and an extension for 4 min at 60 ^{o}C. The annealing temperature was 55 ^{o}C for types C or D, while for types A, B, E, or F was 60 °C. Sequences obtained were analysed using the sequence analysis module SeqMan with the software package DNASTAR Lasergene (GATC, Konstanz, Germany). This was compared with GenBank entries offered by the National Center for Biotechnology Information (NCBI).

4.16 Isolation of *C. botulinum* from PCR-positive samples

Isolation of *C. botulinum* was carried out from the PCR-positive samples after sequencing of PCR products. Aliquots of 100 µL of each enrichment culture in CMM, FAB, and RCM were transferred into tubes containing 5 mL fresh FAB for a further anaerobic incubation of 2 d at 35-37 °C. The cultures were streaked on FAAE and incubated under the same condition for 4 d. Colonies showing positive

lipase reaction were taken and inoculated into FAB tubes and incubated for 2 d. Moreover, the colonies were Gram-stained and microscopically examined. These cultures were confirmed by PCR and MBA. The isolates were frozen at -30 °C, lyophilized in a BETA 1 freeze-dryer (CHRIST, Osterode am Harz, Germany), and stored at 4 °C.

4.17 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was used only for samples that showed non-specific bands in agarose electrophoresis. A total of 10 mL of 6% polyacrylamide gel contains 8 mL of DNA-PAGE [480 mL 2xTBE buffer (Tris Borate EDTA, SIGMA, Deisenhofen, Germany), 98 mL 50% glycerol, and 240 mL deionised water]; 2 mL of 30% AA-Bis (BIO-RAD, München, Germany); 100 µL of 10% ammonium persulfate; and 10 µL of TEMED (N,N,N′,N′-Tetramethylethylenediamine, SIGMA, Deisenhofen, Germany). The electrophoresis was carried out in 1xTBE loading buffer at a constant voltage of 100 V for 1 h. After the PAGE the DNA fragments were stained using silver staining kit of ROTH (CARL ROTH, Karlsruhe, Germany), following the producer's instructions. For each gel a positive and a negative control were carried out in parallel.

Specific bands that were at the same position as the positive control on the agarose gel were cut out and put into 50 µL of buffer EB overnight at 4 ^{o}C. The buffer was taken out and purified using a QIAquick Gel Extraction Kit (QIAGEN, Düsseldorf, Germany). Briefly, three volumes of buffer QG were added to the DNA buffer, mixed well, and pipetted into a QIAquick Spin Column. After centrifugation at 10,000 x *g* for 1 min, the flow-through was discarded and 700 µL buffer PE was added. The QIAquick Spin Column was centrifuged at the same speed for 1 min. After discarding the flow-through, an additional centrifugation at 10,000 x *g* for 1 min was carried out to remove the residual ethanol from the wash buffer PE. Finally, the QIAquick Spin Column was placed into a clean 1.5 mL microcentrifuge tube and 50 µL buffer EB (10 mM Tris·Cl, pH 8.5) was added. After one minute the column was centrifuged in the same way as that in the other steps. The purified

DNA samples were stored at -20 ^{o}C until further analyses such as sequencing, PCR, or restriction enzyme analyses were performed.

4.18 Statistical analysis

The results were subjected to statistical analysis by using SAS (SAS/STAT User Guid, Version 8.1, CARY, NC, USA). Analyses of variance to yield the significance of differences in recovery of *C. botulinum* among types and between two enrichment media were conducted.

5 RESULTS

5.1 Spore production

Strains A 2298, B 2299, D 2301, and F 2303 produced good yields of spores in MAB, SeM, and SM, respectively. The sporulation was 80-100%. The first sporangia as well as spores were observed on the second day of incubation. However, these strains sporulated poorly in other media. No variation in ability to sporulate occurred in case of strain F 2303 in SM or in SM with dried hay added (SM-hay).

Sporulation up to 90% of strains C 2145 and E 2625 was obtained in FAAE, while other C and E strains produced lower than 30%. Substitution of egg yolk by horse blood in FAAB resulted in markedly poor sporulation yield of strains B 2299, C 2145, C 2279, E 2302, and F 2303.

After centrifugation of sporulation cultures, resuspension of the pellets in SSS for further transformation into the spore form from the vegetative cells obtained a marked increase (10-20%) in sporulation of all strains. There was no loss of toxigenicity of the spores.

No spore or very poor sporulation was observed on BA and EYA and in RCM with dried hay added (RCM-hay). On BA, strain C 2300 produced up to 80% spores on the third day of incubation. However, bioassay and PCR showed the loss of toxigenicity.

The media with the highest sporulation yield of the different *C. botulinum* strains are shown in Table 10.

Table 10: Spore production of the *C. botulinum* strains in various media

Strain	**Media**	**Incubation**		**Sporulation rate**
		Time (d)	Temperature (^{o}C)	**(%)**
A 2298	TM	2-4	30-35	90-100
B 2299	MAB	2-4	35-37	80-100
C 2145	FAAE	3-5	35-37	80-90
D 2301	SeM	2-4	35-37	90-100
E 2625	FAAE	3-5	30-35	70-90
F 2303	SM	4-7	30-35	80-100

5.2 Enumeration of *C. botulinum* spores in spiked honey samples

The number of each strain of *C. botulinum* in honey samples inoculated with individual type of spores estimated by the MPN-PCR method is shown in Table 11. There was a significant difference between the numbers of types C and D ($p<0.05$). Type D was significantly different to types B and F, while type C was significantly different to types A and E. No significant difference in the number of types A and E as well as types B and F. Type D revealed the highest recovery, while type C had the lowest recovery.

In honey inoculated with spore mixtures of the *C. botulinum* types, *C. botulinum* type E revealed the highest, whereas type D the lowest number (Table 12). There was a significant difference in MPN between the two types. No significant difference ($p>0.05$) was obtained in the number of types A, B, C, and F.

Table 11: Recovery of *C. botulinum* in honey samples artificially spiked with individual spore types (MPN g^{-1})

Type	n	MPN	
		Mean*	SE*
A	6	112,000^{ab}	35,000
B	6	66,000^{bc}	9,000
C	6	21,000^{c}	10,000
D	6	137,000^{a}	22,000
E	6	89,000^{ab}	17,000
F	6	59,000^{bc}	9,000

*n, number of samples; *, rounded figures; ^{abc}Means with different superscripts differ significantly ($p<0.05$); SE, standard error*

The number of recovered spores of *C. botulinum* estimated by MPN-PCR was similar to, or higher than the number of spiked spores (Fig. 1). Similar results were obtained in honey spiked with mixed spore types except for types B and D recovery rates were lower.

Table 12: Recovery of *C. botulinum* in honey samples artificially spiked with mixed spore types (MPN g^{-1})

Type	n	MPN	
		Mean*	SE*
A	6	3,000^{ab}	800
B	6	2,000^{ab}	1,000
C	6	2,000^{ab}	1,000
D	6	1,000^{b}	200
E	6	4,000^{a}	700
F	6	2,000^{ab}	400

*n, number of samples; *, rounded figures, ^{ab,} Means with different superscripts differ significantly ($p<0.05$); SE, standard error*

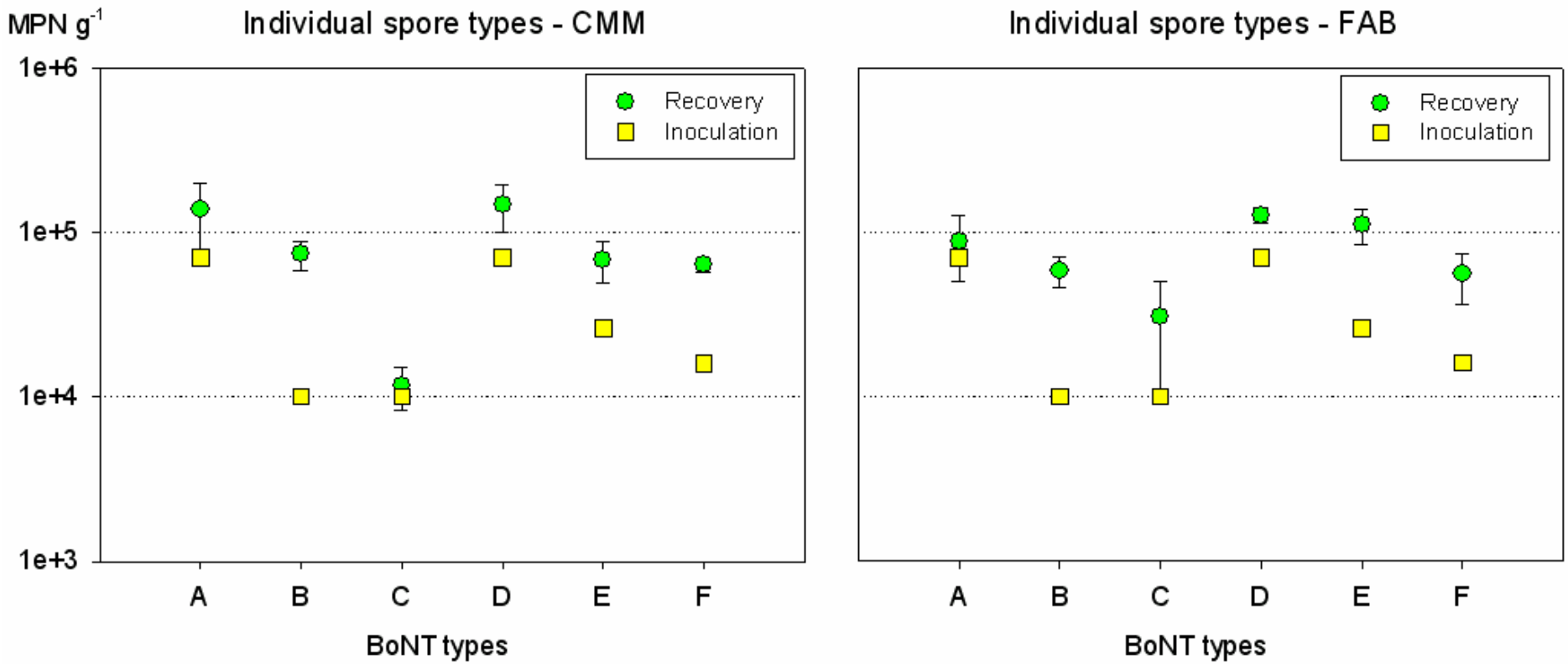

Fig. 1: Comparison of recovery of *C. botulinum* in inoculated honey with individual spore types in different media by PCR

