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Studies on Isolation and Identification of *Clostridium botulinum* Investigating Field Samples Specially from Equine Grass Sickness Cases

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Dedication

To my parents, sisters, brothers, wife and children
with love

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Abbreviations

Ab	Antibody
Ag	Antigen
BoNT	Botulinum neurotoxin
BSA	Bovine serum albumin
CDC	The Botulism Laboratory at the Centers for Disease Control and Prevention
CVRL	Central Veterinary Research Laboratory
dNTP	Deoxynucleotide triphosphate
ED	Equine dysautonomia
EGS	Equine grass sickness
ELISA	Enzyme-linked immunosorbent assay
EYA	Egg yolk agar
GI	Gastrointestinal
GPB	Gelatine phosphate buffer
IBT	Institute of Applied Biotechnology in the Tropics at the Georg-August-University of Göttingen
MB-ELISA	Magnetic bead-enzyme-linked immunosorbent assay
NT	Neurotoxin
NAPs	Neurotoxin associated proteins
OVI	Onderstepoort Veterinary Institute
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phenyl ethanol
RT	Room temperature
SNAP-25	Synaptosomal associated protein-25
SNARE	Soluble NSF-attachment protein receptors
TeNT	Tetanus neurotoxin
TI	Toxico-infectious
VAMP	Vesicle associated membrane protein

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1 INTRODUCTION

Botulism is a non-febrile highly fatal disease of man and animals caused by neurotoxins of *Clostridium botulinum*. It is characterized by partial or complete flaccid paralysis of the muscles of locomotion, mastication and deglutition due to inhibition of the release of the neurotransmitter acetylcholine by botulinum neurotoxins at cholinergic nerve endings (Kriek and Odendaal, 1994). Botulism occurs sporadically, but in intensively farmed animals, it is responsible for high mortalities (Smart *et al*., 1983; Abbitt *et al*., 1984; Trueman, 1992). Animal botulism can be a public health problem, since humans can be intoxicated by ingestion of contaminated meat. Minute quantities acquired by ingestion, inhalation or by absorption through the eye or a break in the skin can cause profound intoxication and death in humans (CDC, 1998).

C. botulinum encompasses a heterogeneous group of obligate anaerobic, spore-forming, Gram-positive rods that produces the most potent biologic toxin known (Rocke, 1993). It produces seven neurotoxins that are pharmacologically similar, but serologically distinct from each other and designated as types A through G (Rocke, 1993). The bacterium is almost ubiquitous and is found in soils and organic matter worldwide. Eventhough *C. botulinum* is nearly ubiquitous in the environment and in the flora of herbivores; it may not always be toxic, perhaps helping to explain the sporadic nature of the outbreaks (Galey, 2001). *C. botulinum* is occasionally present in the animal gut (Ricketts and Greet, 1984) and can be an opportunist due to gut stasis (Hunter *et al*., 1999).

Several forms of botulism in both man and animals were described (Smith and Sugiyama, 1988; CDC, 1998), but in general it is either due to intake of the preformed toxin (classical form) or the toxin is formed inside the animal body after colonization by *C. botulinum* (toxico-infectious form). The classical form of botulism is usually due to ingestion of a food contaminated with botulinum neurotoxins (food-borne botulism), while the toxico-infectious (TI) form is usually due to proliferation of *C. botulinum* in an existing wound (wound botulism) or in the gut (visceral botulism, Böhnel *et al*., 2001). The TI form mainly occurs in human infants (infant botulism) or foals (shaker foal syndrome) and rarely in adult humans or animals (Arnon *et al*., 1978; Bartlett, 1986).

Equine grass sickness (EGS), a disease of horses, ponies and donkeys, was first recognized in the northeast of Scotland in 1907 (Tocher *et al*., 1923), then spread to the other parts of Britain, Europe and some other parts in the world (McCarthy *et al*., 2001), but is still of unknown aetiology. It was suspected to be a toxico-infectious form of botulism due to some similarities between its clinical picture and that of the botulism. The disease is characterized clinically by signs of autonomic dysfunction of the gastrointestinal (GI) tract due to severe and extensive damage in neurons of the autonomic (mainly enteric) nervous system (Pollin and Griffiths, 1992). Many evidences have been produced indicating that *C. botulinum* is most likely the cause (Tocher *et al*., 1923; Hunter and Poxton, 1998; Poxton *et al*., 1998; Hunter *et al*., 1999; Böhnel *et al*., 2003; McCarthy *et al*., 2004b). However, due to the sporadic nature of the disease, other scientists see that *C. botulinum* is ubiquitous and it is unlikely to be linked with the disease (Collier *et al*., 2001). The link of *C. botulinum* with GS should be confirmed by the detection of *C. botulinum* and/or its toxin in GS samples and their prevalence be significantly more than in control samples. However, the more definitive linkage is achieved when the disease is experimentally caused.

Due to the severity of botulism and the great hazard it causes to both man and animals, the diagnostics of *C. botulinum* and its toxin are still insufficient (Robinson and Nahata, 2003; Gessler and Böhnel, 2003); and probably the disease is substantially under diagnosed (CDC, 1998; Böhnel *et al*., 2001). A presumptive diagnosis is made on the basis of the history, clinical signs, negative post-mortem examination and exclusion of similar diseases. A definitive diagnosis depends on: 1) the demonstration of botulinum toxin in the feed or source of intoxication, serum, GI contents or wound of a patient; 2) demonstration of bacterial forms of *C. botulinum* in GI contents, internal organs or feed; or 3) detection of antibody response to *C. botulinum* in recovered patients (Smith and Sugiyama, 1988). The diagnosis of EGS, unlike botulism, can be confirmed by histopathological examination either of the sympathetic ganglia and/or ileal tissue at post mortem or of the ileal biopsies obtained at laparotomy from living animals (Scholes *et al*., 1993). Laboratory diagnostics include the conventional culture methods to isolate the organism, mouse bioassay, molecular and immunological typing, and some other methods. *C. botulinum* is difficult to isolate (Smith and Sugiyama, 1988). It is extremely fastidious,

strict anaerobic, there is no selective medium for all its groups, and it can lose its toxicity during the isolation process. Isolation of *C. botulinum* is especially important for vaccine production, as *C. botulinum* strains are locality specific. The major definitive and standard test used to identify botulism is the toxin neutralization using the mouse bioassay. The bioassay is especially important in cases where the specimen contains only detectable toxin. The case in which PCR is useless and ELISA may be less sensitive. However, it is not suitable for examination of test samples containing other lethal substances (Dezfulian and Bartlett, 1985) and is of insufficient sensitivity relative to the extreme sensitivity of the horse (Galey *et al*., 2000). Horses are suggested to be 1-10,000 times more sensitive than mice (Kinde *et al*., 1991). Recently, sensitive immunoassays such as enzyme-linked immunosorbent assay (ELISA) (Ferreira *et al*., 2003) and polymerase chain reaction (PCR)-based methods (Szabo *et al*., 1994b) were developed for diagnosis of botulism. Immunoassays, unlike the mouse bioassay, can detect the toxin either in active or inactive form. However, ELISA methods are most often less sensitive than the mouse bioassay (Trueman *et al*., 1992). Several reports showed that PCR amplification is a reliable alternative to the standard bioassay method for identifying BoNT-producing clostridia (Szabo *et al*., 1994a and b: Fach *et al*., 1996). PCR is important if the specimen contains viable cells and no detectable toxin by the bioassay or immunoassay (Szabo *et al*., 1994a). Thus, combination of culture method, bioassay, immunoassay and PCR seems to be important for better diagnosis of botulism.

Following the death of the thoroughbred racehorse, Dubai Millennium, from EGS, the Dubai Millennium Research Foundation (DMF) was set up with the intention of further investigating the hypothesis that equine grass sickness is caused by a toxico-infection with *C. botulinum* and to better understand potentially trigger factors. The current study is a part of this project.

2 REVIEW OF THE LITERATURE

2.1 Equine grass sickness

2.1.1 Definition

Equine grass sickness or equine dysautonomia (ED) is a disease of horses, ponies and donkeys of unknown aetiology, characterized clinically by signs of autonomic dysfunction of the GI tract due to severe and extensive damage in neurons of the autonomic (mainly enteric) nervous system (Pollin and Griffiths, 1992). As its name indicates, it occurs almost exclusively in horses while at pasture and rarely reported in stabled animals (Gilmour, 1987).

2.1.2 History and distribution

Equine grass sickness was first identified in the northeast of Scotland at a military training camp at Barry in Angus near Dundee (McLaren, 1918; Spreull, 1922; Tocher *et al*., 1923; Greig, 1924) in 1907 (Tocher *et al*., 1923), where about 100 army horses died because of the disease. The disease was then spread to the other parts of Scotland, England and Wales, which now have the highest incidence, but the disease is well recognized in Northern Europe, specially Sweden, Denmark, Germany and less in France, Belgium, Italy, Holland, Norway, Finland and Switzerland (Cottrell *et al*., 1999; McCarthy *et al*., 2001). In Austria, the first case of histologically confirmed grass sickness (GS) was only very recently reported (Wlaschitz and Url, 2004) in a four-year old pony mare. A single case confirmed histopathologically was reported in the Falklands (Woods and Gilmour, 1991). A condition in horses apparently similar to GS was described by OCHOA and STELLA DE VELANDIA (1978) in Columbia, and more laterally, in the USA and Colombia (Hedderson and Newton, 2004). Also GS was reported to occur in Australia (Stewart, 1977). In addition, a grass sickness-like syndrome prevailing in Argentina and southern Chile, where it is known as '*mal seco*', was discovered in 1912 (Uzal and Robles, 1993), the same period of the first recognition of EGS. The syndrome, because of its typical epidemiology, clinical signs and histopathology to EGS, suggested being also EGS (Uzal and Robles, 1993; Hunter and Poxton, 2001; Araya *et al*., 2002).

2.1.3 Aetiology

Despite the early recognition of the disease, in 1907, the aetiology is still unknown. Numerous epidemiological studies and laboratory investigations to all likely causative agents have been carried, but none is proved to be the cause. Different agents investigated are bacteria, effect of adverse weather conditions on grass (oxidative stress and excitotoxicity), mycotoxins, toxic plants, viruses, insect vectors, nutritional deficiencies, chemicals and metabolic upsets (Tocher, 1923; Anon, 1936; Greig, 1942; Bartosz, 1997; Robb *et al*., 1997; Hunter and Poxton, 2001). However, the nature of the autonomic nervous system neuronal damage suggests that a type of a neurotoxin is involved (Griffiths *et al*., 1994). This suggestion was already confirmed by the experiment done by GILMOUR (1973b) and by POGSON *et al*. (1992). Potential neurotoxins proposed are toxins of *C. botulinum* group III (especially type C), mycotoxins (especially of *Fusarium germinearium*) and secondary metabolites of grasses (Bartosz, 1997; Robb *et al*., 1997; Cottrell *et al*., 1999).

2.1.3.1 Suspected agents

Bacteria. Bacteria are among the agents that were first suspected to be the cause of EGS. A bacterium morphologically and toxicologically resembling *Bacillus botulinus* (now *C. botulinum*) was first isolated from the GI tract of a case of EGS in 1919 and, subsequently from the spleens of a number of horses with GS (Tocher *et al*., 1923). However, TOCHER *et al*. (1923) stated that "any normal animal can swallow a large number of *C. botulinum* spores without any ill effect. There must be a predisposing cause to EGS". They thought it likely to be gastric irritation. Thereafter, many evidences have been produced indicating that *C. botulinum*, especially type C is the most likely candidate (Miller, 1994; Poxton *et al*., 1997; Hunter and Poxton, 1998; Poxton *et al*., 1998; Hunter *et al*., 1999; Hunter and Poxton, 2001; Böhnel *et al*., 2003; McCarthy *et al*., 2004b). More details about the likelihood of *C. botulinum* to be the cause are shown below. *C. perfringens* type A enterotoxin has been detected in serum of suspected GS horses in Columbia that were not confirmed by histopathological examination (Ochoa and de Velandia, 1978). However, GILMOUR *et al*. (1981) found a negative serological association between cases of GS and *C. perfringens* type A in Scotland. The sera from acute and chronic cases of the disease failed to neutralise either crude or partially purified enterotoxin of *C. perfringens*. At an early stage of investigations a diplostreptococcus similar to that associated with

Borna disease was isolated from the CNS of an acute case of GS. However, when horses inoculated with this isolate intracranially, subcutaneously and intravenously remained normal. It was later concluded that this organism was without aetiological significance in GS (Greig, 1942).

Mycotoxins. A number of fungal species, including *Fusarium germinearium* are commonly found more prevalent in pastures where there have been confirmed cases of EGS than in other pastures (Doxey *et al*., 1991; Robb *et al*., 1997). Cultures of the species *F. graminearium* were specially found extremely toxic to neurons in vitro (John *et al*., 1997). Investigation of fungi isolated from the equine alimentary lumen did not identify any particular EGS-associated species, but did identify species with the potential to produce neurotoxins (Doxey *et al*., 1990). UZAL and ROBLES (1997) fed *Fusarium* cultures to four horses for four days; the animals showed signs of mild to moderate colic. Furthermore, *F. germinearium* mycotoxin is a severe gastrointestinal irritant and is immunosuppressive; it probably has its greatest effect on gut-associated lymphoid tissue (Pestka *et al*., 1987), whereby compromising mucosal immunity. This fungus could be a predisposing factor to *C. botulinum* (Collier *et al*., 2001).

Secondary metabolites in grass and increased activity of nitridergic neurons. High incidences of EGS occur shortly after periods of cool, dry weather with irregular ground frosts (Doxey *et al*., 1991), conditions which alter plant metabolism and inhibit plant growth. It is established that plants in such conditions undergo oxidative stress and may have increased concentrations of secondary metabolites including various phenolics, nitrate, glutamate, aspartate, malonate, lipid peroxides, and reduced concentrations of antioxidants including ascorbate, thiols, alpha-tocopherol, beta-carotene, superoxide dismutase, reduced glutathione, glutathione reductase and dehydroascorbate reductase (Draper, 1972; Taylor *et al*., 1972; Bartosz, 1997). It is possible that some of these secondary metabolites could induce neuronal death in EGS (Cottrell *et al*., 1999). Also, it has been suggested that grazing horses ingest high levels of these compounds which in turn can stimulate increased production of nitric oxide from nitridergic neurons within the autonomic nervous system, which can account for many of the clinical signs of ED (Cottrell *et al*., 1999).

Toxic plants. It is suggested that a toxic plant may be the cause, but no evidence has been shown (Robb *et al*., 1997). White clover due to its cyanide-producing properties was proposed and tested by TOCHER *et al*. (1923), who did an experiment to test the toxicity of alsike clover by let horses to feed on it for an entire season. No ill effect was observed. It was proposed that ingestion of highly cyanogenic wild white clover may be an important trigger factor for the disease because it inhibits GI motility and is microbiocidal. In addition, the cyanide content of clover varies with the growth cycle, thereby possibly explaining some of the seasonality of EGS (Hedderson and Newton, 2004). McGORUM *et al*. (2000) found that plants collected immediately after an outbreak of ED had reduced antioxidants and weak prooxidants activities when compared with control plants. Also, ED plants had significant increased concentration of fructose and low molecular weight phenolic compounds. They thought that these changes may contribute directly or indirectly to GS.

Other agents suspected. An extensive survey of the insect populations failed to establish any relation with GS (Anon, 1936). GREIG (1942) took the view that a filterable virus was the most likely cause, but no evidence of association of the disease with any virus has been shown. Also, chemicals, nutritional deficiencies and metabolic upsets are suggested and examined, but showed no link with the disease. Moreover, frequent usage of anthelmintics (viz. ivermectin) is suspected to increase the risk of the disease (Wood *et al*., 1997), which might interfere with gut function in some way that increases the absorption of a neurotoxin (Milne, 1997), but this was not confirmed by WOOD *et al*. (1998).

2.1.3.2 Why *Clostridium botulinum* is most likely the cause?

Clostridia are normally found in the healthy colon, where their numbers are kept in check by other bacteria. However, when they establish themselves in the ileum they become formidable foes (Johnson, 2001). They digest fibre and produce medium length fatty acids that increase water absorption, causing hypertension and drying up the faeces, causing constipation (Johnson, 2001). *C. botulinum* can be an opportunist due to gut stasis (Collier *et al.,* 2001).

Many evidences have been produced indicated that *C. botulinum* is the most likely candidate (Tocher *et al*., 1923; Miller, 1994; Poxton *et al*., 1997; Hunter and Poxton, 1998; Poxton *et al*., 1998; Hunter *et al*., 1999; Hunter and Poxton, 2001; Garrett *et*

al., 2002; Böhnel *et al*., 2003; McCarthy *et al*., 2004b). *Bacillus botulinus* (now *C. botulinum*) was first isolated from the gut of a case of EGS in 1919 and also from the spleens of a number of horses with GS (Tocher *et al*., 1923). TOCHER *et al*. (1923) injected the toxin of his isolate (*C. botulinum*) subcutaneously in horses and it produced signs of acute GS. Also, a vaccination trials utilizing a toxin/antitoxin mixture, derived from known strains of *B. botulinus* (probably type B) involving over 2000 horses on different farms were performed as randomised control trials (Tocher, 1924). Half the horses on each farm were inoculated and the other half acted as controls. Two doses were given in spring in the first year of the study; this resulted in 2.8 % mortality rate in vaccinated animals compared to 9.3 % in controls. Using a vaccine of a higher protective value in the second year, the mortality in controls was 10 % compared to only 1.5 % in inoculated animals. Evidence has been produced for *C. botulinum* type C neurotoxin production in EGS cases (59 % of ileal contents, 47 % of faecal samples) and unaffected control animals (7 % of ileal contents, 3 % of faecal samples) (Hunter and Poxton, 1998). Also, rising titres of specific systemic antibodies to surface antigens of *C. novyi* type A, a non-botulinum-toxin-producing species identical in cell surface composition to group III *C. botulinum*, and BoNT/C were detected in horses that had been in contact with EGS or that were grazing on land where GS had occurred frequently in the past compared to significantly lower levels of antibodies in horses with GS (Hunter and Poxton, 2001). This finding indicates that horses with low levels of systemic immunity to these antigens may be more susceptible. BoNT/C was also detected directly by ELISA in the ileum of 45 % (13/29) of horses with GS compared to 4 % (1/28) of controls, and in the faeces of 44 % (20/45) of horses with GS compared to 4 % (3/77) of controls. The organism was detected indirectly by assay for BoNT/C by ELISA after enrichment in culture medium. *C. botulinum* type C was shown to be present in 48 % (14/29) of ileum samples and 44 % (20/45) of faecal samples from horses with GS, compared to 7% (2/27) of ileum samples and 8 % (6/72) of faecal samples from controls (Hunter *et al*., 1999). From this study (Hunter *et al*., 1999), the BoNT/C was detected directly and/or after enrichment in the GI tract of horses with acute GS (74 %), subacute (67 %) and chronic (67 %) compared to 10 % in controls (Hunter and Poxton, 2001). HUNTER *et al*. (1999) have acknowledged that the association of *C. botulinum* type C with EGS may be a result of the stasis of the GI tract, but together with serological data (Hunter and Poxton, 2001) they hypothesized that the association is causal. Also, recent

studies (McCarthy, 2002; McCarthy *et al*., 2004b) have shown that EGS cases have significant lower serum antibody titre to *C. botulinum* type C and its toxin than horses that either have been in contact with EGS affected horses or have grazed frequently affected pastures. Moreover, in a recent work to investigate two cases of confirmed EGS in a stud in South England (Böhnel *et al*., 2003), the results supported the hypothesis that GS in horses is a clinical form of botulism. Different types and type mixtures (A-E) of *C. botulinum* and BoNT were found. Test samples included grass, soil, compost (biofertilizer), faeces and tissues (from one case). The free BoNT was found for the first time in the growing grass as well as in anaerobic enrichment cultures. BoNT/C and D was demonstrated in one of the compost samples, and three contained toxigenic *C. botulinum* type A-D in mixed forms in enrichment cultures. In tissue samples, BoNT/C and D were detected in spleen, mid-jejunum and small colon; while the enrichment cultures showed toxicity only in the small colon (type D). HUNTER (http:/www.grasssickness.org.uk/research/milk_leonie_hunter.htm) reported that specific antibodies to *C. botulinum* type C were detected in both colostrum and milk of mares which had been in contact with GS. However, he found no significant difference between antibody levels in mares that had been in contact with GS and those had no contact. Unlike antibodies, the toxin was not detected in milk of cattle inspite of the high dose (175 ng/kg body weight) of BoNT/C injected (Moeller Jr. and Davis, 2001). A significant increase in the clostridia numbers in EGS cases compared to controls and isolation of 14 *Clostridium* species (including *C. botulinum* group III) from EGS cases compared to only one (*C. bifermentans*) from control animals was reported by GARRETT *et al*. (2002). Also, GRIEß *et al*. (1996) has observed a prominent increase in numbers and types of clostridial cells in horses with typhlocolitis or colic compared to healthy horses. Only *C. perfringens* was isolated from healthy horses.

C. botulinum neurotoxin type C is suspected for a number of reasons. BoNT/C has a unique neurotoxicity in that it is the only botulinum neurotoxin that can cause overt neuronal degeneration (Williamson *et al*., 1995). It is therefore possible that BoNT/C can cause damage to a wide range of neurons in vivo. *C. botulinum* type C produces three types of toxins: C1, C2 and C3 (Mauss *et al*., 1990; Hara-Ykama *et al*., 1994). BoNT/C1 inhibits the release of acetylcholine at cholinergic nerve terminals leading to flaccid paralysis. It prevents neurotransmitter release by specific proteolysis of

syntaxin (Schiavo *et al*., 1995) and SNAP-25 (Foran *et al*., 1996), which are synatosomal proteins that are involved in synaptic vesicle exocytosis. C2 and C3 have ADP-ribosylating activity (Mauss *et al*., 1990; Hara-Ykama *et al*., 1994). C2 is known to inhibit neutrophil migration which could account for the characteristic absence of inflammatory reaction in EGS, and it is also known to enhance neurosecretory release and hypersecretion, a likely feature of EGS (Rocke, 1993; Cottrell *et al*., 1999). C3 can cause neuronal degeneration in vitro (Williamson and Neale, 1998) and hence it can account for the chromatolysis in EGS. All the three toxin types of *C. botulinum* type C might be involved in producing the typical pathology of EGS (Cottrell, 1999; Hunter *et al.*, 1999). BoNT/C is severely toxic to mouse spinal cord neurons (Williamson and Neale, 1998) and rat hypocampal and cortical neurons (Osen-Sand *et al*., 1996) in culture.

Moreover, *C. botulinum* is suspected due to the clinical similarities observed between EGS, human infantile botulism (Cottrell *et al*., 1999) and shaker foal syndrome, which are also toxico-infections by spores of *C. botulinum*, and forage poisoning in hoses, due to ingestion of preformed botulinum toxins.

2.1.4 Epizootiology

Equine grass sickness, as its name indicates, occurs almost exclusively in animals while at pasture and very rare in stabled animals (Greig, 1942; Scholes *et al*., 1993). It affects horses, ponies and donkeys. Grass sickness was also reported in a common zebra (Ashton *et al*., 1977). Dysautonomias similar to equine dysautonomia, which are also of unknown aetiology, were reported in dogs (Rochlitz and Bennett, 1983), cats (Key and Gaskel, 1982), hares and rabbits (Whitwell, 1997). The neuropathology of the dysautonomias exhibited by these animals is reported to be remarkably similar (Pollin and Griffiths, 1992; Whitwell, 1997). WHITWELL (1997) stated that “it is not unrealistic to hypothesize that the dysautonomias in all these species could arise following exposure to the same or very similar neuronal insult“.

Several risk factors that are associated with the disease were described. The epidemiological data showed that the disease is more prevalent during spring and early summer (April to July) with peak incidence in May, but it can occur throughout the year (Doxey *et al*., 1991c; Milne *et al*., 1994). It is observed that the majority of

outbreaks occur during cool (7-11 °C) and dry weather with irregular ground frosts (Doxey *et al*., 1991c; McCarthy *et al*., 2001). WOOD *et al*. (1998) reported that 66% of cases occurred following a two weeks period of predominantly dry weather, followed by rains. Horses which had recently moved to a different pasture contract the disease more than those which have spent more than two months on the premises (Gilmour and Jolly, 1974: Doxey *et al*., 1991c). It is noted that 45 % of cases are due to moving from one pasture to another (Doxey *et al*., 1991b). Most incidences are associated with pastures where the disease has previously occurred (Gilmour and Jolly, 1974; Newton *et al*., 2004). GILMOUR and JOLLY (1974) reported that a higher proportion of horses which experience GS were receiving either no supplement or only concentrates than of those receiving hay and concentrates. McCARTHY *et al*. (2004) observed that feeding hay or haylage was associated with a decreased risk of disease, change of feeding type or quantity during the 14 days prior to disease was associated with increased risk, and the use of ivermectin was also associated with a significant increased risk of EGS. In contrast, WOOD *et al*. (1998) found no evidence that the supplementary feeding of hay or forage alone was associated with a decreased risk of the disease. It is suggested that the incidence might be reduced by stabling, even part-time (Gilmour and Jolly, 1974). Young animals, usually 2-7-year-old, are more susceptible (Gilmour and Jolly, 1974; Doxey *et al*., 1991c), with peak incidence among 3-5-year-old animals (Doxey *et al*., 1991b). However, GS was reported in horses from one year to over 20 years old and is of very rare occurrence during first year of life (Greig, 1942). Suckling foals below six months of age, despite ingesting significant volumes of grass, rarely develop the disease. Whether this apparent protection is due to maternally derived colostral antibodies, age-related differences in physiology, toxin metabolism or the way ingesta is processed is unknown (Cottrell *et al*., 1999). All breeds appear to be equally susceptible, asses and mules are very rarely kept in the affected districts and it is possible that they may be susceptible (Greig, 1942). In a recent study (Newton *et al*., 2004) to identify risk factors associated with recurrence of EGS on a previously affected premises in UK, there was an increased rate of recurrence with higher number of horses, presence of younger animals, stud farms and livery/riding establishments, loam and sand soils, rearing of domestic birds and mechanical droppings removal. While rate of recurrence decreased with chalk soil, cograzing ruminants, grass cutting on pastures and removal of droppings by hand.

Also, the disease was found associated with increased soil nitrogen content and pasture disturbance (McCarthy *et al*., 2004a). Prior contact with EGS is associated with a ten-fold reduction in the likelihood of the disease (Wood *et al*., 1998). However, GILMOUR and JOLLY (1974) reported no difference in frequency of the disease between animals with previous contact and those which had no previous contact with it. Confirmed recurrence of the disease in the same animal was not reported (Newton *et al*., 2004). In a study conducted by WOOD *et al*. (1998), it was observed that female horses had a three-fold reduction in risk compared to males; however, this was because female horses had far bigger number than males (Hedderson and Newton, 2004). Stresses such as recent purchase, travelling long distances, mixing with strange horses, castration, and foaling have shown to have effect. DOXEY *et al*., (1991b) found that horses in good body condition were more likely to contract the disease. However, a work by WOOD *et al*. (1998) did not support these findings.

Latency of the disease is proposed to be 1-3 weeks, but can be as short as 2-5 days after animals being put out on grass (Guthrie, 1940; Doxey *et al*., 1991c). Incidence rate is low; it is reported that EGS affects 2-4 % of equine population in all breeds throughout the British Isles (Greig, 1942; Gilmour and Jolly, 1974; Gilmour, 1987; Doxey *et al*., 1991b; Newton *et al*., 2004); but the disease is invariably fatal (mortality may exceed 95 %) (Gilmour and Jolly, 1974). It is proposed that the disease is not contagious, but that there appears to be an association with particular premises (Gilmour and Jolly, 1974). MILNE (1997a) found no evidence that the disease can be transmitted naturally between horses.

2.1.5 Pathogenesis

The mechanism of action of the putative neurotoxin remains unknown. It is hypothesized that the putative neurotoxin may be ingested, elaborated in the gut or produced by hepatic metabolism (Sharp, 1987; Hunter *et al*., 1999). However, the neurotoxicity of nitric oxide, which can be synthesized in excess by nitredergic neurons due to their activation by some agent such as grass secondary metabolites, is also suggested (Cottrell *et al*., 1999). Putative neurotoxins mostly proposed are botulinum neurotoxins, pasture mycotoxins and grass secondary metabolites (Cottrell *et al*., 1999). The putative neurotoxin exerts its action either directly or after a

predisposing factor. Tocher *et al*., (1923) proposed gastric irritation as a predisposing factor to *C. botulinum*. COLLIER *et al*. (2001) suggested *Fusarium germinearium* to cause the gastric irritation which was proposed by Tocher and his colleagues. Also, HUNTER *et al.* (1999) and COLLIER *et al.* (2001) thought that *C. botulinum* can be an opportunist due to any factor causing gut stasis. Furthermore, J. K. Miller and I. R. Poxton, Edinburgh, hypothesized that both a nutritional trigger and the intestinal immune status to *C. botulinum* are involved in the pathogenesis of the disease (Cottrell *et al*., 1999).

As many EGS features can be produced by BoNT/C (Cottrell *et al.*, 1999; Hunter *et al.*, 1999), it is worthfull to suspect the actions of BoNT/C. BoNT/C has a unique neurotoxicity in that it is the only botulinum neurotoxin that can cause overt neuronal degeneration (Williamson *et al*., 1995). It is therefore possible that BoNT/C can cause damage to a wide range of neurons in vivo. BoNT/C1 inhibits the release of acetylcholine at cholinergic nerve terminals leading to flaccid paralysis, which could account for the signs of autonomic dysfunction of the GI tract. C2 is known to inhibit neutrophil migration which could account for the characteristic absence of inflammatory reaction in EGS, and it is also known to enhance neurosecretory release and hypersecretion, a likely feature of EGS (Cottrell *et al*., 1999). C3 can cause neuronal degeneration in vitro (Williamson and Neale, 1998) and hence it can account for the chromatolysis in EGS. There are two possible routes by which the putative neurotoxin affects the neurons; by direct action on the perikaryon or via the axon (Griffiths *et al*., 1994b). All the three toxin types of *C. botulinum* type C might be involved in producing the typical pathology of EGS (Cottrell, 1999; Hunter *et al.*, 1999).

It is thought that the neurotoxin reaches the peripheral autonomic ganglia via circulation and/or by retrograde axonal transport (Griffiths *et al.*, 1994b). Evidence of retrograde axonal transport has been shown experimentally by GRIFFITHS *et al*. (1994a). There was evidence suggesting that EGS agent may cross the placenta; when a full-term foal born by caesarean section to a mare with acute grass sickness. The foal lived for only 24 hours and was found, on histological examination, to have some of the lesions associated with grass sickness. These lesions had been caused presumably by a placenta-crossing factor, which was therefore likely to be present in

the blood of acutely affected animals (Gilmour, 1973b). However, a preliminary study done by WHITWELL (1992) showed that the putative neurotoxin does not appear to cross the placenta.

It is suggested that the neuronal damage starts in the enteric nervous system (Bishop *et al*., 1984; Hodson and Wright, 1987), where the putative neurotoxin appears to be non-specific to neuronal type (Sabate *et al*., 1983). There is a correlation between the clinical severity and the extent and distribution of enteric neuronal damage (Doxey *et al*., 1992; Pogson *et al*., 1992; Scholes *et al*., 1993) with the greatest neuronal loss usually observed in the ileum. Damage to peripheral nervous system in EGS involves, beside enteric nervous system, some of paravertebral, prevertebral, dorsal root and ciliary ganglia (Cottrell *et al*., 1999). However, the damage in the CNS is usually less severe, focally, widely distributed and mainly seen in the spinal cord, specific brain stem nuclei and some of cranial nerves (Gilmour, 1973a; Wright and Hodson, 1988).

The agent of EGS exerts its main effects on the GI tract. In acute cases, stomach and small intestine are rapidly distended with fluids, indicating a hypersecretory characteristic of the cause. This abdominal distension causes intestinal ileus, colic and dehydration (Cottrell *et al*., 1999). Also, this hypersecretion leads to reduced circulatory volume and may cause death from cardiac failure. Due to the neuronal damage and consequently partial or complete paralysis of the GI tract, especially in the acute disease, signs of autonomic dysfunction such as dysphagia, failure of oesophageal peristalsis and drooling of saliva are prominent.

Involvement of nitrergic neurons is also suggested in ED and consequently increased nitrergic neuronal activity and increased synthesis of nitric oxide, which has excitotoxic (Patel *et al*., 1996) and anti-inflammatory (Alican and Kubes, 1996) actions, which can account for most of EGS features (Cottrell *et al*., 1999).

2.1.6 Clinical features

Clinical signs of equine grass sickness are described by many authors (Pool, 1927; Edwards, 1987; Pinsent, 1989; Milne *et al*., 1994; Milne, 1996; Mair and Hillyer, 1997) and are mainly due to partial or complete paralysis or stasis of the GI tract and

its autonomic dysfunction. The signs occur in three overlapping forms, acute, subacute and chronic, classified on the basis of duration and severity of clinical signs.

In the acute form the onset is sudden and the signs are severe and usually death ensues or the animal has to be euthanised within two days. The signs of acute form are frequent mild to severe abdominal pain (colic), massive abdominal distension due to accumulation of fluids in the stomach and small intestine, marked difficulty in swallowing (dysphagia), slow mastication, dropping food from the mouth, spontaneous nasogastric reflux of foul smelling green or brown stomach fluid, patchy or generalised sweating, excessive salivation, fine muscle tremors and on rectal examination small firm faecal pellets covered with mucus in the rectum, and the colon is heavily impacted with dry ingesta. In addition, the animal is depressed, with tachycardia (70-120 beats/min), gut sounds are usually decreased, reduced urine output, the rectal temperature is normal or there may be pyrexia of up to 39.5 °C and ptosis of upper eye lids.

Subacute cases show similar, but in general, less severe signs often without nasogastric reflux, most cases either die or have to be put to death within seven days of onset and few of them progress to chronic form. Some body weight loss and tucked-up abdomen appearance usually develops in subacute disease.

In chronic GS the signs are less severe and may develop insidiously, but this form is predominantly characterised by a marked loss of weight and emaciation leading to 'greyhound' appearance and dry rhinitis causing snoring sound from the nasal cavity. Subclinical cases are thought to occur but have not been proven (Doxey *et al*., 1995; Milne, 1997b).

Clinical features of EGS have several parallels to infant botulism in humans (a toxico-infection) (e.g. dysphagia, constipation, diminished appetite, and spectrum of severity (Poxton *et al*., 1999). Various similarities as well as differences have been noted between cases of EGS and botulism (Walker, 1929; McGorum *et al*., 2003). Similarities include muscle tremors, dysphagia, ptosis, salivation, and tucked up abdomen. Differences include the presence of profound myasthenia and dilated

pupils, and the absence of tachycardia, sweating, rhinitis sicca, impacted faeces and histopathological changes in case of botulism.