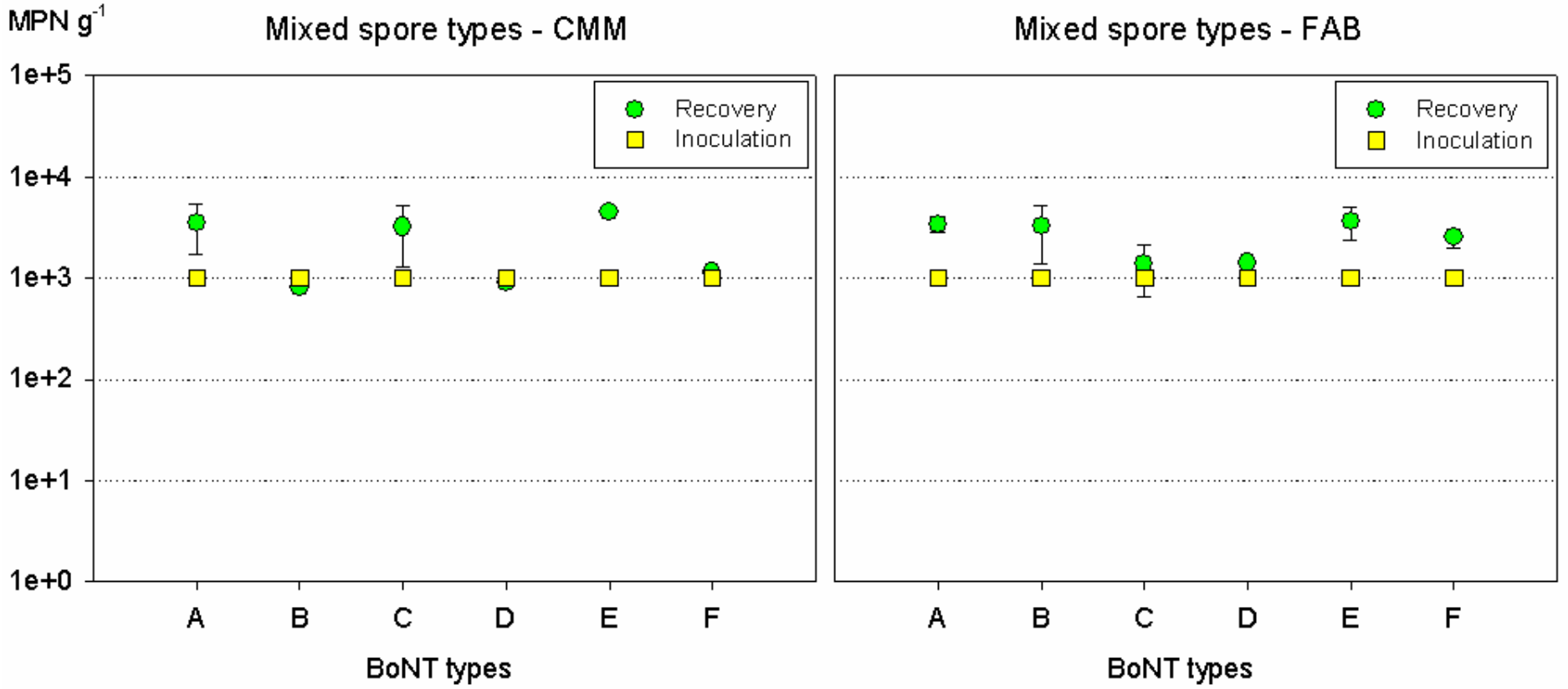

Fig. 2: Comparison of recovery of *C. botulinum* in inoculated honey with mixed spore types in different media by PCR

The confirmation using MBA was carried out with growing tubes at the highest dilution. All tubes that were PCR positive were also positive in MBAs.

Comparison of MPN of *C. botulinum* in honey samples inoculated with individual (group A) and mixed (group B) types of spores between the two enrichment media FAB and CMM was carried out. In both media, no difference in the number of *C. botulinum* in honey samples of group A as well as group B was estimated by MPN-PCR (Table 13 and 14). The two enrichment media CMM and FAB revealed no significant difference in MPN of *C. botulinum* types A to F per gram of honey.

Table 13: Recovery of *C. botulinum* types A to F in honey samples inoculated with individual type of spores from different enrichment media (MPN g^{-1})

Medium	**n**	**MPN**	
		Mean*	SE*
CMM	18	83,000[a]	16,000
FAB	18	78,000[a]	11,000

*n, number of samples; *, rounded figures; [a]Means with the same superscripts are not significantly different (p>0.05); SE, standard error*

Table 14: Recovery of *C. botulinum* in honey samples spiked with mixed types of spores from different enrichment media (MPN g^{-1})

Media	**n**	**MPN**	
		Mean*	SE*
CMM	18	2,000[a]	500
FAB	18	3,000[a]	400

*n, number of samples; *, rounded figures; [a]Means with superscripts are not significantly different (p>0.05); SE, standard error*

5.3 Incidence of *C. botulinum* spores in honey and infant food samples collected from Vietnam and Germany

A total of 46 honey and infant food samples obtained from supermarkets in Germany and other 278 honey and infant food samples from retail stores in

Vietnam were analysed for the presence of *C. botulinum* spores.

5.3.1 Incidence of *C. botulinum* spores in honey and infant foods purchased in Vietnam

The results of PCR analyses are presented in Table 15. Twenty of 179 (11.2%) infant food samples and eight of 99 (8.1%) honey samples were PCR positive. None of rice/soya bean infant foods was PCR positive. Only one of 35 (2.9%) milk infant foods was positive. Five of 50 (10%) pork/chicken samples were found PCR positive. The highest numbers of PCR-positive samples were obtained from fruit/vegetable and shrimp/fish infant foods, 20% and 23.3% respectively.

From the positive samples, *C. botulinum* type C was most frequently obtained from fruit/vegetable and shrimp/fish infant foods. While types B and E were obtained from various types of infant foods, type D was detected in honey samples with the most frequent number of samples (7 of 8 positive samples). More than one type was detected in eight infant food samples (3 types), in two other infant samples (2 types), and in two honey samples (2 types).

Table 15: Incidence of *C. botulinum* spores in honey and infant food samples in Vietnam by PCR

Types of samples		n	Positive (%)	Type (*)
Honey		99	8 (8.1)	B (1), C (2), D (7)
Infant Foods	Pork/Chicken	50	5 (10.0)	B (5), E (4), F (2)
	Milk	35	1 (2.9)	B, E, F
	Fruits/Vegetables	35	7 (20.0)	B (2), C (7), E (2)
	Shrimp/Fish	30	7 (23.3)	B (3), C (7), E (4)
	Rice/Soya bean	29	0 (0.0)	
Total		**278**	**28 (10.1)**	

n, Number of samples tested; (*), Number of *C. botulinum* type detected

5.3.2 Incidence of *C. botulinum* spores in honey and infant foods purchased in Germany

Eight of 46 samples collected in Germany harboured spores of *C. botulinum* type B (Table 16). Of these samples, two honey samples were also type E positive. All infant milk powder samples were negative, while 4 of 9 infant cereal formula, one of 8 potato purée, and one of 7 vegetable formula samples were positive for type B.

Table 16: Incidence of *C. botulinum* spores in honey and infant food samples in Germany by PCR

Types of samples	n	Positive (%)	Type (*)
Honey	13	2 (15.4)	B (2), E (2)
Infant cereal formula	9	4 (44.4)	B
Infant milk powder	9	0 (0.0)	
Potato purée	8	1 (12.5)	B
Vegetable formula	7	1 (14.3)	B
Total	**46**	**8 (17.4)**	

n, Number of samples tested; (*), Number of *C. botulinum* type detected

5.4 Mouse bioassays

Mouse bioassays of the PCR-positive samples were conducted. Except two honey samples collected from Vietnam, all samples were negative regardless the incubation conditions, namely incubation time and temperature. The two honey samples, however, were then BMA negative when the mouse neutralisation test was carried out.

5.5 Isolation of *C. botulinum* from PCR-positive samples

Isolation of *C. botulinum* from PCR-positive samples was carried out by streaking on FAAE. *C. botulinum* was isolated from ten of 28 and one of eight PCR-positive samples from Vietnam and from German respectively (Table 17). However, these isolates were MBA negative. Type F PCR-positive samples presented proteolytic activity in CMM tubes.

Table 17: *C. botulinum* isolates from PCR-positive samples*

Types of samples		Numbers of isolates
Honey		5
Infant foods	Fruits/Vegetables	2
	Shrimp/Fish	4

*, all samples were from Vietnam except for a honey sample from Germany

5.6 Incidence of *C. botulinum* spores in artificially inoculated honey and infant foods

Honey and infant foods were artificially inoculated with a mixture containing an equal amount of *C. botulinum* spores to examine the validation of the method applied in the recent study. Fig. 3 presents the recovery rate of *C. botulinum* spores types A to F in inoculated infant foods at various concentrations of spore inoculation.

At the concentration of 0.1 spores g^{-1} of infant foods, no *C. botulinum* spores could be detected, while at the concentration of 100 spores g^{-1} 100% of samples inoculated with *C. botulinum* spores of types B, C, D, E, and F and 66.67% of samples inoculated with type A were detectable. Types A, B, C, and E could be detected at the concentration of 1 spore g^{-1}, while for types D and F the minimum concentration for detection was at a level of 10 spores g^{-1}.

In honey inoculated with the mixture of *C. botulinum* spores types A to F, all types were not detectable at an inoculation level of 0.1 spores g^{-1} (Fig. 4). Except for type D, all types could be recovered at the concentration of 1 spore g^{-1} with a recovery rate of 14% up to 71%. At the concentration of 100 spores g^{-1} all types were recovered in 66% to 100% of the inoculated samples. The highest recovery rates were observed in type E followed by those in type B. Type D recovered in 11% of the spiked samples at the inoculation level of 10 spores g^{-1}.

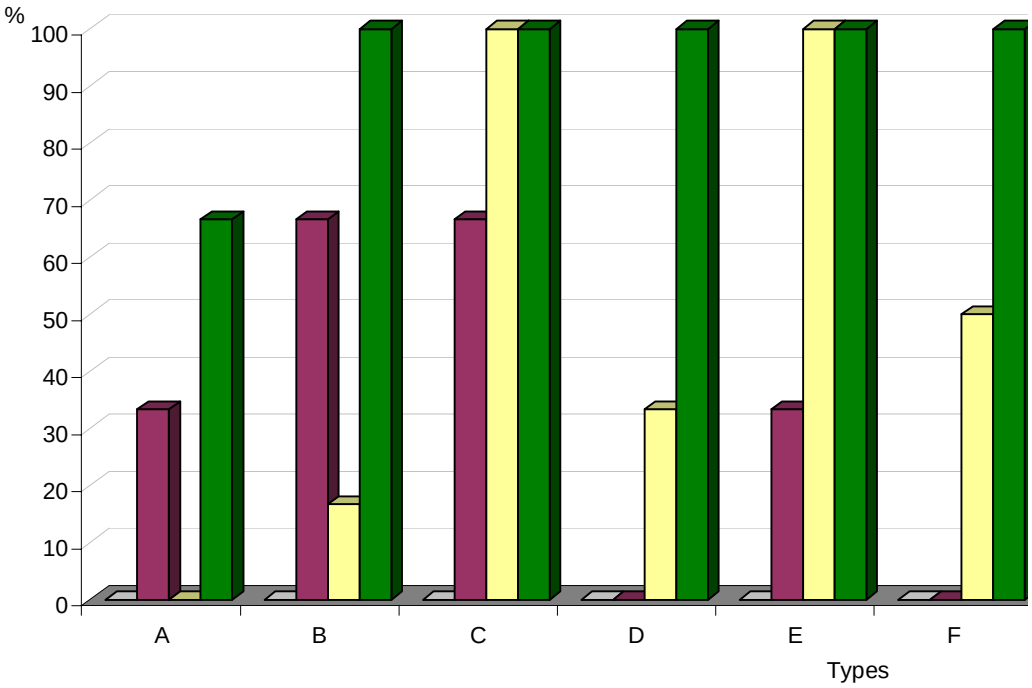

Fig. 3: Recovery rate of *C. botulinum* types in inoculated infant food samples with 0.1 (□), 1 (■), 10 (■), and 100 (■) spores g^{-1}.