2.1.7 Pathology

Post mortem lesions in equine grass sickness are not pathognomonic and are of less significance than the histopathological picture of autonomic neurons. Gross pathology of acute cases shows distension of stomach and small intestine with foul smelling fluid, secondary impaction of colon and caecum with dry ingesta, small faecal boluses covered by sticky mucus are usually found in the rectum, congestion of abdominal organs and very often there is colonic mucosal adhesion of blood products (Cottrell *et al*., 1999). There is usually splenomegaly (Greig, 1942; Mahaffey, 1959) and erosion of the oesophageal mucous membrane. Also, blackish batch at junction between ventricles and auricles and marked congestion in vessels of nasal septum were observed (Tocher *et al*., 1923). Subacute cases have less severe abnormalities, have little or no gastric or small intestine distension, have varying degrees of secondary impaction of large intestine and often have rhinitis sicca. The most prominent feature of chronic cases is cachexia, due to inanition or maldigestion and malabsorption, with stomach and small intestine usually being relatively empty (Cottrell *et al*., 1999). Necrosed or decayed ethmoid bone, which often leads to secondary inflammation of the brain and spinal cord, was also reported (Tocher *et al*., 1923).

The histopathological changes in equine dysautonomia are mainly seen in the autonomic neuronal cells. ED is associated with characteristic degenerative changes in the peripheral autonomic ganglia, neurons of the enteric nervous system, certain brain stem nuclei and often more restricted changes in sensory ganglia (Obel, 1955; Mahaffey, 1959; Pogson *et al*., 1992). The main alterations in the nerve cells are axonal dystrophy, cytoplasmic vacuolation, pyknotic and eccentric nuclei, margination or loss of Nissl bodies, increased numbers of lysosomes and mitochondria which assume a central location and loss of a recognisable Golgi structure (Gilmour 1974, Hodson and Wright, 1987; Griffiths *et al*., 1993). However, all the grades of cell degeneration from early chromatolysis to death and disintegration were encountered (Mahaffey, 1959).

It is suggested that the neuronal damage starts in the enteric nervous system (Bishop *et al*., 1984; Hodson and Wright, 1987), where the putative neurotoxin appears to be non-specific to neuronal type (Sabate *et al*., 1983). A correlation between the clinical severity and the extent and distribution of enteric neuronal damage has been found (Doxey *et al*., 1992; Pogson *et al*., 1992; Scholes *et al*., 1993) with the greatest neuronal loss usually observed in the ileum. Neuronal lesions in acute cases are seen in the stomach and both small and large intestine (Scholes *et al*., 1993; Murray *et al*., 1997) and localised in the distal small intestine in chronic cases (Scholes *et al*., 1993). Damage to peripheral nervous system in EGS involves, beside enteric nervous system, some of paravertebral, prevertebral, dorsal root and ciliary ganglia (Cottrell *et al*., 1999). However, the damage in the CNS is usually less severe, focally, widely distributed and mainly seen in the spinal cord, specific brain stem nuclei and some of cranial nerves (Gilmour, 1973a; Wright and Hodson, 1988). These neuronal elements are not always affected to the same degree (Pogson *et al*., 1992). A study conducted by MARRS *et al*. (2001) showed moderate hepatocellular pathology in conjunction with steatosis and cholestasis in GS cases.

The involvement of nitrergic neurons in EGS is suggested (Cottrell *et al*., 1999). It is observed that the anatomical distribution of neuronal nitric oxide synthase neurons bears a remarkable similarity to the distribution of neuronal damage which occurs in EGS. The relative absence of lesions in the cerebral cortex in EGS (Barlow, 1969) may reflect the fact that the cortex contains few nitrergic neurons (Snyder and Bredt, 1992).

2.1.8 Diagnosis

There are no pathognomonic macroscopic findings at ante mortem or post mortem examination and the diagnosis can only be confirmed by histopathological examination either of the sympathetic ganglia and/or ileal tissue at post mortem or of the ileal biopsies obtained at laparotomy from living animals (Scholes *et al*., 1993). Diagnosis based on the demonstration of non-inflammatory neuronal degeneration (chromatolysis and necrosis) on histopathological examination of enteric tissue is extremely accurate (Scholes *et al.*, 1993). Because laparotomy has its adverse effect on case survival (Milne *et al*., 1994), the ante mortem diagnosis must rely heavily on the epidemiological factors, clinical findings and the elimination of other possible

causes of colic, impaction, dysphagia, food discharge and weight loss (Doxey *et al*., 1991b). MILNE (1991) and DOXEY *et al*. (1995) mentioned that the accuracy of diagnosis based on clinical signs of autonomic failure without ileal biopsy may reach 98 %. GREET and WHITWELL (1986) and MILNE (1996) have suggested the usage of contrast radiography if doubt exists in case of dysphagia and to demonstrate oesophageal dysfunction. Endoscopy also has been proposed as diagnostic aid. Ocular administration of phenylephrine (alpha-adrenergic agonist) as a mean to diagnose GS was studied by HAHN and MAYHEW (2000). A significant greater mean increase in the size of the palpebral fissure was found in GS cases compared to controls.

Laboratory tests showed marked increase in plasma levels of adrenaline and noradrenaline (Hodson *et al*., 1984; Hodson *et al*., 1986) and dopamine-beta-hydroxylase (Griffiths *et al*., 1993), which is associated with noradrenaline synthesis, in blood of grass sickness cases. This increase in catecholamines may account for the generalized sweating and atony of the GI tract. MAHAFFY (1959) found no biochemical or haematological abnormalities. However, examination of blood parameters (Stewart *et al*., 1940; Milne and Doxey, 1990; Doxey *et al*., 1991d) indicated hypovolaemia and increase in plasma protein, sugar and specific gravity. In addition, on examination of urine from GS cases, the specific gravity was higher than normal, glucose was detected, elevated total protein and pH was less than normal (Marrs *et al*., 1999), which were attributed to either kidney damage, increased levels in plasma or dehydration. However, this was criticised by KERR (1999) because only 4 affected horses were studied and the method used to analyse the urine was not optimal.

Differential diagnosis in GS is important to eliminate other possible causes of colic and impaction such as primary colonic impaction/displacement/torsion, colitis, caecal impaction, small intestinal obstruction, intestinal intussusception, peritonitis and enteritis (Milne, 1996). Also, other causes of dysphagia and food discharge such as classic botulism, pharyngitis, pharyngeal paralysis and oesophageal obstruction or ulcers should be considered. Due to some clinical and histological overlap between EGS, EMND (equine motor neuron disease) and EDM (equine degenerative myeloencephalopathy), similarities are suggested, but not confirmed. GERBER

(1994) and FATZER *et al*. (1995) hypothesized that EGS and EMND may be different manifestations of the same underlying disease process. However, DIVERS *et al*. (1994; 1997) took the view that the disease appears to represent a distinct clinical syndrome due to its completely different epidemiology, beside major differences in the clinical picture.

2.1.9 Treatment

The disease is invariably fatal and treatment should not be considered for acute and subacute cases and euthanesia is indicated (Milne, 1997b). Most authors consider that the prognosis is also hopeless in chronic cases and no useful treatment (Greig, 1942; Gilmour, 1988; Pinsent, 1989). On the other hand, there are several reports of successfully treated and recovered chronic cases (Milne and Wallis, 1994; Milne *et al*., 1994; Doxey *et al*., 1995; Milne, 1997; Doxey *et al*., 1998). However, only horses with the mildest chronic form of the disease can be successfully nursed to recovery (Doxey *et al*., 1995).

In general the prognostic indicators to select a case for treatment are the degree of dysphagia, appetite, the severity of colic, the audibility of gut sounds and the severity of rhinitis (Milne *et al*., 1994). Intensive and thorough nursing care is the mainstay in the treatment (Doxey *et al*., 1998). A case having been selected for treatment, the feed should be considered next; a high energy, high protein diet which is easily swallowed should be fed. Diazepam as an appetite stimulant and oral electrolytes can be given (Milne, 1997). Analgesics such as non-steroidal anti-inflammatory drugs, lubricants (purgatives must be avoided) and probiotics to improve gut flora can be given. Also, cisapride, a prokinetic agent, was used for the treatment of chronic GS by MILNE *et al.* (1996) and it was beneficial but expensive and accurate case selection should be undertaken. The rate of passage of digesta and dry matter intake was found significantly increased. Cisapride enhances the release of acetylcholine from the postganglionic nerves of the myenteric plexus of the gut (Lee *et al*., 1984) leading to an increase in gut motility. Use of cisapride in horses with large colon motility dysfunction may be efficacious (Pakestraw, 2003). Moreover, cleaning, grooming, washing of sweaty areas and cleaning of nostrils is important (Milne and Wallis, 1994). In addition, frequent human contact is important in keeping the horse interested and stimulated. The recovery rate at best is approximately 40 %, but the

majority of recovered cases returned to normal working life (Milne and Wallis, 1994; Doxey *et al*., 1998).

2.1.10 Control

Some epidemiological evidences showed that resistance to EGS can occur in older horses; those have been in a particular pasture for longer time or those had a prior contact with the disease (Greig, 1942; Doxey *et al*., 1991b; Wood *et al*., 1998). This resistance may be in a form of an immune response to the aetiological agent (Hunter and Poxton, 2001). Suckling foals below six months of age, despite ingesting significant volumes of grass, rarely develop the disease. This apparent protection may be due to maternally derived colostral antibodies (Cottrell *et al*., 1999). In an experimental study, rising titres of specific systemic antibodies to surface antigens of *C. botulinum* type C and BoNT/C were detected in horses that had been in contact with EGS or that were grazing land where EGS had occurred frequently in the past compared to significantly lower levels of antibodies in horses with grass sickness (Hunter and Poxton, 2001). This finding indicates that horses with low levels of systemic immunity to these antigens may be more susceptible. Due to the association found between EGS and *C. botulinum* by TOCHER *et al*. (1923), a vaccination trial utilizing a toxin/antitoxin mixture, derived from known strains of *B. botulinus* (*C. botulinum*, probably type B) involving over 2000 horses on different farms were performed as randomised control trials (Tocher, 1924). A significant decrease in the mortality in the inoculated animals (1.5 %) compared to controls (10 %) was found. These epidemiological and experimental data indicate that the disease can be controlled by vaccination. Recently, due to the most believed theory that EGS is a toxico-infectious form of botulism, experience of Neogen Corporation (USA) in equine botulism vaccine production and the good facilities for experimental vaccination-challenge studies at the Animal Health Trust's Allen Centre for Vaccine Studies, it was recommended to produce a vaccine from *C. botulinum* type C toxoid against the disease (Hedderson and Newton, 2004). Incidence can be reduced by stabling, even part-time, during spring and early summer especially to young animals (2-7-year-old) and giving hay and concentrate supplement. In addition, pastures of previous outbreaks should not be grazed (Gilmour and Jolly, 1974; Scholes *et al*., 1993).

2.2 Botulism

Botulism is a non-febrile highly fatal disease of animals and man caused by neurotoxins of *Clostridium botulinum*. It is characterized by partial or complete flaccid paralysis of the muscles of locomotion, mastication and deglutition due to inhibition of the release of the neurotransmitter acetylcholine by botulinum neurotoxins at cholinergic nerve endings (Kriek and Odendaal, 1994). Botulism occurs sporadically, but in intensively farmed animals, it is responsible for high mortalities (Smart *et al*., 1983: Abbitt *et al*., 1984; Trueman, 1992). Animal botulism can be a public health problem, since humans can be intoxicated by ingestion of contaminated meat.

2.2.1 Clostridium botulinum

C. botulinum was first isolated by van Ermengem in 1897 in Belgium from salted ham. He named the organism *Bacillus botulinus*, which was later renamed *C. botulinum* (Hauschild and Dodds, 1993). *C. botulinum* encompasses a heterogeneous group of obligate anaerobic, spore-forming, Gram-positive rods (Rocke, 1993) that produces the most potent biologic toxin known. The bacterium is ubiquitous and is found in soils and organic matter worldwide. *C. botulinum* is either not a normal inhabitant of the gut (Ortiz and Smith, 1994), occasionally present (Ricketts and Greet, 1984) or may be present (McLoughlin *et al*., 1988).

2.2.1.1 Nomenclature and taxonomy

Based on the serological properties of the toxin they produce, *C. botulinum* strains are divided into seven types (A-G), named according to their chronological discovery (Table 1). Types A, B, E and F are mainly involved in botulism in man, whereas types C and D are responsible for botulism in animals. Type G, according to CDC (1998), has not been confirmed as a cause of illness in humans and animals. However, SONNABEND *et al*. (1981) reported the isolation of type G organisms and demonstration of its toxin in five human cases with sudden death in Argentina; also, it was found associated with infant botulism (Sonnabend *et al*., 1985). HOLDEMAN and BROOKS (1970) divided *C. botulinum* types A-F into three major groups according to their cultural characteristics and metabolic products (Table 2). Other clostridia that have cultural characteristics similar to those of *C. botulinum* and exhibit high relatedness (Collins and East, 1998) are also included in these groups. Group I (proteolytic) contains strains of type A, proteolytic strains of types B and F, and *C.*

sporogenes; group II (non-proteolytic) contains strains of type E and non-proteolytic strains of types B and F; whereas group III (non-proteolytic) includes strains of types C, D and *C. novyi* type A. A few strains of types C and D are mildly proteolytic, but are nevertheless included into group III. A fourth group (proteolytic) includes strains of type G, but due to its distinct phenotypic and genotypic features, was put as a separate species, *C. argentinense* (Suen *et al*., 1988). Some non-toxigenic strains of *C. subterminale and C. hastiforme* are also members of group IV (Suen *et al*., 1988).

Table 1: Chronological discovery of different types of *C. botulinum* (Kriek and Odendaal, 1994; Sperber, 1982)

C. botulinum type	Discoverer	Year
A and B	G. S. Burke*	1919
Cα	I. A. Bengston	1922
Cβ	H. R. Seddon	1922
D	P. J. Du Toit and E. M. Robinson	1928
E	Bier	1936
F	Moller and Sheibel	1960
G	Giminez and Cicarelli	1970

*Burke described type A and B for the first time, but type B may be the toxin discovered in 1896 by van Ermengem and type A may be the toxin discovered by Landman in 1904 (Sperber, 1982)

Although most C*. botulinum* organisms produce a single type of BoNT, it is now recognized that some strains produce mixtures of two toxin types (viz. AF, AB, BA and BF [Franciosa *et al*., 1994; Cordoba *et al*., 1995]). Comparison of nucleotide sequences of BoNT genes does not agree with the four phylogenetic groups of organisms, suggesting that there has been lateral gene transfer of BoNT genes (Collins and East, 1998). Type C strains consist of two distinct subtypes, Cα and Cβ (Kriek and Odendaal, 1994). Type Cα produces C1 and lesser amounts of C2 and D toxins; Cβ produces C2 toxin and type D produces predominantly type D toxin along with smaller amounts of C1 and C2 (Jansen,1971). C3 (exoenzyme) is produced by both C and D. In a study conducted by OGUMA *et al*. (1986), it was found that

biochemical characteristics of *C. botulinum* type C and D differentiate them into four groups and not three as it is known (Cα , Cβ and D). Also, OCHANDA *et al*. (1984) and OGUMA *et al*. (1984) found that classification of Cα, Cβ and D is not correct according to toxin reactions against antibodies raised against Cα, Cβ and D. They found four groups of reactions based on antigenic structure of toxins and relationship between toxin production and phages. The heterogeneity that exists in types C and D NTs has probably arisen from the mutation or recombination of phage genomes that is thought to occur during the cycles of curing and reinfection of type C and D strains in the environment (Sunagawa and Inoue, 1991). Group III organisms (*C. botulinum* types C and D, and *C. novyi*) toxins are each encoded on separate pseudolysogenic bacteriophages (Eklund *et al*., 1972). Cultures of toxigenic strains can be cured of their prophages and stop producing toxins and can be converted to toxigenic state by reinfection by phages (Oguma *et al*., 1986). The type of the toxin produced is determined by the specific phage with which the bacterium is infected (Eklund and Poysky, 1974; Eklund *et al*., 1974). Type C strains can be reinfected by either C or D bacteriophages, but strains of type D are infected only by the homologous phage. Also, *C. novyi* can be converted to either type C or D *C. botulinum* by phage type. Cured type C organism may continue to produce C2 toxin. This suggests that Cβ strains are derived from Cα strains upon loss of their prophage (Jansen, 1971). A cycle of phage loss and reinfection is thought to occur in vivo (Eklund *et al*., 1974). Some strains of *C. butyricum* and *C. baratii* produce NTs that cross-react with BoNT/E (McCrosky *et al*., 1986) and F (Hall *et al*., 1985) respectively. Some evidence suggests that the toxic factors may be transfered between different clostridia, when transfer of neurotoxigenicity from *Clostridium butyricum* to a non-toxigenic *Clostridium botulinum* type E-like strain occurred (Zhou *et al*., 1993). *C. butyricum* and *C. barrati* toxigenic strains are genetically remote from group I-IV botulinum strains (Suen *et al*., 1988). Thus, six phenotypically distinct groups of clostridia are now known to be capable of producing BoNTs. Undoubtedly, additional serotypes will be identified in the future if for no other reason than mutations in the antigenic properties of existing toxins (Sperber, 1982).

The taxonomic denominator for *C. botulinum* is the production of botulinum neurotoxin, which was first suggested by PRÉVOT (1953, cited by Collins and East, 1998). However, neurotoxin production is not a stable phenotype in many *C.*

botulinum strains; other bacteria (viz. *C. baratii* and *C. butyricum*) are known to produce BoNT; some *C. botulinum* strains produce dual types of BoNTs; and non-toxigenic organisms which contain silent BoNT genes are present (Collins and East, 1998). So, nomenclature, which is rigidly based on BoNT production, is unsatisfactory and a major change is needed (Collins and East, 1998). The future nomenclature should take into account the phenotypic, genotypic and BoNT production characteristics. A sensible solution would be to designate each of the four groups as a separate species (Collins and East, 1998). Reclassification of *C. botulinum* was also suggested by HUNTER and POXTON (2002). For more details about complications of *C. botulinum* nomenclature, the reader is referred to COLLINS and EAST (1998).

Table 2: Phenotypic differences between organisms capable of producing botulinum neurotoxins (from Hatheway, 1998)

Characteristic	Groups			Other BoNT-producing *Clostridium* species		
	I	II	III	*C. argentinense*	*C. butyricum*	*C. baratii*
Toxin types	A,B,F	B,E,F	C,D	G	E	F
Proteolysis	+	-	-	+	-	-
Liquefaction of gelatin	+	+	+	+	-	-
Fermentation of						
Glucose	+	+	+	-	+	+
Fructose	±	+	±	-	+	+
Mannose	-	+	+	-	+	+
Maltose	±	+	±	-	+	+
Sucrose	-	+	-	-	+	+
Trehalose	-	+	-	-	+	-
Lipase	+	+	+	-	-	-
Metabolic acids[b]	A,iB,B,iV,PP	A,B	A,P,B	A,iB,B,iV,PA	A,B	A,B
Optimal growth temperature	35-40 °C	18-25 °C	40 °C	37 °C	30-37 °C	30-45 °C
Minimal growth temperature	10 °C	3.3 °C	15 °C		10 °C	
Spore heat resistance (temperature/D-value)	112 °C/1.23	80 °C/ 0.6-1.25	104 °C/ 0.1-0.9	104 °C/ 0.8-1.12		
Phenotypically related *Clostridium* species	*C. sporogenes*		*C. novyi*	*C. subterminale*		

+, all strains are positive; -, all strains are negative; ±, some strains are positive and some are negative. A, acetic; P, propionic; B, butyric; iB, isobutyric; iV, isovaleric; PP, phenylpropionic; PA, phenylacetic.

2.2.1.2 Botulinum neurotoxins

C. botulinum produces seven neurotoxins that are pharmacologically similar, but serologically distinct from each other; designated as types A through G (Rocke, 1993) according to their chronological discovery (Table 1). They are the most toxic biological substances known. The MLD for mice of BoNT type A per gram of body weight is 1.2 ng i.p., for type B is 0.5-2 ng i.p, C1 is 1.1 ng i.v, C2 is 1.2 ng i.p, D is 0.4 ng i.p, E is 1.1 ng i.p and F is 2.5 ng i.v. (Gill, 1982). MLD of BoNT/C in cattle was found to be between 0.25 and 0.5 ng/kg body weight (Moeller Jr. and Davis, 2001). BoNT is produced as a complex with a group of neurotoxin associated proteins (NAPs). Botulinum neurotoxin complex is the only known example of a protein complex where a group of proteins (NAPs) protect another protein (BoNT) against the acidity and proteases of the stomach (Sharma *et al.*, 2003). BoNTs are proteins which are produced intracellularly as protoxins and released into the culture supernatant in relatively low concentrations during logarithmic phase of growth and increase dramatically when cell growth ceases and bacteria undergo autolysis (Simpson, 1981). The toxic components are activated to the maximum toxic state by proteolytic enzymes. The proteolytic strains can activate their own toxins, while the toxins produced by non-proteolytic types are activated by exogenous proteases, such as trypsin in the host stomach (Sperber, 1982). However, the ability of trypsin to activate BoNT has been demonstrated for types A to G with varying degrees (Simpson, 1981). The toxins are initially synthesized as single chain polypeptides which are enzymatically cleaved to form the active dichain structure (heavy [H] and light [L] chains). The active form of BoNT is about 150 kDa, 100 kDa H chain and 50 kDa L chain. The H chain consists of an amino-terminal 50 kDa domain (H_N) and a carboxyterminal 50 kDa domain (H_C) (Fig. 1). The proteins remain linked through a highly conserved disulfide bond (Pellizzari *et al.*, 1999). Absorption of BoNTs is accomplished via endocytosis, primarily in the small intestine and only small amounts of protein escape digestion to be absorbed; however, the extremely high toxicity of BoNT can make even those small amounts extremely hazardous (Bonventre, 1979). Polypeptide subunits of BoNTs are bound to non-toxic neurotoxin-associated proteins which protect the toxins in the GI tract from acidic degradation. These proteins could conceivably block antigenic sites and prevent recognition of the toxins by the local antibodies.

The mechanism of action of BoNTs results from the specific binding of the heavy chain to the presynaptic membrane of cholinergic nerves (Pellizzari *et al*., 1999). Once bound, different H chain components facilitate internalization of the light chain within the nerve endings. The light chain, a zinc-dependent protease (Fujii *et al*., 1992), degrades with high specificity the synaptic proteins that are involved in the fusion of synaptic vesicles with the plasma membrane, thus inhibiting release of acetylcholine, thereby producing a flaccid paralysis (Moriishi *et al*., 1996). Types A and E degrade SNAP-25; type B, D, F and G degrade VAMP/synaptobrevin; and type C1 degrades HPC-1/syntaxin (Moriishi *et al*., 1996) and SNAP-25 (Foran *et al*., 1996). These three proteins form the synaptic SNARE complex. Once toxin is bound at the motor endplate, improved neuromuscular function is achieved only by the regeneration of new endplates, explaining the usual delay of four to ten days before noticeable clinical improvement occurs after initiating antitoxin therapy (Kriek and Odendaal, 1994). Each neurotoxin type has its own specific receptors, which may explain differences in species susceptibility to different toxin types.

The genes of Type A, B, E and F NTs are likely to be present on the bacterial chromosome (Hauschild and Dodds, 1993), type G NT gene is contained within a plasmid, whereas C and D toxin encoding genes are present within bacteriophages (Rossetto *et al*., 2002). BoNT serotypes exhibit 30-60 % sequence identity (Szílagyi *et al*., 2000). However, serotype-specific antisera have been reported to elicit little or no cross-reactivity (Szílagyi *et al*., 2000). BoNTs within the same physiological group are almost identical, while the extent of diversity between NTs of the same serotype from different physiological groups is greater (e.g. Types A and B proteolytic are more related than types B proteolytic and non-proteolytic) (Henderson *et al*., 1997). BoNT/F gene sequence of *C. baratii* is different from BoNT/F gene sequence of both proteolytic and non-proteolytic strains of *C. botulinum* (Campbell *et al*., 1993).

Recently BoNTs have become extremely useful therapeutic drugs; and the number of indications being treated by BoNTs is greatly increasing; including numerous focal dystonias, spasticities, tremors, blepharospasm, cosmetic applications, migraine and tension headaches, and other maladies (Johnson, 1999; Brin *et al*., 2002). The remarkable therapeutic utility of botulinum toxin lies in its ability to specifically and potently inhibit involuntary muscle activity for extended duration.

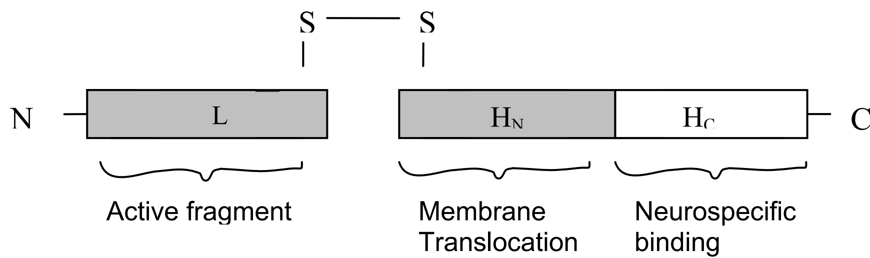


Fig. 1: Schematic structure of botulinum neurotoxin, showing three functionally distinct domains (Hunter and Poxton, 2002). L, light chain; H_N, N-terminal end of heavy chain; H_C, C-terminal end of heavy chain.

2.2.1.3 Growth requirements and cultural characteristics

C. botulinum strains are motile, have peritrichous flagella, Gram-positive rods with subterminal oval spores which cause swelling to cells. Group I strains are straight to slightly curved, about 2-10 µm x 0.5-2 µm. In media with inadequate nutrients, they may increase in length to 20-45 µm (Kriek and Odendaal, 1994). The cells normally occur single and less often in pairs or short chains. They retain the Gram stain very well, often only becoming Gram-negative with the commencement of sporulation. Group II strains are straight rods which measure 1.7-15.7 µm x 0.8-1.6 µm, occurring singly or in pairs. Group III organisms are straight rods, with dimensions between 3.0-22 x 0.5-2.4 µm. Colonies of *C. botulinum* strains are circular with an irregular edge and about 3 mm in diameter, which may increase to 8 mm after an extended period of incubation. Colonies are raised or flat, rough or smooth, and commonly show some spreading. However, the colony morphology of pure cultures of *Clostridium* species may be variable, so that the culture appears mixed. Subculture of single colonies yields the same variable types (Jousimies-Somer *et al*., 2002). The lipolytic enzymes produced by *C. botulinum* strains cause the development of a precipitate in an agar medium containing egg yolk underneath the colony and an irridescent film (pearly layer) covering the colony, both of which are due to the presence of free fatty acids (Kriek and Odendaal, 1994). Some strains of group III also produce lecithinase (zones of precipitation) (Segner *et al*., 1971). In general, colonies of all groups of *C. botulinum* strains are usually surrounded by a narrow margin of complete haemolysis (Smith and Sugiyama, 1988). Anaerobe cultures are characterized by a foul odour due to volatile fatty acids, amine endproducts and hydrogen sulfide production (Jousimies-Somer *et al*., 2002). In general, the phenotypic characteristics vary greatly between Groups I to III and other BoNT-

producing *Clostridium* species (viz. *C. argentinense*, *C. butyricum* and *C. baratii* (Table 2). Group I organisms and *C. argentinense* are proteolytic, while others are non-proteolytic. *C. botulinum* strains produce lipase, while others do not. Different groups of *C. botulinum* and other species vary in their ability to ferment sugars, their optimum and minimum growth temperatures, resistance of spores to heat, and the metabolic acids they produce.

Because no single procedure, no single medium or temperature of incubation is best for all toxin types and cultural varieties of *C. botulinum*, isolation of *C. botulinum* is difficult (Smith and Sugiyama, 1988). Also, *C. botulinum* is highly fastidious, and with the exception of some strains of group III bacteria, it is a strict anaerobe and even traces of oxygen in its environment can inhibit its growth (Kriek and Odendaal, 1994); and the bacteria can lose toxigenicity during isolation process (Smith and Sugiyama, 1988). Deleterious effects of oxygen are also necessary to be avoided during transport and preservation of specimens. Cold temperatures will allow increased oxygen diffusion and reduce number of vegetative forms (CDC, 1998; Jousimies-Somer *et al*., 2002). However, in nature the growth may happen in environment of low oxygen concentration. *C. botulinum* types A and B cultured in association with avian skin flora had similar growth patterns under both aerobic and anaerobic conditions (Dezfulian, 1999). Microbial flora in certain ecological habitats may actually promote the growth of *C. botulinum* through consumption of molecular oxygen and lowering of oxidation-reduction potential (Dezfulian, 1999). Moreover, the isolation of *C. botulinum* in environmental and food samples is frequently complicated by the presence of proteolytic and non-proteolytic non-toxigenic strains that both phenotypically and genotypically resemble *C. botulinum* and exhibit a high relatedness with their toxigenic counterparts (Lindström *et al*., 2001). From the literature, media used to isolate *C. botulinum* include Trypticase Peptone Yeast Extract Glucose, Robertson's Cooked Meat Medium, Brain Heart, Chopped Meat Glucose Starch, Peptone-Yeast-Glucose, Reinforced Clostridial Medium (broth and agar), Egg Yolk Agar, and Blood Agar). Egg-meat medium fortified with 1 % additions of yeast extract, ammonium sulfate and glucose was found the best for growth and sporulation of *C. botulinum* type C (Segner *et al*., 1971). *C. botulinum* type C2 was detected by using trypticase peptone medium enriched with 1 % glucose (Nakamura *et al*., 1978). C2 is produced only during sporulation and not during vegetative

growth. A few selective media have been developed for isolation of *C. botulinum.* A selective medium, *C. botulinum* isolation agar (CBI), containing cycloserine, sulfamethoxazole and trimethoprim as inhibitory agents has been found suitable for isolation of group I *C. botulinum* from human faecal material (Dezfulian *et al*., 1981). CBI was later modified by MILLS *et al*. (1985) to botulinum selective medium, which enabled more rapid isolation of types A and B compared to CBI. Also, a selective and differential medium for isolation of the proteolytic group I strains was developed by SILAS *et al*. (1985). These media improve the isolation of some *C. botulinum* strains (Glasby and Hatheway, 1985), but they did not allow the growth of all *C. botulinum* strains (Whitmer and Johnson, 1988).

The main limiting factors of growth of *C. botulinum* in foods are: 1) temperature, 2) pH, 3) water activity (a_w), 4) redox potential (Eh), food preservatives, and 6) competing microorganisms (CDC, 1998). Low a_w inhibits *C. botulinum* growth. A minimum a_w of 0.94 is needed to support growth and toxin production. The optimum Eh for growth of *C. botulinum* is low (~ -350 mV) but toxin production has been observed at Eh of +250 mV. Because of this range, *C. botulinum* growth and toxin production can occur even in products considered to have a high oxygen level (CDC, 1998). Growth and toxin production occurs in anaerobic condition at pH > 6.0 and not encouraged under acidic conditions such as those found in properly prepared haylage and silage. If the ensiling process is incomplete or circumvented, however, toxin production is possible (Galey, 2001). Usually growth and toxin production is limited or prevented at pH 4.6. However, it was reported to occur below this level (Raatjes and Smelt, 1979). *C. botulinum* grows optimally at a pH range of 7.0-7.6 (Kriek and Odendaal, 1994). However, other pH ranges were reported; e.g. for type A and B, the pH range for growth was found 5.01-8.89 (Sperber, 1982). After being produced, BoNT viability was found not to be affected by pH in the range of 2.7-10.2 (Halouzka and Hubálek, 1992). However, the toxin viability is greatly affected by the change in the temperature. In experimental work, toxin type C was inactivated after two days at 37 °C, two weeks at 28 °C, six months at 5 °C, five years at -20 or -70 °C (Hubálek and Halouzka, 1988). The optimum growth temperature for group I is 37 °C, group II is 28-30 °C and for group III is 40 °C (Smith and Sugiyama, 1988). If all types are being sought and only one incubator temperature can be used, 30 °C for five to seven days should be used (Smith and Sugiyama, 1988). Organic nutrient

requirements for growth and toxin production of groups I and II strains differ (Whitmer and Johnson, 1988). The most essential nutrients for group I were arginine and phenylalanine, which were not required by group II. Group II required carbohydrate energy source. Arginine (0.1 %) is a principal energy source for group I (Smith and Sugiyama, 1988).

The requirements of *C. botulinum* spore germination are much less restrictive than those for growth. Most spores can germinate under conditions in which vegetative growth is impossible (Sperber, 1982). Spore germination was increased by L-alanine and L-lactate or bicarbonate ions (Alberto *et al*., 2003). Germination of spores of proteolytic *C. botulinum* type B was strongly triggered by L-alanine/L-lactate/$NaHCO_3$ (Broussolle *et al*., 2002). PLOWMAN and PECK (2002) found that optimum germination for non-proteolytic strains of type B, E and F occurred in L-alanine/L-lactate. Sodium bicarbonate, sodium thioglycolate and heat shock each enhanced germination, but were not essential. It is recommended by SMITH and SUGIYAMA (1988) to add lysozyme (5µg/ml) to enrichment medium whether heated or not heated to stimulate germination of *C. botulinum* spores. Germination is also promoted by the addition of 0.1 % starch, which binds the long chain fatty acids capable of inhibiting spore germination (Kriek and Odendaal, 1994). Heat treatment can activate dormant spores to germinate. *C. botulinum* type A spores are fully activated for germination by exposing them to a temperature of 80 °C for 10-20 min (Kriek and Odendaal, 1994).