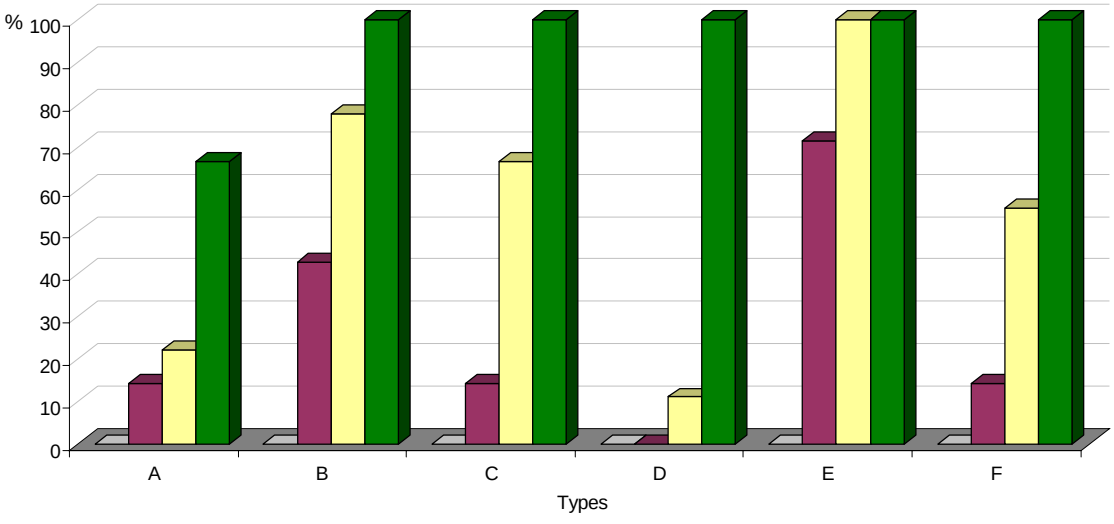

Fig. 4: Recovery rate of *C. botulinum* types in honey samples inoculated with 0.1 (□), 1 (■), 10 (■), and 100 (■) spores g^{-1}.

5.7 Restriction enzyme analysis

From PCR-positive samples, PCR products were confirmed by digestion of the amplified fragments by restriction enzymes. Digestion patterns of the amplified fragments of *C. botulinum* strains and of honey and infant food samples are presented in Fig. 5-9. Digestion patterns of honey and infant food samples were in agreement with those of the *C. botulinum* strains used as positive control samples. However, there were no or very weak patterns obtained from some samples. Four of 20 type B samples and four of 13 type E samples revealed no band. Six of 20 type B and three of 13 type E samples revealed restriction patterns in only one of two parallel analyses of each sample.

5.8 Sequence analysis

Nucleotide sequencing analyses of amplified fragments of PCR-positive samples revealed similarity levels to *C. botulinum* neurotoxin types B, C, D, E, and F (Table 18). The sequences obtained from PCR fragments of PCR-positive samples were 98-100% identical to that of respective *C. botulinum* neurotoxin types.

Table 18: Nucleotide sequencing analysis of PCR-positive samples

Type	Similarity level (%)	GenBank accession number*
B	99.58	M81186
C	99.95	AB200364
D	99.95	S49407
E	99.68	X62089
F	98.13	L35496

* GenBank entries offered by the NCBI

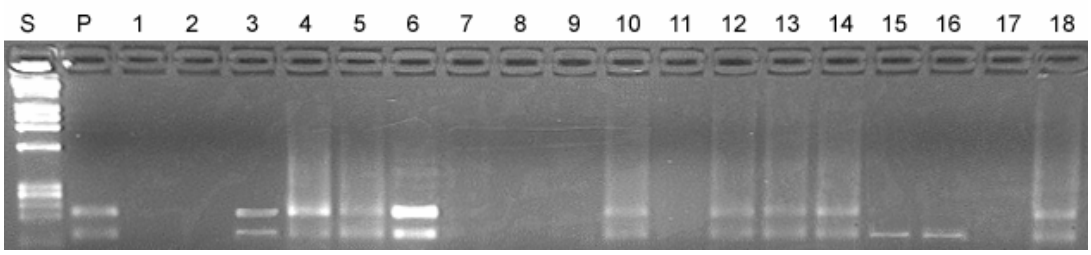

Fig. 5a: Digestion patterns of PCR products of type B with DraI. S, standard DNA; P, positive control; Lanes 1, 3, 4, 5, 6, 7, 9, 11, 13, 14, 15, 17, and 18, samples 1-13; Other lanes, repeated analysis of the samples.

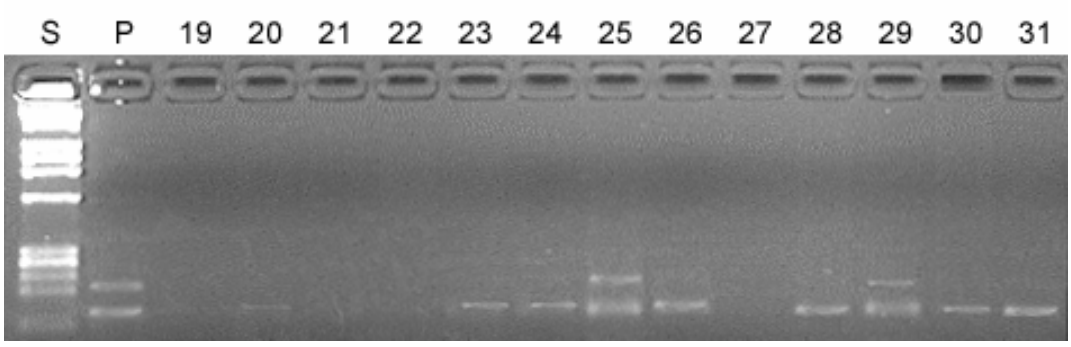

Fig. 5b: Digestion patterns of PCR products of type B with DraI. S, standard DNA; P, positive control; Lanes 19, 21, 23, 25, 27, 29, and 31, samples 14-20; Other lanes, repeated analysis of the samples.

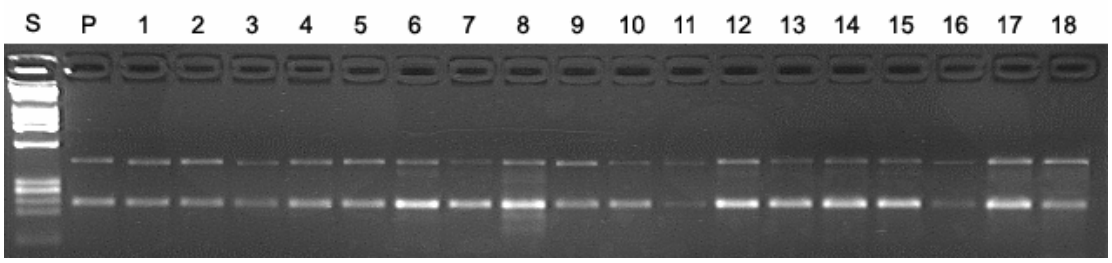

Fig. 6a: Digestion patterns of PCR products of type C with MobII. S, standard DNA; P, positive control; Lanes 1, 2, 3, 4, 6, 7, 9, 10, 11, 12, 13, 14, 15, 17 and 18, samples 1-15; Other lanes, repeated analysis of the samples.

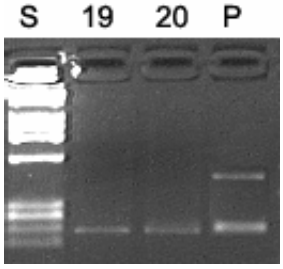

Fig. 6b: Digestion patterns of PCR products of type C with MobII. S, standard DNA; P, positive control; Lane 19 and 20, sample 16.

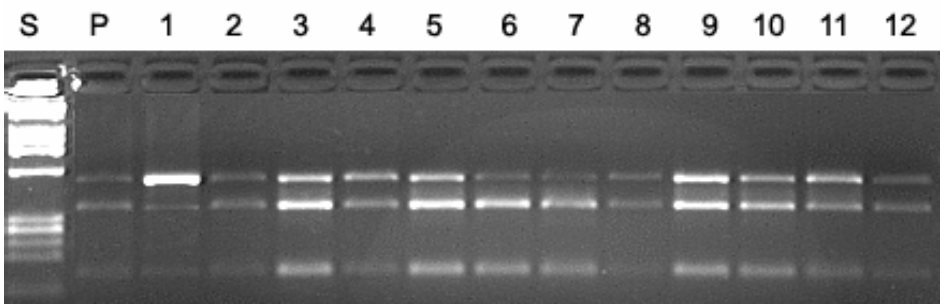

Fig. 7: Digestion patterns of PCR products of type D with MobII. S, standard DNA; P, positive control; Lanes 1, 3, 4, 6, 8, 10, and 12, samples 1-7; Other lanes, repeated analysis of the samples.

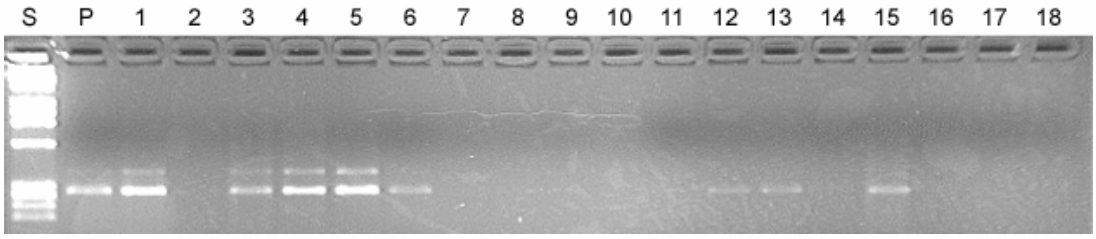

Fig. 8a: Digestion patterns of PCR products of type E with DraI. S, standard DNA; P, positive control; Lanes 1, 3, 5, 6, 7, 9, 11, 13,15, and 17, samples 1-10; Other lanes, repeated analysis of the samples.

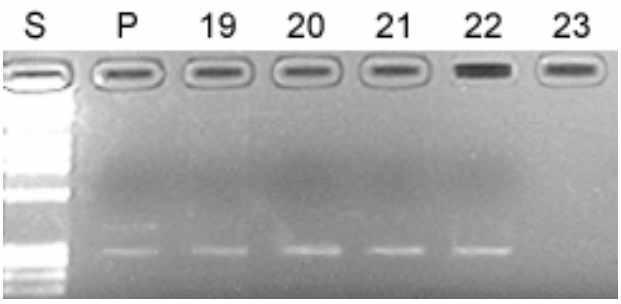

Fig. 8b: Digestion patterns of PCR products of type E with DraI. S, standard DNA; P, positive control; Lanes 19, 21, and 23, samples 11-13; Other lanes, repeated analysis of the samples.

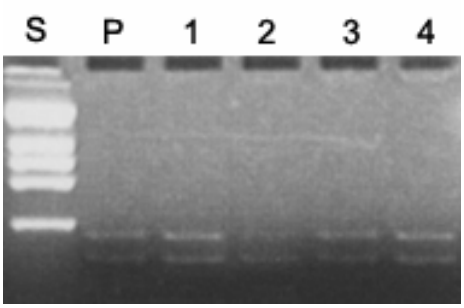

Fig. 9: Digestion patterns of PCR products of type F with DraI. S, standard DNA; P, positive control; Lanes 1, 2, and 3, samples 1-3; Lane 4, repeated analysis of sample 3.

6 DISCUSSION

6.1 Spore production

For strain A 2298, shorter incubation time of sporulation in TM was observed in comparison to *C. botulinum* 62A using the same amount of trypticase but with a substitution of peptone by 1.0% ammonium sulphate (Tsuji and Perkins, 1962). The medium used in this study was also simpler than that developed by Tsuji and Perkins. While very poor sporulation was obtained with all C strains, strain D 2301 sporulated well in SeM, which was used for sporulation of *C. botulinum* type C in the study carried out by Segner et al. (1971).