For isolation of anaerobes, the primary plating media should be prereduced or freshly prepared (preferably less than two weeks old), but plates for subsequent subculturing do not need to be reduced (Jousimies-Somer *et al*., 2002). Also, liquid media for primary isolation have to be heated in a boiling water bath for 10 min to remove oxygen and then cool to room temperature (RT) (CDC, 1998). The inoculation should not be more than ten percent by volume of soil samples or 20 % of food (Smith and Sugiyama, 1988). As *C. botulinum* can be inhibited by other bacteria (Graham, 1978; Szabo *et al*., 1994; Sandler *et al*., 1998; Böhnel and Lube, 2000) such as *C. perfringens* and *C. tetani* (Smith, 1975; Smith, 1978), direct inoculation of specimens on agar media and heat and alcohol treatment are important. Sometimes the isolation is possible only if the specimens are treated with heat or alcohol before

incubation (Smith and Sugiyama, 1988). Direct streaking of specimens on agar medium was successfully used to isolate *C. botulinum* from infant botulism faecal samples (Glasby and Hatheway, 1985). Alcohol and heat treatment select for isolation of spore-forming bacteria, whereby all vegetative cells can be destroyed and this will eliminate the non-spore-formers that can compete with *C. botulinum* and adversely affect its growth (CDC, 1998). Alcohol treatment is better than heat treatment for low heat-resistant group II strains of *C. botulinum* (Smith and Sugiyama, 1988). However, spores of non-proteolytic *C. botulinum* heated at 95 °C for 15 min failed to be inactivated (Lund and Peck, 1994). In a study conducted by DAIFAS *et al*. (2003) to see the effect of ethanol on *C. botulinum* growth, they observed that the ethanol can extend the lag phase, decrease the exponential growth rate, and decrease the final level of growth in the stationary phase. The growth and toxin production was delayed by an ethanol concentration of 4 % and completely inhibited by 6 %. *C. botulinum* spores are resistant to heat, requiring temperatures of greater than 120 °C for destruction. Spores of different groups vary in their heat resistance (Table 2). Heating specimens at 80 °C for 10 min may be of advantage for group I strains as others may be destroyed. Low heating at 60 °C for 15-30 min for group I or intermediate heating of 71 °C for 15 min for group III was used (Segner *et al*., 1971). After heat test, if large numbers of other bacteria are present, it may be impossible to isolate *C. botulinum* from the plate and if so, alcohol treatment for spore selection has to be used (CDC, 1998). The disadvantage of heat and alcohol treatment is that non-sporulating strains may not be isolated (Dezfulian *et al*., 1981) and toxigenic strains may become non-toxigenic (Eklund *et al*., 1972). It appears that the highly toxigenic strains of all types tend to form fewer spores than the less toxigenic ones (Kriek and Odendaal, 1994). Enrichment cultures should preferably be incubated for 5-7 days, the period of active growth giving the highest concentration of bacterial toxin and sporulation (Smith and Sugiyama, 1988; Broussolle *et al*., 2002). Incubation for additional 10 days to detect possible delayed germination of injured spores, if no growth, is also indicated.

Anaerobic incubation can be done in anaerobic chamber, anaerobic jars or by automatic systems such as the Anoxomat system. Recovery of anaerobes from clinical specimens has shown slight increase with the Anoxomat system compared to the conventional anaerobic jars and the anaerobic chamber (Jousimies-Somer *et al*.,

2002). Phenylethyl alcohol in primary plates can inhibit swarming clostridia and facilitate isolation of other colonies (Jousimies-Somer *et al*., 2002). Plates for anaerobes should not be exposed to air until after 48 h of incubation, since anaerobes are most sensitive to oxygen during their logarithmic phase of growth. However, if on selective medium, the plates may be examined after 24 h of incubation since the selected organisms grow rapidly (Jousimies-Somer *et al*., 2002). Group I organisms of *C. botulinum* are more easy to be isolated than organisms of the other groups (Hatheway and McCroskey, 1987). *C. botulinum* was isolated from 111 (out of 336) stool samples from infant botulism cases, which were all group I organisms (Hatheway and McCroskey, 1987). Addition of antibiotics to isolation media to isolate *C. botulinum* was frequently used (e.g. Dezfulian, 1981; Silas *et al*., 1985). It is necessary to pick several lipase positive colonies because some isolates may be non-toxic (CDC, 1988). It may be difficult to isolate *C. botulinum* type E from mixed cultures because of bacteriocin produced by non-toxigenic organisms that are similar to *C. botulinum;* this can be overcome by using a medium containing trypsin, which inactivates the bacteriocin such as trypticase-peptone-glucose-yeast extract-trypsine (CDC, 1998). As freezing may kill vegetative cells (Jousimies-Somer *et al*., 2002, CDC, 1998), isolates must be stored in a sporulating form (5-7 days incubation). *C. botulinum* can lose its toxicity during isolation process (Collins and East, 1998), its isolation with stable toxigenicity is difficult (Hauschild and Dodds, 1993), or even pure toxigenic cultures may become non-toxigenic (Eklund *et al*., 1971).

2.2.2 Botulism in animals

Botulism is a non-febrile highly fatal disease of cattle, sheep, goats, horses, mules, donkeys and rarely pigs. Other animals such as dogs, birds, fish, minks, and certain laboratory animals are also susceptible (Smith and Sugiyama, 1988). Botulism was early reported in cattle by Le Vaillant in 1780-1785 (Kriek and Odendaal, 1994).

Cattle are the most affected species. Sheep and goats are less commonly affected and outbreaks were reported in sheep more than in goats (Van Derlugt, 1995). Botulism can occur from exposure to BoNT by one of three possibilities. Most commonly by ingestion of preformed toxin (intoxication) associated with carcasses, decayed organic matter such as poorly ensiled haylage or silage or coprophagy (in case of poultry). Toxico-infectious botulism is another source of exposure in which

the organism grows in the gut and produces the toxin. This form of botulism was reported in foals (skaker foal syndrome; Rooney and Prickett, 1967), in adult horses (Hartigan, 1985), in chickens (Miyazaki and Sakaguchi, 1978), in pheasants (Kriek and Odendaal, 1994), and in cattle (visceral botulism; Böhnel *et al*., 2001). A similar TI occurs in human infants and adults. The TI form of botulism seems to be increasing. In France, the increasing incidence of animal botulism is supposed to be mainly due to the toxico-infectious form (Popoff and Argente, 1996; cited by Böhnel and Lube, 2000). Similarly, bovine botulism in Germany has increased due to the visceral form (Böhnel *et al*., 2001). The visceral form of botulism may occur without evidence of a major paralysis of locomotoric muscles (Böhnel *et al*., 2003). The third form of botulism is wound botulism (Swerczek, 1980), which results from infection of an anaerobic wound leading to toxin production. The wound can be inside the animal such as ulcers or necrotic lesions, which are encountered e.g. in shaker foal syndrome (Swerczek, 1980) or outside the animal as in case of tetanus. Animals are mostly affected by *C. botulinum* types C or D toxins and rarely by type A or B (Schocken-Iturrino *et al*., 1989; Seifert, 1996). Botulism in cattle has been associated with pica (aphosphorosis) (Kriek and Odendaal, 1994). Grazing pastures which have been spread with poultry litter occasionally containing decomposing chicken carcasses were the most common source of bovine botulism (Fach *et al*., 1996). Ensiled poultry litter was also reported to be implicated in outbreaks of bovine botulism (McLoughlin *et al*., 1988). Avian botulism is caused mostly by type C and involves large numbers of wild ducks and geese (Smith and Sugiyama, 1988). C2 toxin has been implicated in some cases of type C botulism in broiler chickens where diarrhoea and enteritis have been observed alongside the neuroparalysis signs of botulism (Ohishi and Gupta, 1987, cited by Hunter and Poxton, 2002).

A substantial quantity of ingested botulinum toxin is either not absorbed or destroyed by digestive processes. Small non-lethal doses of toxin ingested over a period of time will also cause intoxication, as BoNT has a cumulative action (Böhnel *et al*., 2001). Apparently there is a varying susceptibility to various BoNTs in man and different animal species, breeds and individuals (Smith and Sugiyama, 1988). However, the old belief that some types may not be toxic for different animal species or man is invalid (Kriek and Odendaal, 1994; Seifert and Böhnel, 1995). Absorption of toxin is accomplished via endocytosis, mainly in the duodenum (Miyazaki and Sakagushi,

1978), but also in the rumen (stomach), jejunum and ileum. The flaccid paralysis caused by the toxin develops as a result of the inhibition of acetylcholine release at the neuromuscular junctions. A progressively developing flaccid paralysis results; paralysis of muscles of respiration being the eventual cause of death. The paralysis in botulism spares CNS and sensory nerves. In addition, to neurotoxin, several other metabolic by-products contribute to pathogenesis (Seifert and Böhnel, 1995). The non-toxic components in BoNT are thought to be important in the pathogenesis of botulism as they protect the neurotoxin from proteases and acidity in the GI tract; the larger the progenitor complex the higher the oral toxicity (Hunter and Poxton, 2002).

Severity of signs depends on type and quantity of toxin ingested and accordingly the disease has been divided into peracute, acute, subacute and chronic forms. The incubation period in cattle usually varies from 2-6 days, but deaths for up to 17 days may occur (Kriek and Odendaal, 1994). The duration of illness may be as short as 24 h in peracute cases or up to 7 days or longer with chronic cases. Affected animals manifest partial or complete paralysis of muscles. Paralysis usually starts in hind quarter and then spreads to forelimbs, head and neck. The appetite is usually not affected, few animals may be constipated, ruminal tympany may develop, tongue often protrudes from the mouth, profuse salivation, and difficulty in chewing and swallowing occur; however, dysphagia is not a consistent finding. Tucked up abdomen is usually observed in chronic form. Signs due to type B toxin are markedly different from type C and D toxins (Kriek and Odendaal, 1994; Seifert, 1996). Anorexia, regurgitation of feed and water, profuse salivation, no ataxia, tail and tongue are not paralysed, are signs observed with type B.

No specific treatment other than administration of specific or multivalent hyperimmune serum in the early stages of the disease is possible. However, the use of specific toxoids or antitoxoids is complicated by the possibility that more than one type of BoNT may be present in any case of botulism and it is difficult to determine the specific toxin early, if at all. So, polyvalent or bivalent antitoxin is usually used, e.g. C and D which are mixed immediately before injection. As the antisera are expensive and not easily attainable, they are generally used selectively for the treatment of valuable stock. Vaccination against botulism has been effectively used in South Africa (Kriek and Odendaal, 1994). Because the concentration of BoNT

required to induce clinical signs is low, recovered animals do not develop immunity (Coleman, 1998). Good nursing is essential when treating animals suffering from botulism. Rehydration, laxative in constipated animals, support of laterally recumbent animals especially ruminants, are important. Correction of phosphorous deficiency (pica), removal of source of intoxication, not to feed suspected feed or improperly made silage or haylage. No poultry litter should be used or to vaccinate before feeding it at first and thereafter immunize regularly, or sterilize it. Oral antibiotic use in botulism is controversial. Some authors claim that oral antibiotics (e.g. metronidazole, penicillin) do not eliminate *C. botulinum* from the intestinal tract of foals, while others note that these drugs may increase toxin release by killing the vegetative form of *C. botulinum* or may disturb the normal GI tract flora, favouring clostridial overgrowth (Semrad and Peek, 2002). Metronidazole is also reported to predispose laboratory animals and humans to botulism. Aminoglycosides, tetracyclines, procaine penicillin should be avoided as they may potentiate neuromuscular blockade (Semrad and Peek, 2002). So, antibiotics may be given only for secondary infections.

2.2.3 Botulism in horses

Horses are extremely sensitive to botulinum toxin. Amounts of toxin which are sublethal in mice may rapidly cause the death of adult horses if administered i.v. (Kinde *et al*., 1991). Exposure of the horse to botulism may occur from ingesting preformed toxin in contaminated feed (forage poisoning) or rarely may result from enteric (adult horses or very young horses [shaker foal syndrome]) or wound infection with *C. botulinum* and subsequently production of toxins in vivo (Galey, 2001). Clinical paralysis and death may result within hours from exposure to a high toxin level or, as in many cases, may develop and progress over a period of two to three weeks or more.

Forage poisoning was confirmed to be caused by *C. botulinum* by Graham and Brueckner in 1919 (Kriek and Odendaal, 1994). Forage is often the source, especially poorly ensiled silage or haylage and that contaminated with carrion. Forage poisoning most often is caused by consumption of feed contaminated with type B toxin (Galey, 2001). In North America, horses are affected most frequently by type B botulism (> 85% of cases), occasionally by type C, and only rarely by type A

botulism (Galey, 2001). Type B and D have been reported in cases of equine botulism in England (Ricketts and Greet, 1984). Type C2 is also reported together with C1 causing botulism in horses (Kinde *et al*., 1991). In general forage contaminated with carrion tends to have type C or D toxin (Galey, 2001).

Toxico-infection occurs mainly in very young horses (skaker foal syndrome) in the intestine, which is predisposed by ulcer or necrosis, and appears to be potentiated by excessive amounts of corticosteroids contained in the fat of the milk of mares (Swerzek, 1980). TI botulism in foals (similarly in human infants) thought to be due to lack of an established normal gut flora that permits germination of spores and growth of *C. botulinum* in the GI tract (Bartlett, 1986). Shaker foal syndrome usually is attributed to type B toxin. However, a case of type C in a two-month old foal was reported (Semrad and Peek, 2002). It is sporadically occurs in foals between two weeks and eight months of age; mainly in the fast growing period at 2-4 weeks of age (Swerczek, 1980). Signs of shaker foal syndrome are stiff gait, muscular weakness and tremors (shaking prior to recumbency) without ataxia, dysphagia, decreased tail tone may also be noted, dyspnoea with extension of head and neck is seen as the disease progressed, recumbency and death that occurs more often 24-72 h after the onset of clinical signs (Swerczek, 1980). TI botulism in adult horses sporadically occurs (Swerczek, 1980; Hartigan, 1985) and is always associated with an altered GI environment; for example following GI disease, abdominal surgery, or recent antibiotic therapy (Chia *et al*., 1986). On one particular thoroughbred horse farm, 91 cases of TI botulism occurred among foals, yearlings, and adult horses and only two animals survived, which showed typical clinical signs of botulism (Szabo *et al*., 1994). The equine grass sickness disease now is strongly considered as a TI form of botulism (Hunter *et al*., 1999; Böhnel *et al*., 2003).

Wound botulism in horses is mainly reported due to type B (Whitlock and Buckley, 1997). It has been associated with castration, inguinal or injection abscesses, trauma and surgery in adult horses and in foals with omphalophlebitis, umbilical hernias and with infected leg wounds (Whitlock and Buckley, 1997; Semrad and Peek, 2002).

The clinical signs of equine botulism are the same regardless of the pathogenesis; however, subtle differences may be produced by the various toxin types (C versus A

and B). The time of onset of clinical signs of botulism of horses depends on the dose of toxin ingested or absorbed from GI tract or a wound. The first clinical signs are observed from 12 h to ten days following ingestion of the toxin. Generalized muscle weakness, dysphagia or both are first appear, decreased excercise tolerance and colic follow in some cases. Anorexia, labored breathing and increased salivation, mydriasis and ptosis may happen, decreased tail tone is often, decreased tongue tone, stiff stilled gait, but not ataxic, muscle tremor mainly in type C, increased heart and respiratory rate, urinary retention, constipation and ileus are common signs in recumbent horses with agonal signs and some peddling (Galey, 2001). Aspiration pneumonia may be a complication. Death is caused by respiratory paralysis. Recovery may occur in mildly affected horses.

Definitive diagnosis of botulism in the laboratory is elusive. No pathognomonic gross or histological lesions and circulating toxin levels are often low (Galey, 2001). Analytical methods, such as the mouse bioassay, are of insufficient sensitivity relative to the extreme sensitivity of the horse (Galey *et al*., 2000). Horses are suggested to be 1-10,000 times more sensitive than the mouse bioassay (Kinde *et al*., 1991). Source material may have higher and more easily detectable toxin levels, but it is often unavailable for testing. So, diagnosis often depends on clinical signs, lack of post-mortem lesions and elimination of other potential neurological diseases. The major definitive test used to identify botulism toxin uses the mouse protection bioassay. Samples of source material (faeces, gut contents, liver) can be assayed. Recently, sensitive immunoassays such as ELISA test were developed for BoNT detection (e.g. Doellgast *et al*., 1993; Ferreira *et al*., 2003). However, immunoassays are most often less sensitive than the mouse bioassay. Culture of faecal material or intestinal contents and PCR assays may help to identify the bacterium (Szabo *et al*., 1994; Fach *et al*., 1996). However, presence of *C. botulinum* naturally among gut flora makes this detection questionable if no toxin is detected in the sample.

Treatment largely depends on supportive care and injection of antiserum (polyvalent or monovalent antitoxin), if done early before recumbency occurs it may be of benefit (Rocke, 1993). Horses with mild disease may recover without antitoxin therapy. Antibiotic therapy to prevent bacterial growth e.g. in wound botulism or to treat complications of paralysis like aspiration pneumonia may be used. But drugs that

potentiate neuromuscular weakness (aminoglycosides, tetracyclines and procaine penicillin) should be avoided. Mineral oil may be used to combat ileus and constipation. In addition histamine blockers may be used to prevent gastric ulcers. Severely affected and recumbent animals may require ancillary therapy, including mechanical ventilation, bladder catheterization and oral alimentation (Whitlock and Buckley, 1997). Xylazine and diazepam can be used to sedate horses and foals that struggle excessively in recumbency. However, as with many diseases, the best way to deal with botulism is prevention. Good husbandry, check forage for carrion or toxic plants or other foreign matter, not to feed poor-quality hay to horses and prevention of wounds to occur and if be cleaned and treated, are important measures. Vaccination of valuable animals is done especially in endemic areas, however, the vaccines (toxoids) are not easy to obtain and are expensive. In southern Africa botulism is currently well controlled by vaccination, although sporadic outbreaks of the disease still occur (Kriek and Odendaal, 1994). The use of antisera in horses has been shown to reduce mortality rates from 80-30 % (Swerczek, 1980).

2.2.4 Botulism in humans

Humans are very sensitive to botulism. Minute quantities acquired by ingestion, inhalation or by absorption through the eye or a break in the skin can cause profound intoxication and death (CDC, 1998). Lethal dose for man is $1x10^{-9}$ mg/kg (Bonventre, 1979). With a few exceptions of type F botulism (Hatheway, 1993), the majority of human botulism cases worldwide are due to types A, B, and E toxins (CDC, 1998). There have been few reports of human botulism caused by type C (Segner *et al*., 1971). It has been proposed that the human GI tract does not have receptors for BoNT/C thereby preventing translocation into the circulation (Maksymowych and Simpson, 1996). Recently, there have been reports of human botulism caused by other BoNT-producing *Clostridium* species (viz. *C. baratii* BoNT/F; [Hall *et al*., 1985] and *C. butyricum* BoNT/E [McCroskey *et al*., 1986]). Five clinical forms of botulism have been described in humans: 1) classic or food-borne botulism; 2) wound botulism; 3) infant botulism; 4) hidden botulism; 5) inadvertent botulism (Cherington, 1998). The main sources of food-borne botulism are meat, fish, milk products and non-acid vegetables such as beans, peas and beets. Wound botulism was first discovered in 1943 when *C. botulinum* type A was recovered from a patient who died of flaccid paralysis (Hatheway, 1990). Wound botulism is the least common form of

botulism, rarely reported (Whitlock and Buckley, 1997); now it is associated mainly with illicit intravenous drug use (Baymiller, 2001). Only group I strains have been incriminated in this condition in humans (Smith and Sugiyama, 1988). In infant botulism spores of *Clostridium botulinum* are ingested and germinate in the intestinal tract. Infant botulism, first recognized in 1976 (Arnon, 1978), is now the most frequently reported form. Honey or Corn syrup in bottle feedings, dust and other materials in the environment seem to be important sources of spores (Doddsm 1993; CDC, 1998). Most cases of infant botulism were due to type A or B organisms of proteolytic group I, but organisms from other groups were also reported (Dodds, 1993); in addition to incrimination of *C. butyricum* and *C. baratii* (Hall *et al*., 1985; Aureli *et al*., 1986). Hidden botulism, the adult variant of infant botulism, occurs in adult patients who usually have an abnormality of the intestinal tract that allows colonization by *Clostridium botulinum*. Inadvertent botulism is the most recent form to be described. It occurs in patients who have been treated with injections of botulinum toxin for dystonic and other movement disorders.

Independent of the toxin type, the clinical manifestation of all forms of botulism is similar. This typically includes a descending flaccid paralysis with dysphagia, a dry mouth, double vision, difficulty in swallowing, dilated pupils, dizziness and muscle weakness. These are accompanied by the paralysis of the more peripheral parts of the body, and finally by paralysis of respiratory muscles which may lead to death.

The treatment of human botulism includes the administration of a therapeutic trivalent antitoxin (A, B and E) and intensive symptomatic treatment, particularly respiratory support.

2.2.5 Diagnostics of botulism

In view of the severity of botulism and the great hazard it poses to the food industry, the diagnostics of *C. botulinum* and its toxin are insufficient and still poorly developed (Robinson and Nahata, 2003; Gessler and Böhnel, 2003); and probably it is substantially underdiagnosed (CDC, 1998; Böhnel *et al*., 2001). A presumptive diagnosis is made on the basis of the history, clinical signs, negative post-mortem examination and exclusion of similar diseases. A definitive diagnosis depends on 1) the demonstration of botulinum toxin in the feed or source of intoxication, serum, GI

contents or wound of a patient; 2) demonstration of bacterial forms of *C. botulinum* in GI contents, internal organs or feed; or 3) detection of antibody response to *C. botulinum* in recovered patients (Smith and Sugiyama, 1988). Laboratory diagnostics include the conventional culture techniques to isolate the organism, biochemical testing, molecular and immunological typing, and some other methods.

2.2.5.1 Culture and isolation

The culture method is complicated by the fact that no growth media selective for both proteolytic and non-proteolytic *C. botulinum* are available. Moreover, the presence of non-toxigenic strains, closely resembling *C. botulinum*, in foods and environmental samples greatly complicates the isolation of *C. botulinum* (Lee and Riemann, 1970; Broda *et al*., 1998). Several other complications, which make the isolation of *C. botulinum* difficult, are mentioned above (2.2.1.3). The samples are cultivated as such, as well as treated with heat or ethanol in order to eliminate vegetative bacteria but not bacterial spores (Smith and Sugiyama, 1988). Strict anaerobic techniques, including deoxygenation of culture media and anaerobic incubation, are required for the successful cultivation of *C. botulinum*. Detection of the organism from food that does not contain demonstrable toxin is inconclusive because of the abundant spores in the environment and in processed foods and their raw materials (Hyytiä *et al*., 1999). However, isolation of *C. botulinum* from human faeces or gastric specimen also provides good confirmatory evidence, since *C. botulinum* is rarely, if ever encountered in human specimens in the absence of botulism (Dowell *et al*., 1977). Successful isolation of *C. botulinum*, using selective (Dezfulian *et al*., 1981; Silas, 1985; Mills *et al*., 1985) and non-selective (Segner *et al*., 1971; Hatheway and McCroskey, 1987) media has been reported. Group I organisms of *C. botulinum* are more easy to be isolated than organisms of the other groups (Hatheway and McCroskey, 1987).

2.2.5.2 Biochemical identification

Several commercial biochemical test systems were used to identify or confirm identification of *C. botulinum* (e.g. API 20 A, Minitek Anaerobe, RapID ANA II, Anaerobic-Tek, MicroScan, Rapid ID 32 A). Contradictory reports on their ability to identify *Clostridium* spp. have been published (Lindström, 2003). Commercial biochemical tests have been shown to fail in identifying both group I and II organisms of *C. botulinum* (Lindström, 2003). Neither were they capable of distinguishing

between *C. botulinum* groups I and II from their non-toxigenic counterparts. These test systems are therefore not suitable for the identification of *C. botulinum* (Lindström, 2003). Organisms of the same group and the related bacteria can not be distinguished from each other by biochemical properties (Hunter and Poxton, 2002). The biochemical properties of the strains of the same group may vary considerably (Oguma *et al*., 1986). In some texts, type C strains are described as being negative in lecithinase (Oguma *et al*., 1986). However, SEGNER *et al*. (1971) reported that all type C strains examined were positive in lecithinase reaction. As for the fermentation of sugars, widely variable patterns have been reported (Oguma *et al*., 1986). Variations in fermentation patterns have been shown even between strains of the same type (Segner *et al*., 1971).

2.2.5.3 Cellular fatty acid analysis

Different types of *C. botulinum* could be differentiated according to the types of fatty acids they produce, by gas liquid chromatography (GLC) (Reiner and Bayer, 1978; Gutteridge *et al*., 1980; Ghanem *et al*., 1991). However, the organisms of the same group and the related bacteria may not be distinguished from each other (Ghanem *et al*., 1991).

2.2.5.4 Mouse bioassay

The mouse bioassay has been used for the detection of botulinum toxins and identification of toxigenic *C. botulinum* (Smith and Sugiyama, 1988; CDC, 1998). The method was described as the standard method for detecting, identifying, and typing of BoNTs. However, animal testing is increasingly restricted and the method is cumbersome, expensive, time consuming and is not suitable for examination of test samples containing other lethal substances (Dezfulian and Bartlett, 1985). The presence of other bacteria in faeces and necrotic lesions often prevents the isolation of *C. botulinum* and detection of NT in the mouse bioassay (Szabo *et al*., 1994). Detection of toxin in food and animal or human body is a definitive diagnosis (Whitlock and Buckley, 1997) and necessary because the mere presence of the organism is not a significant finding. However, the method is not sensitive enough to detect the very low levels of toxin in the test sample. Serum collected from horses affected with toxico-infectious botulism invariably tests negatively since the concentration of toxin in the serum is generally so low that the toxin can not be detected by conventional methods (Swerczek, 1980). Mice are injected i.p. with the

test toxin mixed with antitoxin and with the toxin alone. Survival of mice protected with botulinum antitoxin and death of unprotected mice constitutes a positive assay. Trypsinization is only rarely necessary for detecting BoNT (CDC, 1998). Trypsin activation of the culture supernatant is generally required when strains from group II *C. botulinum* are concerned (Duff *et al*., 1956). EKLUND and POYSKY (1972) found no significant increase in C1 and D toxin compared to A, B, E and F by addition of trypsin. However, trypsin activation of culture fluids was found necessary for the demonstration of most strains of types B, C, D and E (Smith, 1978) or types B to F often require trypsinization of their culture fluids (Smith and Sugiyama, 1988). Also, lethal activity of some strains of type D was found to be enhanced by trypsin (Moriishi *et al*., 1989). C2 is produced as a protoxin and requires trypsin to be activated (Eklund and Poysky, 1972). It will be necessary to prepare a freshly trypsinized fluid, as the continued action of trypsin may destroy the toxin (Solomon and Lilly, 1998). The test should be performed with two mice per each test (CDC, 1998; Smith and Sugiyama, 1988), but, may be for ethical reasons one mouse per test was also used (Sandler *et al*., 1993). Botulinum intoxication usually kills mice in 6-24 h, but delayed deaths occasionally occurre (CDC, 1998). Signs of botulism in mice begin with ruffling of the fur, followed in sequence by labored abdominal breathing, wasp-like narrowed waist, weakness of limbs, paralysis, and death due to paralysis of respiratory muscles (Smith and Sugiyama, 1988; CDC, 1998). False-positive results due to non-botulinum lethalities, e.g. endotoxins from Gram-negative bacteria, infection, tetanus toxin, chemicals, and trauma can be a considerable nuisance. Non-specific death of mice was found to be reduced by high centrifugation and/or filtration of liquid culture (Solomon and Lilly, 1998), dilution of culture supernatant (Hatheway and McCroskey, 1987), overnight deep freezing of supernatant (Smith and Sugiyama, 1988), use of antibiotics (Sandler *et al*., 1993), addition of BSA, which eliminates non-specific reactions through the interaction of constituent serum immunoglobulin M with endotoxin material from Gram-negative microbiota (Solberg *et al*., 1985) and preinjection of mice with antitetanus in case of soil samples (Smith and Sugiyama, 1988).

High detectability of different types of *C. botulinum* from naturally contaminated samples by the mouse bioassay was reported. For example, 67.5 % (n=77) (Zechmeister *et al*., 2002), 52 % (n=2,200) (Sandler *et al*., 1993) for Type C; 54 %

(n=122) (Böhnel, 1999) for all types. However, due to development of new molecular methods with increased sensitivity, the mouse bioassay is considered less sensitive (Szabo *et al*., 1993; Szabo *et al*., 1994b). The method failed to identify clinical cases of botulism in animals (Thomas, 1991; Trueman *et al*., 1992). Inconclusive results are sometimes obtained (Szabo *et al*., 1994; Böhnel, 1999). Cross-reactions were observed between e.g. type C and D (Jansen, 1971; Fach *et al*., 1996), F and E (Yang and Sugiyama, 1975). Some strains have been found to produce mixtures of 2 types of toxins, A+F, A+B and B+F (Franciosa *et al*., 1994; Cordoba *et al*., 1995), and in this case it is necessary to use polyvalent antitoxins. Intestinal contents and faecal samples are the most suitable for testing by mouse bioassay. Liver, spleen and serum are also used, however, serum of sensitive hosts is rarely found positive (Swerczek, 1980; McLoughlin *et al*., 1988).

2.2.5.5 Molecular detection methods

The molecular detection of *C. botulinum* typically involves the detection of the *BoNT* gene, indicating the presence of the organism in a sample. The molecular approaches include the sensitive and specific polymerase chain reaction and the use of molecular probes (Campbell *et al*., 1993; Franciosa *et al*., 1994). A labelled molecular probe may be further hybridised to a homologous DNA sequence and visualized immunologically. Detection of organisms in environmental samples by PCR has become more common as the need to monitor specific pathogens (Hielm *et al*., 1996) or genetically modified organisms released into the environment (Steffan and Atlas, 1988) arises. Several reports showed that PCR amplification is a reliable alternative to the standard bioassay method for identifying BoNT-producing clostridia (Szabo *et al*., 1994a and b; Fach *et al*., 1996). PCR can be used to detect the bacteria in situ (without enrichment) (Williamson *et al*., 1999), after enrichment (Szabo *et al*., 1994b) or the toxin gene can be detected even in non-vegetative forms (Szabo *et al*., 1993). Detection of toxin gene in spores is important in case of environmental samples. In situ detection is important because culture enrichment of samples can result in competition between microbial populations that may inhibit the growth of the target organism (Sandler *et al*., 1998). Samples suitable for PCR are mainly intestinal contents, faeces and source of affection. Serum is not suitable as the bacteria are normally not found in the circulation. However, detection of *C. botulinum* after enrichment of serum samples by both PCR and bioassay was reported (Szabo *et al*., 1994b). PCR is more rapid than the bioassay and uses no

animals. Most of PCR protocols employ toxin type-specific primers as a single pair in the PCR and not more than one serotype may be detected at a time. Few other protocols employ more than one pair of primers at a time for simultaneous detection of more than one serotype at a time (multiplex PCR. e.g. Lindström *et al*., 2001). The disadvantage of PCR detection directly from a sample is the possible detection of dead cells due to intact DNA after cell lysis. This problem is overcome by combining enrichment procedures with the PCR protocol (Hielm *et al*., 1996). Alternatively, reverse transcription-PCR (RT-PCR) in which gene expression is detected rather than the gene itself, may be employed to distinguish viable and dead bacterial cells (McGrath *et al*., 2000). To increase the sensitivity of PCR assays, nested PCR protocols, which involve several subsequent amplifications, were developed (Kakinuma *et al*., 1997). The reported sensitivities of PCR vary form 10-12.5 fg of DNA (corresponding to 3-5 cells per reaction volume) (Szabo *et al*., 1993; Fach *et al*., 1993) to 0.3 ng of DNA (Craven *et al*., 2002). PCR and PCR-based methods detectability of naturally infected samples varied between authors and groups of *C. botulinum*. For example, for types C and D, 31.2 % (n=160) (Fach *et al*., 1996); for C, 88.9 % (n=18) (Williamson *et al*., 1999); for type B, 94 % (n=66) (Szabo *et al*., 1994b) were reported. PCR is found more sensitive than mouse bioassay by several authors (Fach *et al.*, 1993; Szabo *et al*., 1993; Szabo *et al*., 1994b). However, false-negative results were shown by PCR (Fach *et al*., 1996). This was partly attributed to the presence of direct toxin and absence of *C. botulinum* cells and spores or in non-detectable number plus PCR inhibitors. Nonspecific or unexpected (false-positive) results by PCR were also reported. For example type B gene was detected in two *C. subterminale* cultures (Franciosa *et al*., 1994). Cross-reactivity between some toxin genes was reported. For example, type B toxin gene, in addition to type A, was detected in 43 type A strains, compared to only one by bioassay (Franciosa *et al*., 1994).

Several limiting factors, which can affect the PCR results, were reported. A major limiting factor in the application of PCR for natural samples is the isolation of template DNA of sufficient quality and concentration into a practical volume for PCR analysis (Szabo *et al*., 1994a). PCR inhibition was thought due to high concentration of template DNA and this was found correct after being treated by diluting the extracted DNA ten folds (Fach *et al*., 2002). WILLIAMSON *et al*. (1999) found that

further purification of extracted DNA was critical for successful amplification of BoNT/C1 gene by PCR. Without this purification step, organic materials which co-purified with the DNA during the extraction process inhibited the enzymatic activity of the *Tag* DNA polymerase, preventing amplification. Specific primers for specific amplification of a unique fragment of a BoNT gene are also critical for successful PCR. If primers are not highly specific, non-specific or no products may be produced (Campbell *et al*., 1993).

Other molecular methods used in diagnosis of botulism include the pulsed-field gel electrophoresis (PFGE), which has an excellent discriminatory power and reproducibility (Hielm *et al*., 1998). A PCR-based method, randomly amplified polymorphic DNA assay (RAPD), which is less reproducible but can be quickly performed, was also used (Hyytiä *et al*., 1999). Also, the rRNA gene restriction pattern analysis (ribotyping) has been used to identify *C. botulinum* (Hielm *et al*., 1999).

2.2.5.6 Immunological detection methods

Several immunoassay methods have been reported for the detection of botulinum neurotoxins or their antibodies. However, many of these assays such as immunodiffusion assay (Ferreira *et al*., 1981), passive haemagglutination assay (Evancho *et al*., 1973), and radioimmunoassay (Boroff and Shu-Chen, 1973) have poor sensitivities or specificities, which decrease their diagnostic value. The most widely used immunoassay method is enzyme-linked immunosorbent assay (ELISA). However, many assays developed are less sensitive and specific than bioassay (Szílagyi *et al*., 2000). Many reports have shown that ELISAs developed for detection of botulinum toxins failed to detect the toxin in samples from a sound clinical diagnosis of botulism (Abbitt *et al*., 1984; Thomas, 1991; Trueman *et al*., 1992). To improve the sensitivity and specificity of ELISA, a variety of modifications to the test have been established (Doellgast *et al*., 1993; Roman *et al*., 1994; Szílagyi *et al*., 2000). Modified ELISAs (ELISA-based methods) include ELISA-ELCA (enzyme-linked coagulation assay) (Roman *et al*., 1994), chemiluminescence immunosorbent assay (CLISA) (Ligięza *et al*., 1994), and immunomagnetic separation (magnetic bead-ELISA) (Liu *et al*., 2001; Kourilvo and Steinitz, 2002). The principle advantage of magnetic beads is the separation and concentration of target antigens in complex media and increase of the reaction kinetics due to a potentially greater surface area

compared to solid phase in immunoassays (Bruno *et al*., 1996). Accordingly various ligands are incubated with magnetic beads and then assessed using either specific primary antibodies and a secondary enzyme-conjugate antibody or a specific primary enzyme-conjugate antibody. Some ELISA and ELISA-based methods with sensitivities approximately the same as that of mouse bioassay (Doellgast *et al*., 1993; Szílagyi *et al*., 2000; Zechmeister *et al*., 2002; Ferreira *et al*., 2003) or more sensitive than bioassay (Roman *et al*., 1994) were established and described as a convenient alternative to the mouse bioassay. ELISA methods can be used to assay BoNTs directly in clinical specimens or foods (Potter *et al*., 1993; Rocke *et al*., 1998), after enrichment (Doellgast *et al*., 1993), in toxigenic colonies (Dezfulian, 1993); or to assay the botulinum antibodies in sera (Ricketts and Greet, 1984; Jubb and Ellis, 1993). However, due to the possibility to find antibodies to BoNTs in normal animals in areas where botulism is endemic, false-positive results may be encountered by ELISAs developed for detection of antibodies (Gregory *et al*., 1996). Cross-reactivity between different BoNTs and related clostridia was reported. For example, cross-reactivity was reported between types C and D, and *C. novyi* (Thomas, 1991), and A and B (Dezfulian *et al*., 1984; Franciosa *et al*., 1994).