The increased sporulation of all strains in SSS was observed, while toxigenicity of the *C. botulinum* spores was not affected. The detailed effect of SSS on sporulation and toxigenicity of *C. botulinum* spores was not reported in previous studies. Kihm et al. (1990) found that *C. botulinum* 113B sporulated well in media containing 0.01 to 1.0 mM Fe, Cu, Mn, or Zn.

Similar poor sporulation on BA was also reported by Roberts (1965). While in DSM spores were well produced by *C. perfringens* (Duncan and Strong, 1968), *C. botulinum* strains grew poorly and produced no spores. On BA, strain C 2300 produced up to 80% spores on the third day of incubation. However, bioassay and PCR showed the loss of toxigenicity.

Bacteria from stock cultures should be transferred into large volumes of medium, FAB in this study, before they are inoculated into sporulation media. This method brings a large number of cells to maturity and eventual sporulation at approximately the same time. Similar observation was reported by Perkins in production of different clostridia spores (1965).

6.2 Enumeration of *C. botulinum* spores in spiked honey samples

Compared to the numbers of *C. botulinum* spores inoculated in spiked honey samples, the MPN results were either the same or higher reflecting the high recovery rate of *C. botulinum* in both media CMM and FAB used. High recovery rates up to 100% or even higher than the inoculated number were obtained by

MPN-PCR estimation of *Listeria monocytogenes* in fermented sausages (Martin et al., 2004) and of *Vibrio parahaemolyticus* in seafood (Miwa et al., 2003).

MPN method has been used for the enumeration of low levels of bacteria, especially bacteria in water. MPN method, however, unlike bacterial plate count, tends to yield a higher bacterial count (Eller et al., 1967; Peeler et al., 1992). Different researchers described quantification of microorganisms using MPN-PCR technology in various types of samples: in soil (Fredslund et al., 2001), in water (Savill et al., 2001; Chern et al., 2004), and in food (Mantynen et al., 1997; Miwa et al., 2003; Martin et al., 2004). MPN-PCR was used to enumerate *C. botulinum* spores in soil amended with spiked compost (Gessler and Böhnel, 2006). A MPN-PCR protocol for detection and enumeration of *C. botulinum* types A, B, E, and F in fish and sediment samples was developed by Hielm et al. (1996). A quantitative PCR-MPN analysis was applied for determination of *C. botulinum* types A, B, E, and F in river lampreys caught in Finnish rivers (Merivirta et al., 2006). The technique was also applied for enumeration of *C. botulinum* spores in honey (Nevas et al., 2002). Enumeration of *C. botulinum* spores using the MPN-PCR method in this study was completed within 5 d, or 8 d when further growing tubes were examined.

The method utilised a large amount of culture media and required much laboratory work for media preparation, pipetting, and conducting PCR with a large number of reactions. However, the MPN-PCR is a simple method and does not require high technological equipment that is not always available in developing countries like Vietnam. The advantage of the MPN-PCR method is that only viable and toxigenic *C. botulinum* spores in honey are enumerated by the multiple tube cultures and PCR detection. The PCR confirmation applied in MPN-PCR method yielded significant labour and time savings. Furthermore, compared to conventional MBA confirmation for quantification of *C. botulinum* in food samples using MPN method, which is followed by mouse bioassays (Sugiyama, 1978; Midura et al., 1979; Huhtanen et al., 1981; Nakano et al., 1990; Nakano and Sakaguchi, 1991), the MPN-PCR method is obviously more convenient and reliable, and avoids facilities as well as ethical issues using experimental animals.

6.3 Incidence of *C. botulinum* spores in honey and infant food samples collected from Vietnam and Germany

A total of 46 honey and infant food samples obtained from supermarkets in Germany and other 278 honey and infant food samples from retail stores in Vietnam were analysed for the presence of *C. botulinum* spores.

Honey has been known to contain *C. botulinum* spores and considered a potential source of infant botulism (Midura and Arnon, 1976; CDC, 1998; Arnon et al., 1979; Midura et al., 1979). Other sweeteners used to supplement infant foods such as corn syrup and other syrups were also investigated (Kautter et al., 1982; Hauschild et al., 1988; Lilly et al., 1991). One of 354 light and one of 271 dark corn syrups were presumptively positive for type *C. botulinum* type A spores, while subsequent testing of the entire contents of both bottles and all other 113 syrups were negative (Lilly et al., 1991). In a study analysing 150 honey, 43 syrup, and 40 dry cereal samples in the USA conducted by Hauschild et al. (1988), *C. botulinum* type A spores were detected in a honey sample associated with an infant botulism case and type B in a sample of rice cereal. From ten categories of infant foods in the USA, including dry cereals, non-fat milk, pasteurised cow's milk, canned fruits and fruit juices, granulated cane sugar, fresh carrots, corn syrup, and honey, with a total of 910 samples, only two of 100 honey samples and eight of 40 corn syrup samples contained *C. botulinum* spores, type A and B, respectively (Kautter et al., 1982). However, *C. botulinum* was not detected in other infant food samples in New York City such as honey, dry cereal, canned formula, evaporated milk, non-fat dry milk, and canned baby food (Guilfoyle and Yager, 1983). The results obtained from these studies reflect the low incidence of *C. botulinum* spores in honey and infant food samples.

The incidence of *C. botulinum* spores in honey has been investigated in several studies applying various methods. The incidence obtained in this study was 8.1% in honey from Vietnam and 15.4% in honey from Germany. The results were consistent with those (11%) reported by Nevas et al. (2002) applying PCR to detect 190 honey samples from Finnish and imported honey. Relatively similar incidence levels were obtained by other investigators: 7% in Argentina (Monetto et al., 1999), 7.1% in Brazil (Schocken-Iturrino et al., 1999), 7.5% in

the USA (Huhtanen et al., 1981), 8.5% in Japan (Nakano et al., 1990), 10% in the USA (Midura et al., 1979), and 14% in Denmark (Nevas et al., 2005a). Lower incidence from 0.9% to 3% was reported in other studies (Hauschild et al., 1988; Kautter et al., 1982; De Centorbi et al., 1997; Rall et al., 2003). One of 52 honey samples (1.9%) in Germany examined by PCR was reported (Mäde et al., 2000). No *C. botulinum* spores were detected in 282 German honey samples (Flemming and Stojanowic, 1980; Hartgen, 1980). *C. botulinum* spores were not detected in one honey sample originating from Vietnam in the survey conducted by Delmas et al. (1994).

Honey and corn syrup in rare cases were considered the only foodstuffs associated with infant botulism until an infant botulism case derived from milk formula was described (Brett et al., 2005). The infant consumed infant formula milk powder, and *C. botulinum* type B was isolated from one of five unopened packets from the same batch. *C. botulinum* type A was detected from an opened container of dried rice pudding and *C. botulinum* type B from opened infant formula milk powder. It was suggested that multiple *C. botulinum* were present in both infant food and the intestine during infant botulism.

The results obtained from infant milk formula samples of the study are consistent with those in studies conducted by Kautter et al. (1982) and Guilfoyle and Yager (1983). Outbreaks of botulism involving milk and dairy products are rare. Less than 1% of the total number of foodborne botulism outbreaks associated with such products has been recorded since 1899. Type A, which was predominant, and type B have been associated with these outbreaks. Involvement of home and commercially prepared products in these outbreaks was equal (Collins-Thompson and Wood, 1993).

High incidence of *C. botulinum* spores was found in pork/chicken, fruits/vegetables, and shrimp/fish infant food samples from Vietnam and in infant cereal formula from Germany in comparison to other types of infant food samples tested. This raises the possibility of *C. botulinum* spore contamination of the ingredients encompassing all the main food groups like fish, meat, poultry, cereals, fruits, and vegetables. Fish has been reported in many food surveys to have the highest level of contamination with *C. botulinum*. Types A, B, C, D, E, and F were identified in fish, shellfish, shrimp, and crabs. A large number of

foodborne botulism outbreaks were associated with fish (Dodds, 1993a). Surveys for *C. botulinum* in meats and poultry showed low levels of contamination compared to fish, since contamination of farm environment is likely lower than aquatic environment (Dodds, 1993b). *C. botulinum* spores types A and B were detected in fruits and vegetables, particularly those harvested from the soil which was contaminated.

While types B, E, and F were predominantly detected in honey samples, there have been few studies in which type A, C, and D were found. Type A was detected in honey from Argentina (Nakano et al., 1990; De Centorbi et al., 1994; De Centorbi et al., 1997; Monetto et al., 1999) and from Japan (Nakano et al., 1990), type C in honey from China, and Japan (Nakano et al., 1990), and type D from Brazil (Schocken-Iturrino et al., 1999). Honey samples containing more than one type of *C. botulinum* spores in a sample were reported in various studies (Nakano et al., 1990).

There were two out-of-date infant food samples purchased in Vietnam, one of which was *C. botulinum* positive. These samples had the expiry dates of one year before the date on which they were purchased for analysis. The presence of the out-of-date foods raises a question about the control of foods purchased in markets in Vietnam.

Although the positive samples in this study were MBA negative for *C. botulinum* toxin, they were not recommended for feeding infants less than one year of age.

Quorum sensing, a mechanism of cell-cell communication of bacteria with their peers as a result of bacterial population density has been reported (Withers and Nordstrom, 1998; Bassler, 1999; Hastings and Greenberg, 1999; Augustin et al., 2000; Gonzalez et al., 2001; Miller and Bassler, 2001). The important role of quorum sensing in food microbiology has been reviewed (Smith et al., 2004). Quorum sensing is used to regulate many diverse physiological activities including symbiosis, competence, antibiotic production, biofilm formation, and sporulation (Miller and Bassler, 2001). For pathogenic bacteria like *C. botulinum* the quorum sensing is also a way to coordinate production of virulence factors such as toxin. Foodborne pathogens such as *E. coli* and *Salmonella typhimurium* use quorum sensing to process cell density information and metabolic cues. The role of cell-cell-signalling by autoinducer 2 (AI-2) for toxin

production in *C. perfringens* was described by Ohtani et al. (2002). Zhao et al. (2006) found evidence for signalling molecules providing the first step in characterising quorum sensing in *C. botulinum.* Little is known, however, on the role of quorum sensing in the growth, sporulation, and/or toxin production of *C. botulinum* present in food.

6.4 PCR analyses

The sensitivity of the multiplex PCR, using DNA templates from pure *C. botulinum* cultures, was 10 cells for type B and 100 cells presenting in a reaction mixture for types A, E, and F. The enrichment of samples before PCR is required to obtain more reliable results due to normally low numbers of *C. botulinum* spores present in naturally contaminated samples (Lindström et al., 2001). PCR for type D using primers DS-11 and DS-22 could detect 2.5 pg of DNA templates, approximately 875 cells (Takeshi et al., 1996). These protocols were found rapid, specific, and reliable for the detection of *C. botulinum*.

The detection of *C. botulinum* is markedly improved by means of the multiplex PCR assay. In our study, the DNA templates for the PCR assays were prepared using the NucleoSpin Tissue kits. This DNA extraction method showed marked improvement in PCR products obtained and visualised in agarose electrophoresis in comparison with the DNA extraction described in the PCR protocol developed by Lindström et al. (2001). In a preliminary study, the DNA extraction using NucleoSpin Tissue kits revealed clearer and less unspecific bands compared to the other DNA extraction method. There were also cases in which the former method revealed clear positive bands while no band was observed in samples prepared with the latter method.

A method for detection of *C. botulinum* in honey was developed by Mäde et al. (2000) applying PCR after enrichment. The amplified products were verified by DNA sequencing. The procedure was found easy to conduct without using animal experiment. However, only *C. botulinum* types A, B, E, F, and G were detected in this study.

The sensitivity of PCR used to detect *C. botulinum* types A, B, E, F, and G in food was compared to that of MBA, the correlation between the two methods was 95.6% for artificially contaminated food samples (Fach et al., 1995). The

PCR method was found more rapid than MBA and avoided handling laboratory animals.