Recently, the sensitivity of immunoassay methods was greatly extended by development of immuno-PCR methods (Sano *et al*., 1992; Wu *et al*., 2001). It is an antigen detection system, in which a DNA specific molecule is used as the marker. Immuno-PCR allows the detection of protein amounts as low as a few hundred molecules. Using a microtitre plate technique for the detection of PCR products, immuno-PCR has been found most suitable for detection of *C. botulinum* in large number of samples (Fach *et al*., 2002). Immuno-PCR sensitivity was found approx. 10^5 more sensitive than ELISA by SANO *et al*. (1992), which was described as the most sensitive method; and 1000-fold more sensitive than the ELISA by WU *et al*. (2001).

3 OWN INVESTIGATIONS

3.1 Aims of the study

The general objective of the present study was to investigate field samples, especially from equine grass sickness cases (a disease suspected to be caused by *C. botulinum*), for isolation and identification of suspected *C. botulinum* organisms.
The specific aims were as follows:

1. Usage of enriched media and culture techniques to isolate organisms that are phenotypically similar (in colony and cell morphology) to *C. botulinum* from field samples obtained from: i) EGS cases (animal and environmental) samples together with control animal samples; and ii) classical botulism-suspected cases, suspected feed and soil, to compare results of isolation.
2. Identification of *C. botulinum*-like isolates by mouse bioassay, polymerase chain reaction (PCR) and magnetic bead-enzyme-linked immunosorbent assay (MB-ELISA).
3. Comparison of results of mouse bioassay with those from PCR and MB-ELISA.
4. Comparison of results of EGS cases samples with those of control animals to see if *C. botulinum* could be linked to EGS.

3.2 Materials and methods

3.2.1 Culture methods and isolation

3.2.1.1 Test samples

The samples investigated in the study are:

- Equine grass sickness samples
    - British samples (the main samples in the study)
    - Non-British samples (from Germany, Austria, Dubai)
- Botulism samples (bovine, equine, human, chicken and soil samples)

The total number of samples investigated was 288; sample distribution according to source is shown in Fig. 2. The GS samples were collected and sent from Britain, where the disease is more prevailing. The samples were kindly provided by Prof. B.

McGorum and Dr. R. Pirie (University of Edinburgh). After receival, the samples were kept frozen at -80 °C until being processed. The GS samples were collected from 7 acute GS cases, both from animals and their environment; together with control samples from four healthy horses.

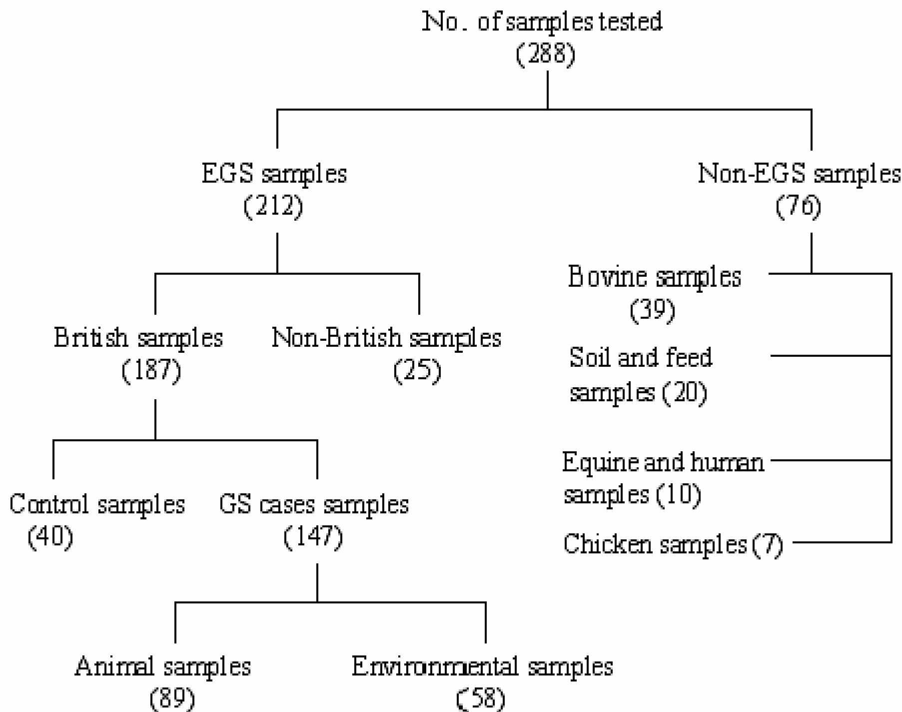


Fig. 2: Diagram to show number and distribution of test samples according to source

The GS samples from animal origin included tissues and contents of stomach, duodenum, jejunum, ileum, caecum, small and large colon, rectum, liver, spleen, tonsil, pharynx and faeces. The samples from the animal environment are different sorts of mud and water, different types of grass and plants, hay, soil, worm casts, a rabbit carcass, and faeces of a bird, pheasant and rabbit. Besides the EGS samples from Britain, samples from five GS suspected cases from Germany (faeces and intestinal contents), a GS confirmed case from Austria (contents of jejunum, ileum, small and large colon) and samples from a horse fed suspected soil in Dubai (liver, spleen, and contents of stomach, jejunum, ileum, caecum and small colon) were also investigated. The samples from Dubai were kindly provided by Dr. U. Wernery, CVRL. Fig. 3 shows the distribution of EGS samples according to sample type. Botulism (non-EGS) samples (Fig. 4) are mainly bovine (intestinal contents, faeces,

and few tissue samples) and soil samples. In addition to few samples from horses (faeces), animal feed, chickens (intestine and liver) and man (stool and tissues), botulism samples were taken from the samples routinely sent to the reference laboratory for diagnosis of botulism in the institute (Institute of Tropical Animal Health, Georg-August-University, Göttingen). All the test samples in this study were tested for the presence of BoNTs by the standard mouse bioassay method by direct or after enrichment testing during the routine diagnostic work of the institute. According to the records of the institute, 81 samples (44 GS and 37 botulism) had evidence for BoNTs (Table 3).

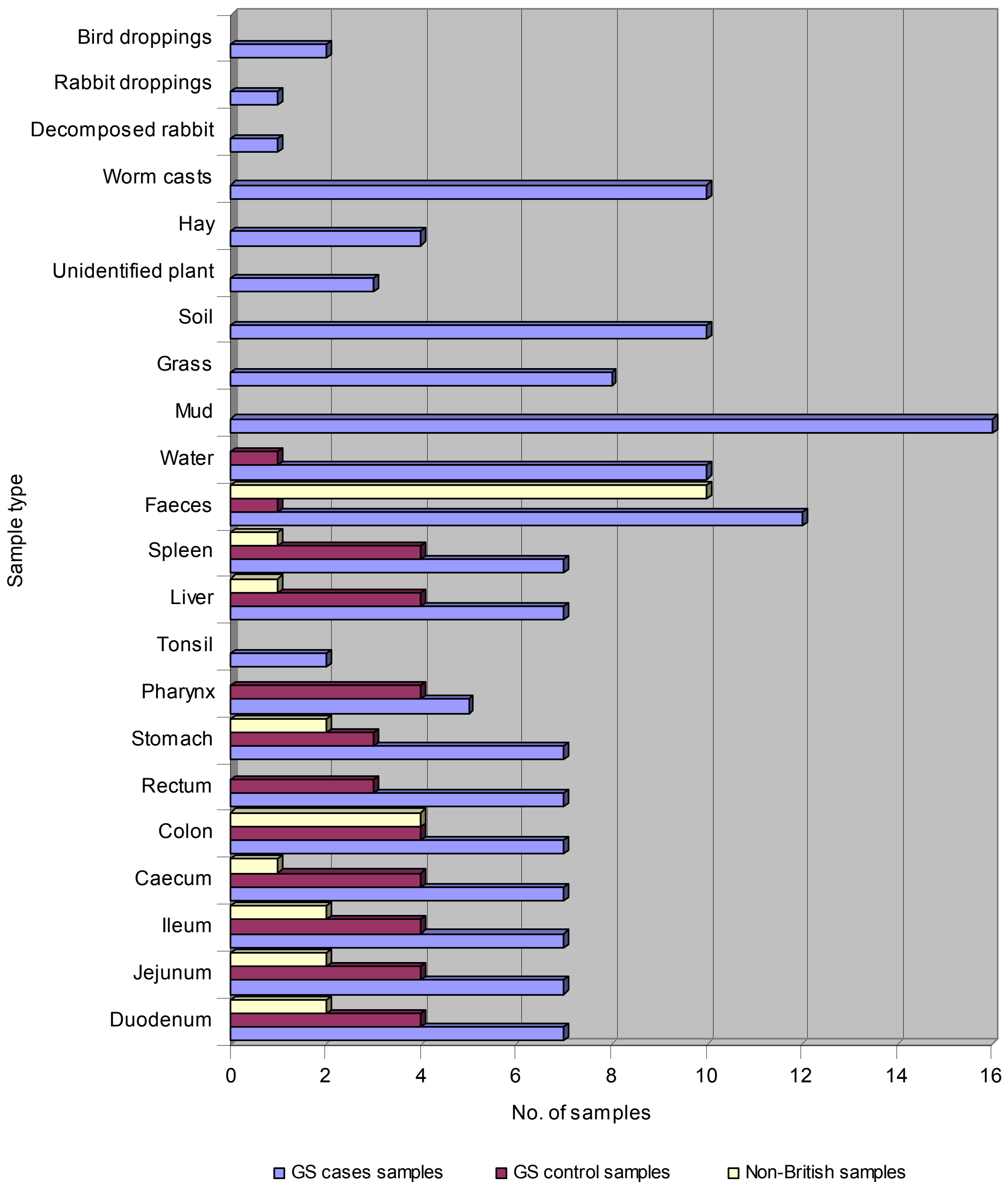


Fig. 3: Distribution of EGS samples according to sample type

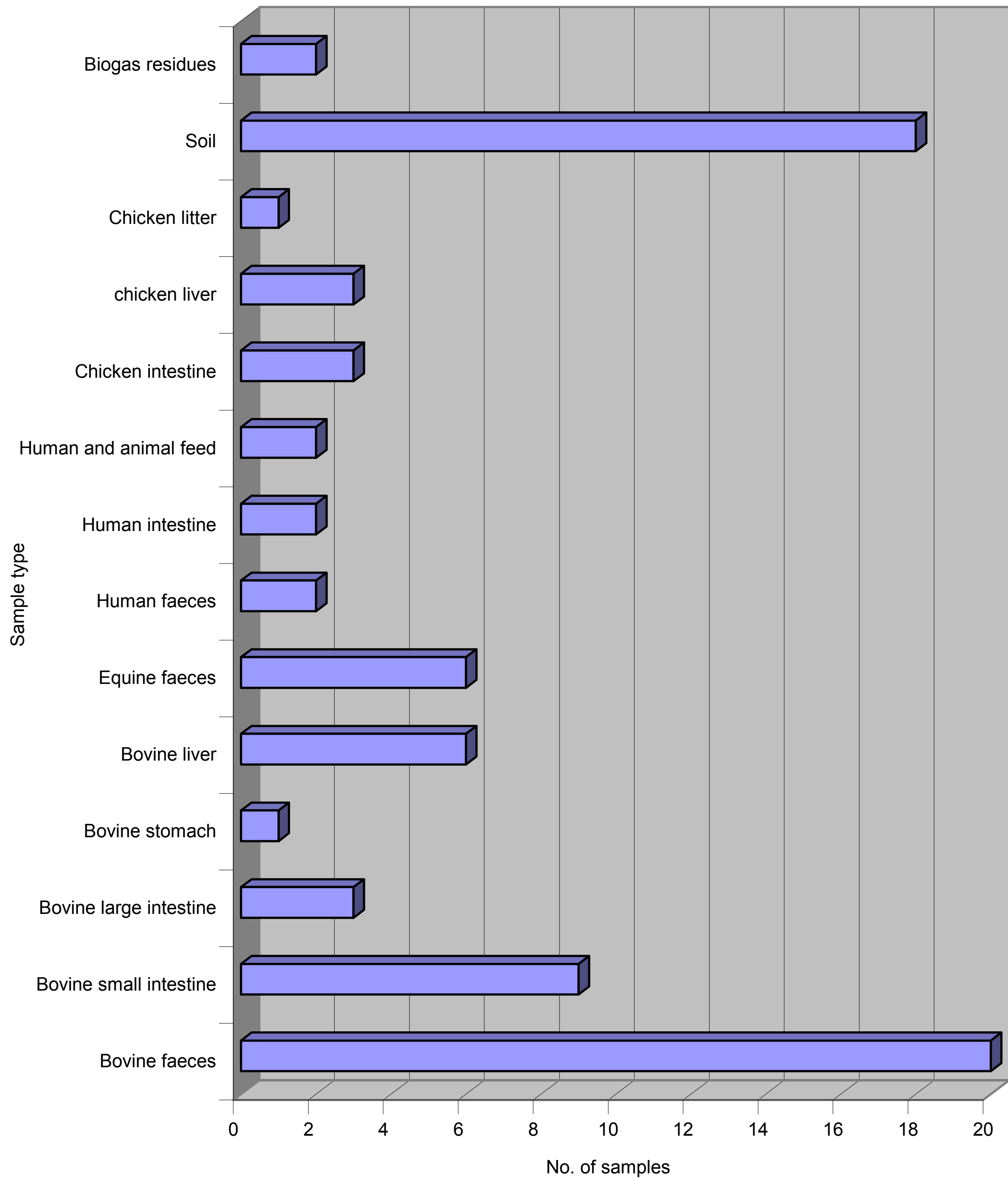


Fig. 4: Distribution of non-EGS samples according to sample type

Table 3: Mouse bioassay results of test samples*

Toxin neutra-lization	EGS samples							Non-EGS samples				Total
	British			Others			Total	Direct	After enrichment		Total	
	Direct	After enrichment		Direct	After enrichment							
		Heated (60 °C/30 min)	Non-heated		Heated (60 °C/30 min)	Non-heated			Heated (60 °C/30 min)	Non-heated		
ABE	3	1	5	3	2	0	14	7	0	3	10	24
CD	3	5	7	1	0	0	16	16	2	4	22	38
ABE/ CD	3	4	5	1	1	0	14	2	2	1	5	19
Total	9	10	17	5	3	0	44	25	4	8	37	81

* These data were obtained from the records of the routine diagnostic work of the institute.

3.2.1.2 Culture media

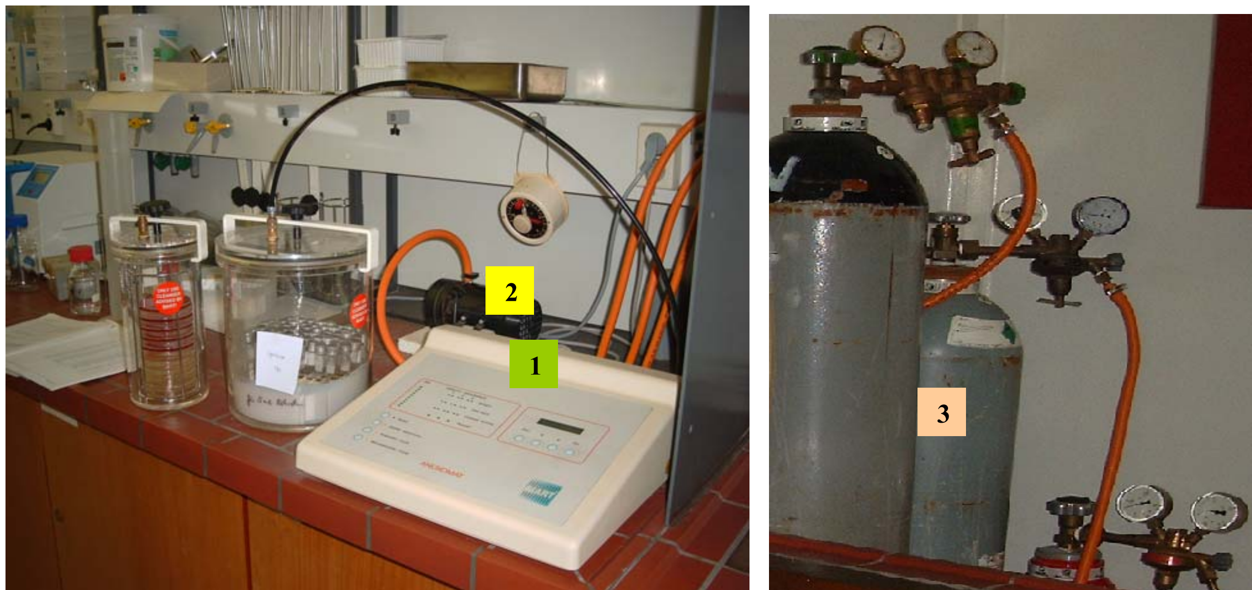
Ingredients of all media were obtained from Merck (MERCK, Darmstadt) unless otherwise stated. Ingredients (except haemin, vitamin K1, blood and egg-yolk emulsion), for all media, were added to 1l demineralised water and dissolved in a boiling water bath. Haemin and vitamin K1 were added after dissolution and before autoclaving. Blood and egg yolk were added under aseptic conditions to sterile agar media. The pH of all media was adjusted to 7.0 ± 0.2 and all media were autoclaved at 121 °C for 15 min. Working solutions of resazurin, haemin and vitamin K1 were prepared according to HOLDEMAN *et al*. (1977) with some modifications (section 7.2). Defibrinated horse blood was obtained from OXOID, Wesel; egg-yolk emulsion from BECTON DICKINSON, Sparks; haemin, L-arginine, trizma base and sodium bicarbonate from SIGMA-ALDRICH CHEMICALS, Deisenhofen. Composition of different media is shown in section 7.1.