6.5 Mouse bioassays

Mouse bioassays of the PCR-positive samples were conducted. The prolongation of incubation time in MBA to increase the possibility of obtaining higher numbers of positive samples was explained by the presence of the small number of spores in the samples producing a low concentration of toxin that only affected the mice after a longer incubation time (Schocken-Iturrino et al., 1999). However, the results obtained in MBAs conducted in this study presented that all PCR-positive samples were not toxic. The concentration of *C. botulinum* spores in the samples might be so low to produce an efficient toxin level to the mice. In the study conducted by Fach et al. (1995), five of 66 samples inoculated with *C. botulinum* type B were PCR positive, but MBA negative, reflecting the possibility of PCR to detect the *C. botulinum* that was present but did not grow sufficiently to produce enough toxin to kill the mice. Another possible reason is the presence of silent genes in the *C. botulinum* spores detected. Franciosa et al. (1994) reported that genes encoding type B neurotoxin were detected by PCR in 43 of 79 strains of *C. botulinum* type A. However, only one of these strains produced a demonstrable type B toxin. A confirmation of the presence of silent or unexpressed botulinum toxin type B genes was conducted using PCR-restriction fragment length polymorphism analysis and specific oligonucleotide probes (Cordoba et al., 1995). The presence of silent genes clusters in many *C. botulinum* strains has been reported. Genes encoding type B botulinum neurotoxin in some type A strains of *C. botulinum* exhibiting no type B toxin activity were detected by Hutson et al. (1996). Further studies need to be conducted to investigate possibility of *C. botulinum* spores with silent toxin encoding genes in strains of other types present in honey and infant food samples.

6.6 Isolation of *C. botulinum* from PCR-positive samples

Isolation of *C. botulinum* from PCR-positive samples was carried out by streaking on FAAE. *C. botulinum* was isolated from only a part (11 of 36) of

PCR-positive honey and infant food samples. The growth of other bacteria than that of *C. botulinum* demonstrated their inhibitory effect on the growth of *C. botulinum*. The presence of inhibitory bacteria resulting in the difficulty of *C. botulinum* isolation has been widely reported (Graham, 1978; Sandler et al., 1998; Böhnel and Lube, 2000). The lipase-positive isolates were found PCR positive, but MBA negative, demonstrating a possible toxicity loss. The toxicity loss in pure *C. botulinum* isolates was reported by Eklund et al. (1971). FAA modified with either horse blood or egg yolk, was found to be better for isolation of *C. botulinum* than BA and EYA, and recommended for use (Saeed, 2005).

6.7 Incidence of *C. botulinum* spores in artificially inoculated honey and infant foods

The method applied in this study detected 1 spore of *C. botulinum* in 1 g of honey and infant foods. It may allow detecting *C. botulinum* spores in naturally contaminated honey and infant samples. The dilution of samples with enrichment media before PCR analyses increased the possibility of detection of *C. botulinum* spores in the samples and reduced the concentration of inhibitory substances that might affect the germination and growth of *C. botulinum* spores and the PCR. The centrifugation and supernatant filtration (SF) before PCR developed by Nevas et al. (2002) detected 0.1 spores of types A and B *C. botulinum* in 1 g of honey, but required much laboratory work and equipment for centrifugation and filtration. The use of dialysis for enumeration of *C. botulinum* spores in honey was described by Sugiyama et al. (1978). The method requires much time and laboratory work. Two methods, dilution and centrifugation, were developed by Midura et al. (1979) for isolation of *C. botulinum* spores in honey. The two methods were followed by MBAs and mouse neutralisation tests. Both methods could detect seven to nine spores in 1 g honey.

6.8 Restriction enzyme analysis

The identification of PCR products of *C. botulinum* types A to F using restriction enzyme digestion profiles was performed by Takeshi et al. (1996). It was found that amplified products were easily confirmed by their digestion patterns. In our study, majority of PCR products showed similar restriction fragments to those of positive control samples. From the samples of type B, 11 of 20 samples

revealed the same fragments as those of the positive sample. Five other samples revealed only one fragment, which could be in agreement with that of entry M81186. Only the 472-bp fragment could be shown, while the other two fragments with 68 bp and 153 pb were too small to be visualised on agarose gel. There was an agreement between the digested fragments of the positive control sample with those obtained from the type C samples. However, compared to the estimated digestion fragments of the entry X53751, restriction patterns obtained were partly different. Digestion patterns of PCR products of type D with MobII were in agreement with those of the positive control, but not in agreement with those of the entry S49407. Their sizes were between 310 bp and 564 bp. In comparison with positive control, some PCR fragments of type E revealed different digestion fragments. However, they were in agreement in sizes and numbers of patterns with the estimated digestion fragments of *C. botulinum* type E X62089 from the sequence data of NCBI. The fragments obtained from samples of type F were in agreement with those of positive control sample, but not in agreement with those of the entry L35496. In some cases, digestion patterns were different or no pattern was observed. The reason is that the concentration of PCR products might be too low or the size of the patterns obtained was so small that they could not be visualised on agarose gel. From our observation, the visualisation of restriction enzyme profiles on agarose gel is not optimal, especially for food samples that contain very low numbers of a target foodborne bacterium. To overcome this problem, polyacrylamide gel electrophoresis may be used.

6.9 DNA sequencing analysis

Although all of the PCR-positive samples, except two honey samples, were MBA negative, DNA sequences of their PCR products showed high level of sequence identity with GenBank entries for *C. botulinum*. The complete nucleotide sequences of the seven botulinum neurotoxin types type A to F have been analysed (Binz et al., 1990a; Binz et al., 1990b; Thompson et al., 1990; Kimura et al., 1990; Poulet et al., 1992; Whelan et al., 1992a; Whelan et al., 1992b; Willems et al., 1993; Hutson et al., 1994; Elmore et al., 1995). All the sequences were identical with those of the GenBank entries. Their positions

were within the neurotoxin encoding genes. One honey sample, although it was type C PCR positive, revealed the sequence that was not identical with the reference *C. botulinum* strains. This sample was not examined by restriction enzyme analysis and considered negative for *C. botulinum*.

7 Conclusion

The recent study achieved spore production of *C. botulinum* types A to F using various media, although none of the media used favoured sporulation of all *C. botulinum* types. The applied MPN-PCR method can be used to enumerate *C. botulinum* spores in honey despite the high concentration of sugar in honey. The enrichment media CMM and FAB were equally suitable for recovery of *C. botulinum* with similar yields. Further studies need to be conducted to assess the validity of the MPN-PCR method in naturally contaminated honey.

For detection of *C. botulinum* spores in honey and infant food samples, the dilution of samples in enrichment media ensured the germination and growth of the spores. It also reduced the concentration of inhibitory substances for PCR analyses. Therefore, the detection of *C. botulinum* spores using PCR analysis was improved. DNA sequencing analysis of PCR products allows confirmation of the amplified fragments and avoids handling laboratory animals.

Demand for convenient and minimally processed baby foods has been increasing worldwide. Spores of *C. botulinum* are likely to be present in raw materials used to produce baby foods, and are able to survive the production process of many of these products. Studies on *C. botulinum* and its spores in a wide category, as well as a large number of samples from soil, water, raw food materials, and foods should be conducted locally.

There have been no studies on *C. botulinum* spores in honey and infant foods in Vietnam and there is no information about infant botulism risk involved with consumption of honey and infant foods. Investigations on the presence of *C. botulinum* spores in honey and infant foods should be conducted on a broader basis. It should probably also be checked in other items normally fed to infants, including contamination levels of raw materials used in infant food processing.

In Vietnam, it is necessary to educate the general public about the causes and prevention not only of infant botulism, but also of foodborne and wound botulism. The public should be educated on preventive interventions such as effective

processing and preparation of commercially and home-made foods; as well as canned and preserved foods; proper use of all canned foods; cessation of feeding honey to, and addition of honey to foods for infants less than 12 months; advice regarding the potential risk of *C. botulinum*; medical care for infected wounds and safe use of needles in drug addicts. Moreover, with recognition of the threat posed by bio-terrorism, botulism outbreaks due to intentional causes must be recognised and planned for. Careful epidemiological and law enforcement investigations play a vital role in determining the cause of a botulism outbreak.

Vietnam is now on its way to becoming a member of the World Trade Organisation (WTO). With the increasing trend of global food trading, the contamination of pathogenic bacteria like *C. botulinum* in honey, infant foods, and foods in general, poses a botulism risk to customers in many countries. The Vietnamese government should compulsorily establish new legislation for food quality control in Vietnam. With respect to the work of Vietnamese policy makers, this study calls for a new quality standard for honey and infant foods. All such products should be (systematically) examined for the presence of *C. botulinum* spores in addition to other aspects of quality. This will require a great change in Vietnamese food hygiene laws.

8 SUMMARY

This study was carried out to develop a procedure for producing spore suspensions of different strains of *C. botulinum* A, B, C, D, E, and F was developed by applying different media. Moreover, a MPN-PCR method was developed to enumerate *C. botulinum* spores in honey samples, targeting the neurotoxin encoding genes.

Another object of the study was the detection of viable *C. botulinum* spores in honey samples and infant foods purchased from retail stores and supermarkets in Ho Chi Minh City, Vietnam, and from retail supermarkets in Germany. The purpose of this survey was to determine if honey and infant foods in these market areas would present a potential threat to infants in Vietnam and Germany.

The first phase of the study was carried out producing spores of different strains of *C. botulinum* A, B, C, D, E, and F. This was carried out using different media to enumerate spores of these strains in honey samples. Different strains sporulated best in different media. Type A strain 2298 sporulated well in trypticase medium (TM), type B strain 2299 in modified Anellis Broth **(**MAB), type D 2301 in Segner Medium (SeM), type F 2303 in sporulation medium (SM), type C 2300 and type E 2625 sporulated well on Fastidious Anaerobe Agar with Egg Yolk (FAAE). The sporulation rate was 80-100% in the liquid media and 70-90% on agar plates. The use of salt solution for sporulation (SSS) after harvesting the spores from the sporulation media increased the sporulation rate by 10-20%, but did not affect the toxigenicity of the spores.

A MPN-PCR method was developed to enumerate *C. botulinum* spores in honey. Sterilised honey samples were spiked with a known number of spores of different strains of *C. botulinum*. Each strain was inoculated separately and there was also a mixture of all the strains. Two media, FAB and CMM, were used as enrichment media. The retrieved number of individual strains and mixed strain spores in the spiked samples after incubation was similar and proportional to the initial spore inoculation. This was regardless of the strain

type, or if the sample had mixed strains of spores. The results were similar between the two enrichment media.

In the second phase, honey and infant food samples were collected from supermarkets in Ho Chi Minh City, Vietnam and in Göttingen, Germany. These were tested for the presence of *C. botulinum* spores using PCR. For confirmation of the amplified fragments, DNA sequencing, restriction enzyme reaction, and MBA were carried out.

Twenty of 179 (11.2%) infant food samples and 8 of 99 (8.1%) honey samples collected from Vietnam were PCR positive. The highest numbers of PCR-positive samples were shrimp/fish (23.3%) and fruit/vegetable (20%) infant foods. None of rice/soya bean infant foods was PCR positive. Out of 50 pork/chicken samples, five were found PCR positive (10%). Only one of 35 (2.9%) milk infant foods was positive.

C. botulinum type C was most frequently found in fruit/vegetable and shrimp/fish infant foods, while type D was the most commonly detected in honey samples. Types B and E were obtained from various types of infant foods. More than one type was detected in ten infant foods and in two honey samples.

Of 46 samples collected in Germany; eight, from which six were infant foods and two were honey samples, harboured spores of *C. botulinum*. All infant milk powder samples were negative, while four of nine infant cereal formula, one of eight potato purée, and one of seven vegetable formula samples were positive for type B *C. botulinum*. Two honey samples were found PCR positive for types B and E.

Although all PCR-positive samples were MBA negative, comparison of the sequences of their amplified PCR products with published sequences of *C. botulinum* strains provided by the NCBI showed a high sequence homology of 98-100%. One type C PCR-positive sample showed a sequence that was not identical with *C. botulinum* Gene Bank entries. Further studies need to be conducted to investigate the possibility of *C. botulinum* spores with silent toxin encoding genes in strains of other types present in honey and infant food samples.

8 ZUSAMMENFASSUNG

Die Studie wurde durchgeführt, um ein Verfahren zu entwickeln, mit dem Sporen der unterschiedlichen *C. botulinum* Typen A, B, C, D, E und F in optimierten Medien hergestellt werden können. Eine Methode zum quantitativen Nachweis von *C. -botulinum*-Sporen in Honigproben wurde erarbeitet. Dieses Verfahren war die Grundlage für eine vergleichende Untersuchung über das Vorkommen von *C. botulinum* Sporen in Honig und Säuglingsnahrung. Proben dieser Lebensmittel wurden bei Einzelhändlern und Supermärkten in Ho Chi Minh Stadt, Vietnam und in Supermärkten in Deutschland gekauft. Das Ziel dieser Untersuchung war, festzustellen, ob Honig und Säuglingsnahrung eine mögliche Bedrohung für Säuglinge und Kleinkinder in Vietnam und Deutschland darstellen können.

Zunächst wurde die Sporenproduktion der unterschiedlichen *C.-botulinum*-Typen A, B, C, D, E und F mit verschiedenen Nährmedien optimiert. Während Typ A in TM, Typ B in MAB, Typ D in SeM und Typ F in SM gut sporulierte, versporten Typ C und Typ E besser auf FAAE. Die Versporungsrate lag 80-100% in den flüssigen Medien und bei 70-90% auf Nährbodenplatten. Wurden die Sporen nach der Ernte in SSS resuspendiert, war erhöhte sich die Versporungsrate um 10-20%. Die Toxigenität der Sporen wurde nicht beeinflusst.

Eine MPN-PCR Methode wurde entwickelt, um *C.-botulinum*-Sporen quantitativ im Honig zu bestimmen. Autoklavierte Honigproben wurden mit einer definierten Anzahl an Sporen der unterschiedlichen *C.-botulinum*-Typen entweder mit jedem Typ einzeln oder mit einer Mischung aller Typen versetzt. Zwei Kulturmedien, FAB und CMM, wurden als Anreicherungsmedien verwendet. Die Zahl der wieder gefundenen Sporen entsprach der zugegebenen Menge, unabhängig davon, ob nur ein Typ oder die Sporenmischung eingesetzt worden war. Zwischen den beiden Anreicherungsmedien wurden keine signifikanten Unterschiede beobachtet.

Im zweiten Abschnitt wurde Honig und Säuglingsnahrung in Supermärkten in Ho Chi Minh Stadt, Vietnam, und in Deutschland gekauft und auf das

Vorkommen von *C.-botulinum*-Sporen mittels PCR untersucht. Die amplifizierten Fragmente wurden durch Restriktionsenzymanalyse und DNA-Sequenzierung verifiziert, die Kulturen im Mäusebioassay geprüft.

Zwanzig von 179 (11.2%) Proben der Säuglingsnahrung und 8 von 99 (8.1%) der Honigproben, die in Vietnam gesammelt worden waren, waren in der PCR positiv. Die höchste Anzahl von PCR-positiven Proben fanden sich bei Garnelen-/Fisch- (23.3%) und Obst-/Gemüse-Säuglingsnahrung (20%), während keine der Reis-/Soja-Nahrungen PCR-positiv war. Von 50 Schweinefleisch-/Huhn-Säuglingsnahrungsproben waren 5 (10%) PCR-positiv. Nur eine von 35 (2.9%) Milch-Säuglingsnahrungsproben war positiv.

C. botulinum Typ C wurde am häufigsten in der Frucht/Gemüse- und der Garnelen/Fisch-Säuglingsnahrung gefunden, während Typ D in den Honigproben am häufigsten ermittelt wurde. Die Typen B und E wurden in verschiedenen Lebensmitteln gefunden. Mehr als ein Typ wurde in 10 Säuglingsnahrungs- und in zwei Honigproben nachgewiesen.

Acht (davon 6 Säuglingsnahrungs- und 2 Honigproben) von 46 in Deutschland gekauften Proben enthielten *C.-botulinum*-Sporen. Alle Säuglingsmilchpulverproben waren negativ, während 4 der 9 Getreidenahrungsproben, eine von 8 Kartoffelpurée- und eine von 7 Gemüsenahrungsproben positiv für Typ B waren. Zwei Honigproben waren in der PCR positiv für Typen B und E.

Obwohl alle PCR-positiven Proben im Mäusebioassay negativ waren, zeigten die Sequenzen ihrer PCR-Produkte eine hohe Homologie von 98-100% mit Datenbanksequenzen (GeneBank). Eine Säuglingsnahrung, die PCR-positiv für Typ C war, zeigte eine Sequenz, die nicht mit *C. botulinum* identisch war. Weitere Studien müssen durchgeführt werden, um ein mögliches Vorhandensein von *C.-botulinum*-Sporen mit stillen kodierenden Genen in Honig- und Säuglingsnahrungs-Proben zu untersuchen.

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10 APPENDIX

All chemicals are from MERCK (Darmstadt, Germany), otherwise will be stated

10.1 Media

Duncan-Strong Medium (M45, FDA) modified

	g L^{-1}
Yeast extract	4.0
Proteose peptone	15.0
Soluble starch	4.0
Sodium thioglycolate	(1.0)*
Na_2HPO_4 $7H_20$	10.0
Distilled water	ad 1000 ml

pH 7.5 $\pm$ 0.2 at 25 °C

Autoclave at 121 °C for 15 min.

***,** amount of Sodium thioglycolate used only in case it was added to the medium.

Fastidious Anaerobe Agar with Blood (FAAB)

	g L^{-1}
Casein peptone	10.0
Meat peptone	10.0
Yeast extract	3.0
Dextrose (or D-Glucose monohydrate)	1.0
Soluble starch	1.0
Sodium chloride	5.0

Sodium pyruvate	1.0
Sodium bicarbonate	0.4
L-Cysteine hydrochloride	0.5
L-Arginine	1.0
Trizma base	0.25
Haemin	0.01
Vitamin K1	0.001
Sodium succinate	0.5
Agar	14.0
Distilled water	ad 1000 ml

pH 7.2 $\pm$ 0.2 at 25 ^{o}C

After autoclaving at 121 ^{o}C for 15 min, 50 mL of defibrinated horse blood (OXOID, Wesel) were aseptically added.
Haemin, L-Arginine, trizma base, and sodium bicarbonate were obtained from SIGMA (SIGMA-ALDRICH, Germany).

FAA is an enriched non-selective solid medium used for cultivation and isolation of obligate and facultative anaerobes, especially those that present in low numbers. Hemin and vitamin K1 enrichments enhance the growth of *Bacteriodes sp*. The low level of glucose prevents the production of high levels of acids and alcohols which would inhibit colonial development.

Fastidious Anaerobe Agar with Egg Yolk (FAAE)

	g L^{-1}
Casein peptone	10.0
Meat peptone	10.0
Yeast extract	3.0
Dextrose (or D-Glucose monohydrate)	1.0
Soluble starch	1.0

Sodium chloride	5.0
Sodium pyruvate	1.0
Sodium bicarbonate	0.4
L-Cysteine hydrochloride	0.5
L-Arginine	1.0
Trizma base	0.25
Haemin	0.01
Vitamin K1	0.001
Sodium succinate	0.5
Agar	14.0
Distilled water	ad 1000 ml

pH 7.2 $\pm$ 0.2 at 25 ^{o}C

After autoclaving at 121 ^{o}C for 15 min, 50 mL of egg-yolk emulsion (BECTON DICKINSON, Sparks) were added aseptically.
Haemin, L-Arginine, trizma base, and sodium bicarbonate were obtained from SIGMA-ALDRICH, Germany.

Fastidious Anaerobe Broth (FAB)

	g L^{-1}
Casein peptone	10.0
Meat peptone	10.0
Yeast extract	3.0
Dextrose (or D-Glucose monohydrate)	1.0
Soluble starch	1.0
Sodium chloride	5.0
Sodium pyruvate	1.0
Sodium bicarbonate	0.4
L-Cysteine hydrochloride	0.5

L-Arginine	1.0
Trizma base	0.25
Haemin	0.01
Vitamin K1	0.001
Sodium succinate	0.5
Agar	14.0
Distilled water	ad 1000 ml

pH 7.2 $\pm$ 0.2 at 25 ^{o}C

Autoclave at 121 °C for 15 min.

Haemin, L-Arginine, trizma base, and sodium bicarbonate were obtained from SIGMA (SIGMA-ALDRICH, Germany).

Modified Anellis Broth (MAB)

	g L^{-1}
Casein peptone	50.0
Meat peptone	5.0
$MnSO_4$. $4H_2O$	0.082
$CaCl_2$	0.055
K_2HPO_4	1.25
$(NH_4)_2SO_4$	20.0
Distilled water	ad 1000 ml

pH 7.5 $\pm$ 0.2 at 25 ^{o}C

Autoclave at 115 °C for 30 min.

After autoclaving, 10 mL of sterile 7.5% $NaHCO_3$ solution were added to 1 L MAB. The $MnSO_4$ was obtained by either autolaving at 121°C for 15 min or by filtration with 0.2 µL PES (polyethersulfone) membrane filter (SARTORIUS, Germany).

Reinforced Clostridial Medium (RCM)

	g L^{-1}
Yeast extract	3.0
Peptone from meat	10.0
Meat extract	10.0
D-Glucose monohydrate	5.5
Soluble starch	1.0
Sodium chloride	5.0
Sodium acetate	4.93
L-Cysteine HCl	0.55
Agar	0.5
Distilled water	ad 1000 mL

pH 6.8 $\pm$ 0.2 at 25 ^{o}C

Blood Agar (BA) has the basis of RCM with addition of 15 g L^{-1} agar. After autoclaving at 121 ^{o}C for 15 min, 50 mL of defibrinated horse blood (OXOID, Wesel) were added aseptically.

If hay was added to this medium, 50 g of dried hay were soaked in 1000 mL distilled water overnight. This hay-water would be used to prepare RCM-hay (Reinforced Clostridial Medium with hay).

Segner Medium (SeM) modified

	g L^{-1}
Yeast extract	10.0
$(NH_4)_2SO_4$	10.0
Glucose	10.0
Egg meat medium (Difco)	80.0
Distilled water	ad 1000 mL

pH 7.0-7.2

Autoclave at 121°C for 15 min.

Sporulation medium (SM)

	g L^{-1}
Nutrient broth (Difco)	6.0
Yeast extract	1.0
Meat peptone	2.0
$MnSO_4$. H_2O	0.0005
Marine broth (Difco)	0.5
Inosin (5mM)	1 mL
Distilled water	ad 1000 mL

pH 7.8 ± 1

Autoclave at 121 °C for 15 min.

If hay was added to this medium, 50 g of dried hay were soaked in 1000 mL distilled water overnight. This hay-water would be used to prepare SM-hay (Sporulation Medium with hay).

Trypticase Peptone Broth (TPB)

	$g\ L^{-1}$
Casein peptone	50.0
Meat peptone	5.0
Distilled water	ad 1000 mL

Autoclave at 121 °C for 15 min.

10.2 Solutions and buffers

Gelatine phosphate buffer

Solution A	
Gelatine	4 %
$Na_2HPO_4.\ 2H_2O$	50 mM
Distilled water	ad 1000 mL

Solution B	
$NaH_2PO_4.\ H_2O$	50 mM
Distilled water	ad 1000 mL

Solution A and B were mixed together and heated at 60°C in a water bath to dissolve gelatine. pH value was adjusted to 6.2 and the buffer was sterilized at 121°C for 15 min.

Phosphate buffered saline

NaCl	120 mM
$Na_2HPO_4.\ 2H_2O$	20 mM
$NaH_2PO_4.\ H_2O$	6 mM

pH 7.4 at 25°C

Autoclave at 121 °C for 15 min.

Salt solution for sporulation (SSS)

	mg L^{-1}
$CaCl_2 . 2H_2O$	2.65
$MgSO_4 . 7H_2O$	8.2
$MnSO_4 . H_2O$	8.8
Distilled water	ad 1000 mL

1 x TBE

Tris borate	90 mM
EDTA	2 mM

pH 8.3

DNA-polyacrylamide gel (6%)

Set clean electrophoresis glass plates with sealer. Mix DNA-PAGE buffer and acrylamide solution. Warm the mixture at 37 ^{o}C for 3 min. Evacuate the mixture briefly to remove small bubbles.

1. Add ammonium persulfate (APS) and TEMED into the acrylamide mixture. Immediately, pour the mixture into the glass plates and stick a comb into the gel solution. Avoid bubbles under the comb teeth. Remove the excess gel solution. Allow to stand at least 30 min after the gel gets solidified at room temperature.
2. Remove the comb from the gel.
3. Set the gel plates on to an electrophoresis chamber. Pour running buffer (1xTBE buffer) into the chamber.

4. Apply PCR products (10-16 µL) that were mixed with PCR staining solution (2 µL) and load electricity at constant 100 V for mini-gels.

5. The running time is 60-80 min.

DNA-PAGE buffer

	mL
50% glycerol	9.8
2xTBE buffer	48.0
Distilled water	24.0

Gel composition

DNA-PAGE buffer (mL)	8
30% acrylamide-bis (mL)	2
10% APS (µL)	100
TEMED (µL)	10
Total volume (mL)	10

10.3 DNA sequencing analyses

Table 19a: Sequence identity rate of PCR products of type B samples

Name	Samples	Ref. No. GenBank	Position	Identical rate
G 28	B1f	gi|144734|gb|M81186.1|CLOBOTB	596-689	98%
	B1r	gi|144734|gb|M81186.1|CLOBOTB	513-610	97%
	B2f	gi|144734|gb|M81186.1|CLOBOTB	546-694	99%
	B2r	Bad sequence		
G 30	B3f	gi|144734|gb|M81186.1|CLOBOTB	537-694	99%
	B3r	gi|144734|gb|M81186.1|CLOBOTB	488-653	100%
G 31	B4f	gi|144734|gb|M81186.1|CLOBOTB	535-694	100%
	B4r	gi|144734|gb|M81186.1|CLOBOTB	490-632	100%
G 33	B5f	gi|144734|gb|M81186.1|CLOBOTB	535-694	100%
	B5r	gi|144734|gb|M81186.1|CLOBOTB	513-639	100%
G 35	B6f	gi|144734|gb|M81186.1|CLOBOTB	517-694	100%
	B6r	gi|144734|gb|M81186.1|CLOBOTB	513-654	100%
G 36	B7f	gi|144734|gb|M81186.1|CLOBOTB	526-694	100%
	B7r	gi|144734|gb|M81186.1|CLOBOTB	490-654	100%
	B8f	Bad sequence		
	B8r	gi|144734|gb|M81186.1|CLOBOTB	490-622	100%
VR 34	B9f	gi|144734|gb|M81186.1|CLOBOTB	538-694	100%
	B9r	gi|144734|gb|M81186.1|CLOBOTB	490-589	100%
	B10f	gi|144734|gb|M81186.1|CLOBOTB	535-694	100%
	B10r	gi|144734|gb|M81186.1|CLOBOTB	513-609	100%
VR 35	B11f	Bad sequence		
	B11r	Bad sequence		
	B12f	gi|144734|gb|M81186.1|CLOBOTB	535-694	100%
	B12r	gi|144734|gb|M81186.1|CLOBOTB	513-631	97%
VR 37	B13f	gi|144734|gb|M81186.1|CLOBOTB	535-694	100%
	B13r	gi|144734|gb|M81186.1|CLOBOTB	490-592	100%
VR 38	B14f	gi|144734|gb|M81186.1|CLOBOTB	537-694	100%
	B14r	gi|144734|gb|M81186.1|CLOBOTB	513-633	100%
VR 40	B15f	gi|144734|gb|M81186.1|CLOBOTB	525-694	100%
	B15r	gi|144734|gb|M81186.1|CLOBOTB	490-657	100%
	B16f	gi|144734|gb|M81186.1|CLOBOTB	517-694	100%
	B16r	gi|144734|gb|M81186.1|CLOBOTB	513-654	100%
N 13	B17f	Bad sequence		
	B17r	gi|144734|gb|M81186.1|CLOBOTB	513-561	100%
MO 7	B18f	gi|144734|gb|M81186.1|CLOBOTB	535-694	100%
	B18r	gi|144734|gb|M81186.1|CLOBOTB	490-603	100%
G 25	B19f	gi|144734|gb|M81186.1|CLOBOTB	534-695	100%
	B19r	gi|144734|gb|M81186.1|CLOBOTB	490-639	100%
	B20f	gi|144734|gb|M81186.1|CLOBOTB	534-694	100%
	B20r	Bad sequence		
G 27	B21f	gi|144734|gb|M81186.1|CLOBOTB	595-692	98%
	B21r	gi|144734|gb|M81186.1|CLOBOTB	513-621	99%

Table 19b: Sequence identity rate of PCR products of type B samples (cont.)

Name	Samples	Ref. No. GenBank	Position	Identical rate
	B22f	gi|144734|gb|M81186.1|CLOBOTB	525-694	100%
	B22r	gi|144734|gb|M81186.1|CLOBOTB	513-653	99%
NF 14	B23f	gi|144734|gb|M81186.1|CLOBOTB	541-694	100%
	B23r	gi|144734|gb|M81186.1|CLOBOTB	490-653	100%
	B24f	gi|144734|gb|M81186.1|CLOBOTB	535-694	100%
	B24r	gi|144734|gb|M81186.1|CLOBOTB	490-638	100%
NF 16	B25f	gi|144734|gb|M81186.1|CLOBOTB	566-694	98%
	B25r	gi|144734|gb|M81186.1|CLOBOTB	488-654	99%
	B26f	gi|144734|gb|M81186.1|CLOBOTB	531-694	100%
	B26r	gi|144734|gb|M81186.1|CLOBOTB	488-654	100%
NF 19	B27f	gi|144734|gb|M81186.1|CLOBOTB	531-694	100%
	B27r	gi|144734|gb|M81186.1|CLOBOTB	513-654	100%
	B28f	gi|144734|gb|M81186.1|CLOBOTB	526-694	99%
	B28r	gi|144734|gb|M81186.1|CLOBOTB	513-633	99%
NF 23	B29f	gi|144734|gb|M81186.1|CLOBOTB	535-694	100%
	B29r	gi|144734|gb|M81186.1|CLOBOTB	490-654	99%
	B30f	gi|144734|gb|M81186.1|CLOBOTB	531-694	100%
	B30r	gi|144734|gb|M81186.1|CLOBOTB	513-654	99%
NF 25	B31f	gi|144734|gb|M81186.1|CLOBOTB	525-694	99%
	B31r	gi|144734|gb|M81186.1|CLOBOTB	513-646	99%
	B32f	gi|144734|gb|M81186.1|CLOBOTB	540-694	100%
	B32r	gi|144734|gb|M81186.1|CLOBOTB	490-654	99%

Table 20: Sequence identity rate of PCR products of type C samples

Name	Samples	Ref. No. GenBank	Position	Identical rate
NF 14	C1f	gi|60360958|dbj|AB200364.1|	156-549	100%
	C1r	gi|60360958|dbj|AB200364.1|	98-508	100%
NF 15	C2f	gi|60360958|dbj|AB200364.1|	139-539	100%
	C2r	gi|60360958|dbj|AB200364.1|	111-507	100%
NF 16	C3f	gi|60360958|dbj|AB200364.1|	138-544	100%
	C3r	gi|60360958|dbj|AB200364.1|	98-508	100%
NF 18	C4f	gi|60360958|dbj|AB200364.1|	139-339	100%
	C4r	gi|60360958|dbj|AB200364.1|	98-507	99%
	C5f	gi|60360958|dbj|AB200364.1|	139-539	100%
	C5r	gi|60360958|dbj|AB200364.1|	98-508	100%
NF 19	C6f	gi|60360958|dbj|AB200364.1|	139-549	100%
	C6r	gi|60360958|dbj|AB200364.1|	98-508	100%
NF 20	C7f	gi|60360958|dbj|AB200364.1|	138-549	100%
	C7r	gi|60360958|dbj|AB200364.1|	101-508	100%
	C8f	gi|60360958|dbj|AB200364.1|	139-549	100%
	C8r	gi|60360958|dbj|AB200364.1|	98-508	100%
NF 21	C9f	gi|60360958|dbj|AB200364.1|	139-549	100%
	C9r	gi|60360958|dbj|AB200364.1|	98-490	100%
NF 22	C10f	gi|60360958|dbj|AB200364.1|	139-539	100%
	C10r	gi|60360958|dbj|AB200364.1|	98-508	100%
NF 23	C11f	Bad sequence		
	C11r	gi|60360958|dbj|AB200364.1|	98-441	99%
NF 24	C12f	gi|60360958|dbj|AB200364.1|	139-549	100%
	C12r	gi|60360958|dbj|AB200364.1|	107-517	100%
NF 25	C13f	gi|60360958|dbj|AB200364.1|	139-539	100%
	C13r	gi|60360958|dbj|AB200364.1|	98-508	100%
NF 26	C14f	gi|60360958|dbj|AB200364.1|	139-539	100%
	C14r	gi|60360958|dbj|AB200364.1|	98-508	100%
NF 27	C15f	gi|60360958|dbj|AB200364.1|	155-549	100%
	C15r	gi|60360958|dbj|AB200364.1|	98-474	100%
	C16f	gi|60360958|dbj|AB200364.1|	146-540	100%
	C16r	gi|60360958|dbj|AB200364.1|	98-493	100%
NF 28	C17f	gi|60360958|dbj|AB200364.1|	139-459	100%
	C17r	gi|60360958|dbj|AB200364.1|	98-517	100%
MO 93	C18f	gi|60360958|dbj|AB200364.1|	139-539	100%
	C18r	gi|60360958|dbj|AB200364.1|	111-517	100%
MO 94	C19f	Not identical		
	C19r	Not identical		
MO 96	C20f	gi|60360958|dbj|AB200364.1|	139-538	100%
	C20r	gi|60360958|dbj|AB200364.1|	98-484	100%
	C21f	gi|60360958|dbj|AB200364.1|	172-242	100%
	C21r	gi|60360958|dbj|AB200364.1|	98-517	100%

Table 21: Sequence identity rate of PCR products of type D samples

Name	Samples	Ref. No. GenBank	Position	Identical rate
MO 90	D1f	gi|260238|gb|S49407.1|	254-696	100%
	D1r	gi|260238|gb|S49407.1|	224-677	100%
	D2f	gi|260238|gb|S49407.1|	254-709	100%
	D2r	gi|260238|gb|S49407.1|	225-677	100%
MO 91	D3f	gi|260238|gb|S49407.1|	254-696	100%
	D3r	gi|260238|gb|S49407.1|	225-677	100%
MO 92	D4f	gi|260238|gb|S49407.1|	270-709	100%
	D4r	gi|260238|gb|S49407.1|	213-674	99%
	D5f	gi|260238|gb|S49407.1|	259-696	100%
	D5r	gi|260238|gb|S49407.1|	227-677	100%
MO 93	D6f	gi|260238|gb|S49407.1|	281-709	100%
	D6r	gi|260238|gb|S49407.1|	213-639	100%
	D7f	gi|260238|gb|S49407.1|	259-696	100%
	D7r	gi|260238|gb|S49407.1|	225-677	100%
MO 94	D8f	gi|260238|gb|S49407.1|	259-703	100%
	D8r	gi|260238|gb|S49407.1|	227-682	100%
	D9f	gi|260238|gb|S49407.1|	270-696	100%
	D9r	gi|260238|gb|S49407.1|	227-677	100%
MO 95	D10f	gi|260238|gb|S49407.1|	270-709	100%
	D10r	Bad sequence		
	D11f	gi|260238|gb|S49407.1|	282-702	100%
	D11r	gi|260238|gb|S49407.1|	225-682	100%
MO 96	D12f	gi|260238|gb|S49407.1|	400-699	100%
	D12r	Bad sequence		
	D13f	gi|260238|gb|S49407.1|	254-696	100%
	D13r	gi|260238|gb|S49407.1|	227-677	100%

Table 22a: Sequence identity rate of PCR products of type E samples

Name	Samples	Ref. No. GenBank	Position	Identical rate
VR 34	E1f	gi|40393|emb|X62089.1|CBNEUTOXE	430-773	100%
	E1r	gi|40393|emb|X62089.1|CBNEUTOXE	384-727	100%
	E2f	gi|40393|emb|X62089.1|CBNEUTOXE	nd	
	E2r	gi|40393|emb|X62089.1|CBNEUTOXE	nd	
VR 35	E3f	gi|40393|emb|X62089.1|CBNEUTOXE	414-773	100%
	E3r	gi|40393|emb|X62089.1|CBNEUTOXE	390-749	100%
	E4f	gi|40393|emb|X62089.1|CBNEUTOXE	417-773	100%
	E4r	gi|40393|emb|X62089.1|CBNEUTOXE	384-673	100%
VR 37	E5f	gi|40393|emb|X62089.1|CBNEUTOXE	438-773	99%
	E5r	gi|40393|emb|X62089.1|CBNEUTOXE	384-742	99%
VR 38	E6f	gi|40393|emb|X62089.1|CBNEUTOXE	413-771	99%
	E6r	gi|40393|emb|X62089.1|CBNEUTOXE	390-732	100%
VR 40	E7f	gi|40393|emb|X62089.1|CBNEUTOXE	413-773	100%
	E7r	gi|40393|emb|X62089.1|CBNEUTOXE	384-751	99%
	E8f	gi|40393|emb|X62089.1|CBNEUTOXE	413-773	100%
	E8r	gi|40393|emb|X62089.1|CBNEUTOXE	390-738	100%
G 25	E9f	gi|40393|emb|X62089.1|CBNEUTOXE	nd	
	E9r	gi|40393|emb|X62089.1|CBNEUTOXE	nd	
	E10f	gi|40393|emb|X62089.1|CBNEUTOXE	467-773	100%
	E10r	gi|40393|emb|X62089.1|CBNEUTOXE	384-701	99%
G 27	E11f	Bad sequence		
	E11r	Bad sequence		
	E12f	gi|40393|emb|X62089.1|CBNEUTOXE	513-773	99%
	E12r	gi|40393|emb|X62089.1|CBNEUTOXE	387-691	99%
NF 14	E13f	gi|40393|emb|X62089.1|CBNEUTOXE	452-773	100%
	E13r	gi|40393|emb|X62089.1|CBNEUTOXE	386-701	100%
	E14f	gi|40393|emb|X62089.1|CBNEUTOXE	497-773	100%
	E14r	gi|40393|emb|X62089.1|CBNEUTOXE	384-718	99%
NF 15	E15f	gi|40393|emb|X62089.1|CBNEUTOXE	447-773	99%
	E15r	gi|40393|emb|X62089.1|CBNEUTOXE	418-691	99%
	E16f	gi|40393|emb|X62089.1|CBNEUTOXE	427-773	100%
	E16r	gi|40393|emb|X62089.1|CBNEUTOXE	384-720	100%
NF 16	E17f	gi|40393|emb|X62089.1|CBNEUTOXE	447-773	100%
	E17r	gi|40393|emb|X62089.1|CBNEUTOXE	384-719	100%
	E18f	gi|40393|emb|X62089.1|CBNEUTOXE	448-773	99%
	E18r	gi|40393|emb|X62089.1|CBNEUTOXE	384-654	100%
NF 19	E19f	Bad sequence		
	E19r	gi|40393|emb|X62089.1|CBNEUTOXE	384-702	100%
	E20f	gi|40393|emb|X62089.1|CBNEUTOXE	449-773	100%
	E20r	gi|40393|emb|X62089.1|CBNEUTOXE	384-719	100%
NF 23	E21f	gi|40393|emb|X62089.1|CBNEUTOXE	449-773	99%
	E21r	gi|40393|emb|X62089.1|CBNEUTOXE	384-695	99%
	E22f	gi|40393|emb|X62089.1|CBNEUTOXE	438-773	100%
	E22r	gi|40393|emb|X62089.1|CBNEUTOXE	384-711	100%

Table 22b: Sequence identity rate of PCR products of type E samples (cont.)

Name	Samples	Ref. No. GenBank	Position	Identical rate
NF 25	E23f	gi|40393|emb|X62089.1|CBNEUTOXE	427-773	99%
	E23r	gi|40393|emb|X62089.1|CBNEUTOXE	384-731	100%
	E24f	gi|40393|emb|X62089.1|CBNEUTOXE	446-773	100%
	E24r	gi|40393|emb|X62089.1|CBNEUTOXE	387-717	100%

Table 23: Sequence identity rate of PCR products of type F samples

Name	Samples	Ref. No. GenBank	Position	Identical rate
VR 34	F1f	gi|529982|gb|L35496.1|CLOBONT	390-908	100%
	F1r	gi|529982|gb|L35496.1|CLOBONT	366-884	100%
VR 35	F2f	gi|529982|gb|L35496.1|CLOBONT	390-908	100%
	F2r	gi|529982|gb|L35496.1|CLOBONT	366-861	99%
VR 40	F3f	gi|529982|gb|L35496.1|CLOBONT	390-908	99%
	F3r	gi|529982|gb|L35496.1|CLOBONT	686-839	90%
	F4f	gi|529982|gb|L35496.1|CLOBONT	394-908	98%
	F4r	gi|529982|gb|L35496.1|CLOBONT	378-837	99%

10.4 Staining solutions and procedures

Modified Gram stain (Hucker's modification)

Solution 1 consists of a) 20.0 g crystal violet
200 mL ethanol (96-99%)
b) 8 g ammonium oxalate
800 mL demineralised water

Solution 2: 5 g iodine
10 g KI
1 L demineralised water

Solution 3: 200 ml acetone
250 mL ethanol

Solution 4: 20 mL Ziel-Neelsen carbol fuchsin
180 mL demineralised water

Method

First, an inoculum is taken from a culture using an inoculation loop and put on a clean microscope slide, and then allowed to air dry to produce a sparse single layer of bacteria. If the culture is solid, it is diluted and well mixed with a drop of sterile water on the slide. The air-dried smears are heat-fixed by passing the slide, inoculum side up, through a bunsen flame 1-2 times, without allowing the slide to become hot to the touch, stained by solution 1 (primary stain) for 30-90 s, and then rinsed off with water for a maximum of five seconds. Lugol's iodine (solution 2) is added as a mordant for 60-120 s and then rinsed with water. Solution 3 is added as a decolourising agent, and the smear is rinsed with water immediately to prevent over-decolourisation. Then solution 4 is added as a counter stain. The smear is washed, air-dried, and examined under a microscope with an oil immersion lens. Gram positive bacteria are black-blue or purple, and the Gram negative bacteria are pinkish-red.

Spore stain (Schaeffer-Fulton stain)

Staining procedure

A smear is prepared following the steps described in the Gram staining. The air-dried, heat-fixed smear is flooded with 0.5% (w/v) malachite green and heated on a flame to near-boiling for 5 min. The smear is then rinsed in running water, counterstained with safranin (0.5% aqueous) for 30 s, and air-dried. Being inspected under a microscope, endospores stain green, while vegetative cells pinkish-red.

11 ACKNOWLEDGEMENTS

I would like to acknowledge my greatest debt to Professor Dr. Dr. Helge Böhnel, the director of the Institute of Tropical Animal Health and Institute of Applied Biotechnology, University of Göttingen, for accepting me as a Ph.D student. I have received inspiring supervision, encouragement, understanding, patience, and unfailing kindness throughout this study.

I would like to thank Prof. Dr. Dr. Udo ter Meulen for accepting to be my coreferee and his recommendation letters for my scholarship applications.

I am particularly grateful to Dr. Frank Gessler for his ongoing immensely helpful comments, criticisms, discussions, and suggestions. This was especially for the practical and theoretical aspects during the study and for his critical reading of the thesis.

We would also like to thank Prof. Dr. Dr. Bertram Brenig, the director of the Institute of Veterinary Medicine, Georg-August-University Göttingen, for his approval for the DNA sequencing analyses at the institute. Thanks are extended to PD Dr. sc. agr. Christoph Knorr and Viola Raupach for their useful guidance and support in DNA sequencing and analyses.

We are grateful to Christian Wagner, Michaela Schmidt, Katrin Hampe and Deborah Fischer, the staff members of the Institute of Tropical Animal Health and Institute of Applied Biotechnology, University of Göttingen, for their co-operation and technical assistance.

Thanks are also due to Dr. Anke Grosse-Herrenthey, Dagmar Nölkes, Dr. Sibylle Pagel-Wieder, Kamal Eldin Hassan Ali Eltom, Dr. Elhassan Mohammed Ali Saeed, Aung Aung, Sibylle Reinmuth, Dr. Abdulkhalig Babiker, and Heinz Hunfeld for help, co-operation, critical suggestions, and the pleasant working atmosphere.

I am grateful to Antje Clarke, the former institute’s secretary, for her

encouragement and help in all aspects. I also thank Ursula Otto for her assistance and sympathy.

I am deeply indebted to Dr. Keith Wing Shing who unconditionally and enthusiastically took time from his busy schedule to proof read the manuscripts of this thesis and to give me valuable advice and encouragement.

Particular acknowledgments go to MSc. Tang Thi Kim Hong, Nguyen Minh Hien, and Le Huu Ngoc for helping me collect and sub-sample the honey and infant foods. I would like to take this opportunity to thank all my friends for helping me, for sharing enjoyable times, and for encouraging me throughout the study, especially during the hardest time after my father's death.

I wish to express my deepest appreciation to my family including my father, my mother and my sister for love, unfailing support and encouragement. Sadly, my father could not see the completion of my Ph.D. work.

I would like to gratefully acknowledge financial support for my Ph.D. study from Friedrich-Ebert-Stiftung and the Institute of Applied Biotechnology, Georg-August-University of Göttingen. The completion of this study would not have been possible without this invaluable support. I gratefully acknowledge the International Ph.D. Programme of Agricultural Sciences (IPAG) for accepting me to attend this programme.

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