3.2.1.3 Culture and isolation procedures

At the beginning, to choose one medium to work with as most likely the best one for growth of *C. botulinum*, different media were used as described below. Twenty-three samples (20 samples from botulism suspected cases, mainly bovine tissues and faecal samples together with few faecal samples from horses, and three soil samples) were tested. After culture of samples, isolation of suspected colonies according to culture procedures shown below and testing of isolates for toxicity in mice, a single broth and agar medium was selected to work with for the rest of samples. Several broth media (CM, EM, CMGS, BH, RCM, FAB and fFAB) and two agar media (FAA and BA) were used. The selection of best broth and agar media was done according to toxicity of suspected colonies isolated and best growth. Best growth and more toxic isolates were shown by fFAB and FAA, which were then used for culture and isolation. Also, fFAB and FAA were found better than BA and EYA (DIFCO, Detroit) for growth of reference strains of *C. botulinum* types A-F. Because strains of group I and III of *C. botulinum* are more important in this study and they can optimally grow at 37 °C and also because the number of test samples is large so that it is difficult to work with more than one temperature, a single temperature for growth (37 °C) was used. The anaerobic conditions were fixed automatically (90 % N_2, 5 % H_2, 5 % CO_2; ANOXOMAT® SYSTEM, MART, Lichtenvoorde, Fig. 5). Liquid medium for all the work was used as 5-ml volume in screw-cap tubes.

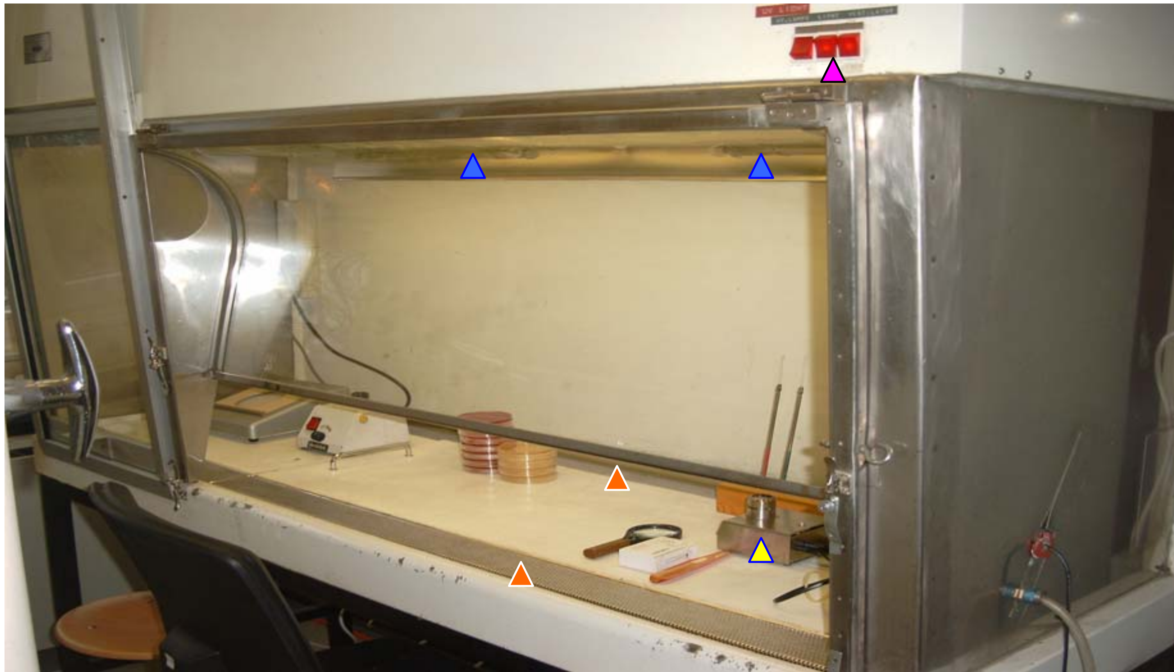
Culture and isolation procedure was conducted in an aerobic chamber (STOLTENBERG-LERCHE, Düsseldorf, Fig. 6), which is with facilities to keep the inside environment sterile. About 0.5-1 g of each specimen was inoculated into two tubes of 5 ml medium, one heat-treated (sometimes heat-treatment was done after enrichment) and the other not and incubated anaerobically for three days (rarely five days). Heat test was conducted mainly at 60 °C for 30 min and rarely 80 °C for 10 min or 70 °C for 15 min. Heat test was performed for about half the number of the test samples. Direct streaking of plates was done for small portion of the test samples and the plates were incubated for two days. After inoculation, smears for Gram stain (modified Gram stain, Hucker, 1979, section 7.3) were made from specimens (if liquid or moist). After incubation, liquid cultures were streaked either only on FAA/E (FAA with egg yolk) or sometimes on both FAA/E and FAA/B (FAA with blood) and incubated anaerobically for two days. To obtain well-separated colonies, only one loopful was streaked on dried plates and in a four-quadrant fashion. Alcohol test was performed for about one third of test samples and mostly after broth enrichment. One ml of ethanol (99 %) was added to one ml of enrichment culture of the specimen in sterile tube, mixed and left for one hour at RT. Then centrifuged and the pelleted material was washed with sterile PBS and centrifuged. Plates were streaked by the pelletted material and incubated for three days. Each enrichment broth culture was smeared for Gram stain. After incubation, the streaked plates, either directly or after liquid enrichment, were observed for various colonial morphologies, and smears for Gram stain were prepared mainly from colonies with characteristics consistent with *C. botulinum* (pearly layer, zones of precipitation, raised or flat, smooth or rough). Plates were examined with a hand lens and sometimes with a photomicroscope. After examination of Gram-stained smears, the suspected colonies were subcultured on agar plates (mainly FAA/B) for purification. Purification mostly needed more than one subculturing process. Gram stain and subsequent subculturing was mostly done from the same single colony. The primary plates were often reincubated along with the subcultures for additional 48 h and inspected again for new morphotypes. Gram-stained smears prepared from specimens, enrichment broth culture or colonies were observed for cell morphology, Gram reaction, and spores (shape and location). Also, wet smears to test for motility of isolates were examined. After plates being apparently of pure colonies, a well-

isolated one or more than one colony was put into 2 tubes (mostly FAB fortified with BH granules) and incubated anaerobically for two days or less often for five days. After the incubation, one tube was used for toxicity testing and toxin neutralization in mice and the second was stored at -20 °C. Isolates were also tested for their ability to grow in oxygen or contamination in aerobic conditions at 37 °C. Photos from important colonies and their smears were taken (ZEISS microscopes).



1, Anoxomat, it automatically controls evacuation and filling of jars with gases, it checks for catalyst validity and leakage or tightness in jars. 2 , Pump for evacuation and filling of gases. 3 Cylinders for N_2, CO_2 and H_2.

Fig. 5: Anoxomat® system (MART)



▲, Keys for ventilation, light and U. V. lamps; ▲, mesh for sucking of air comming from outside; ▲, two U. V. lamps inside at the back for overnight use; ▲, device for gas and electric enlighting (flame).

Fig. 6: Aerobic chamber of STOLTENBERG-LERCHE

3.2.2 Mouse bioassay

The isolates tested for lethality for mice and afterwards toxin neutralization were the lipase- and lipase-/lecithinase-positive rod-shaped isolates, beside some lecithinase-positive and lipase-/lecithinase-negative rods. Apparently pure isolates were subcultured into liquid medium (mainly fFAB) and incubated anaerobically at 37 °C for two to five days. After incubation, cultures were centrifuged at 4000 *g* for 20-30 min. To test for toxicity, white mice of the institute‘s breeding station weighing 18-25 g were injected i.p. with 0.5 ml of each isolate culture supernatant (Smith and Sugiyama, 1988; CDC, 1998). Convulsions and death of mice due to non-specific reactions from the medium constituents was oftenly observed during the first five-ten minutes after i.p. injection, when a medium containing meat particles was used, especially if meat proteolysis has taken place. This has been encountered by using FAB fortified with BH granules for toxicity test and the medium containing meat particles was used for primary isolation only. Then no such reaction was observed during the first hour. Mice were observed for four days for signs of botulism (ruffled fur, wasp waist, laboured respiration, paralysis) or death. Trypsinization was performed only if the isolate was lipase-positive rods and found negative without

trypsin. Trypsin solution (20 mg trypsin [SIGMA-ALDRICH CHEMICALS] dissolved 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 45 min.

Toxin neutralization tests were carried out for lethal isolates. Polyvalent antitoxins type ABE (AVENTIS BEHRING, Marburg), and monovalent antitoxins types C and D (ONDERSTEPOORT VETERINARY INSTITUTE (OVI), Onderstepoort) were mainly used. Also in some instances, monovalent antitoxin types A-E (ID-DLO, Lelystad) and types B and E (IBT) were used. Types F and G were not tested. Antitoxins were rehydrated and used according to manufacturer's instructions. For ethical reasons, number of animal tests had to be kept low; so, initially polyvalent ABE or mixture of types C and D antitoxins were used, and then splitting into monovalent antitoxins was done when necessary. The antitoxin was mixed with 0.5 ml of supernatant and incubated at 37 °C for 30 min. The toxin-antitoxin mixtures and only toxin were injected into mice accordingly (Smith and Sugiyama, 1988; CDC, 1998). Mice were watched for four days. If mice injected with the toxin alone died and those injected with the toxin-antitoxin mixture not, the test was considered positive for that antitoxin. If all mice died, the test was repeated by using diluted toxins. Toxin dilution was made in gelatine phosphate buffer (GPB, pH 6.2).

3.2.3 Polymerase chain reaction

To test for *C. botulinum* types A, B, E and F toxin genes, a multiplex PCR assay, which was developed by LINDSTRÖM *et al*. (2001), was conducted. For *C. botulinum* type C toxin gene detection, a PCR method developed by Institute of Applied Biotechnology (IBT), University of Göttingen (unpublished data) was used. While for detection of *C. botulinum* type D toxin gene, a method established by TAKESHI *et al*. (1996) was adopted.

3.2.2.1 Primers

One set of oligonucleotide primers specific for each of type A, B, E and F (Lindström *et al*., 2001), type D (Takeshi *et al*., 1996), and type C (selected by IBT, unpublished data) were used. The sequence of the primer pair of type C (CP3-01 and CP3-02) used is: 5´-CTG AAA AAG CCT TTC GCA TT-3´ and 5´-TTG TGC CGC AAA AGT ATT GT-3´. Additional two sets of primers specific for type C neurotoxin gene, CS-11

and CS-22 described by TAKESHI *et al*. (1996) and BCS-1 and BCS-2 (TAKARA BIO, Otsu) were also used for some isolates.

3.2.2.2 Isolates and culture

The isolates tested by PCR were only those which showed positive toxin neutralization test by mouse bioassay (Table 6). The isolates for the PCR were cultured in either fFAB, CM, RCM or sometimes in two of them and incubated anaerobically for 48 h at 37 °C.

3.2.2.3 DNA isolation

DNA of all isolates was extracted according to a method developed by IBT, unpublished data. One millilitre of culture broth was aseptically pipetted into a sterile 1.5 ml eppendorf tube. The tubes were then centrifuged at 5,000 x *g* for three min at 4 °C. The supernatant was discarded and 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to the pelleted material and vortexed. Then the tubes were boiled for ten minutes to release the bacterial DNA and then centrifuged as before. A volume of 3 µl of each supernatant was used as template in the PCR mixture.

3.2.2.4 PCR for types A, B, E and F

Master mix. A master mix of reagents of 50 µl was used, which contained 5 µl of buffer II (INVITROGEN, Germany), 1 µl of each primer (0.3 µM) (SIGMA), 1 µl of DNA polymerase (DYNAZYME^{TM}), 3.3 µl of $MgCl_2$ (0.33 mM) (INVITROGEN), 3 µl of template DNA, and sterile deionised water to 50 µl. Buffer II (1.25 ml) contains: 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 15 mM $MgCl_2$, 2 mM of each dNTP, thermostable AccuPrime^{TM} protein, and 10% glycerol.

PCR amplification. A programmable thermal cycler (TGRADIENT, Biometra, Göttingen) was used for the PCR. Reaction mixtures were subjected to initial denaturation at 94 °C for 10 min and 27 cycles of denaturation (95 °C for 30 s), annealing (60 °C for 25 s), extension (72 °C for 85 s), and final extension at 72 °C for 3 min. DNA mixture of reference strains of *C. botulinum* types A, B, E and F as positive control and a negative control containing all of the reagents except template DNA were included in each PCR amplification.

3.2.2.5 PCR for types C and D

Master mix. Fifty-µl master mix of reagents was used, which contained 5 µl of buffer II (INVITROGEN), 1 µl of each primer (for either type C or D), 1 µl of DNA polymerase (DYNAZYME™), 3 µl template, and sterile deionised water to 50 µl. Also, the following master mix was used for part of the isolates: 25 µl of Bioron master mix (BIORON, Ludwigshafen), 1 µl of each primer, 6.6 µl $MgCl_2$ (0.66 mM) (INVITROGEN), 3 µl of template, and sterile deionised water to 50 µl. Bioron master mix (1.25 ml) contains: T*aq* DNA polymerase (0.1 unit/µl), antibodies to *Taq* DNA polymerase, 32 mM $(NH_4)_2SO_4$, 130 mM Tris-HCl, 0.02% Tween-20, 3 mM $MgCl_2$, dNTPs (0.4 mM of each of dATP, dCTP, dGTP, dTTP).

PCR amplification. Using the aforementioned thermal cycler, the reaction mixtures were subjected to initial denaturation at 94 °C for 10 min and 30 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 1 min), extension (72 °C for 1 min), and after the 30th cycle the extension was continued for a further 10 min at 72 °C to ensure that the final extension step was complete. DNA of reference strain of *C. botulinum* type C or D as positive control and a negative control containing all of the reagents except template DNA were included in each PCR amplification.

3.2.2.6 Gel electrophoresis

Two percent agarose (MERCK) was used to visualize the PCR products. Agarose was boiled to dissolve in 1 x TBE buffer (89 mM Tris-base, 2 mM EDTA, and 89 mM boric acid per litre, pH 8.0). DNA fragments were made visible by adding ethidium bromide to a final concentration of 0.55 µg/ml of the molten agarose.

Eighteen-microlitre portions of the resulting amplification reaction mixture were electrophoresed (BIO-RAD, USA). Standard DNA fragments (DNA molecular weight marker VI, FINNZYMES, Espoo) were used as molecular weight markers. Seven microlitres of the standard DNA were put into each raw in the gel. Positive controls were included in each raw and a negative control in one well per gel. Electrophoresis was carried out for 30 min at 75 V or 120 V for 1 h. The amplification products were visualized and photographed with a U.V. transilluminator (MWG-BIOTECH, Germany).

To avoid contamination, sample preparation, PCR amplification, and electrophoresis were done in three different rooms.

3.2.4 Magnetic bead-ELISA

Only 28 isolates were assayed by MB-ELISA for types C and D neurotoxins (the isolates which were neutralized by botulinum antitoxins type CD mixture with mouse bioassay). The isolates were grown in fFAB anaerobically at 37 °C for two or five days. Cultures were centrifuged at 4000 x *g* for 30 min and supernatants were assayed by an immunomagnetic assay based on enzyme-linked immunosorbent assay (MB-ELISA), developed by IBT, unpublished data. Monoclonal mouse-antibody and polyclonal goat-antibody (biotinylated) against type C and D BoNTs were used as capture and detecting antibodies, respectively and to form toxin-antibody complex; magnetic beads coated with sheep anti-mouse IgG, as secondary capture antibody, were added and presence of specific reactants was indicated by enzyme-substrate system (streptavidin-HRP-TMB).

Procedure. Standard toxins, to serve as positive controls, were included in each test. The standard toxins were used as undiluted, 1:10, 1:100, and 1:1000 dilutions in GPB (pH 6.2). The test toxins were added as 1:10 in GPB and undiluted. The test and standard toxins were pipetted as 1.4 ml each into 4-ml-tubes (NEOLAB, Heidelberg). Then a biotinylated polyclonal goat-antibody (0.1µg/ml) and monoclonal mouse-antibody (0.3 µg/ml), specific for types C and D neurotoxins, were added and reactants were shaken by a rotatory shaker (VORTEX) at low speed overnight at 37 °C. After incubation, 1.4 ml casein buffer (1.375 %, STREOSPECIFIC DETECTION TECHNOLOGIES (SDT), Germany) were pipetted into each tube and then 10 µl of dynabeads coated with sheep anti-mouse IgG (Dynabeads M-280, DYNAL BIOTECH, Oslo) were added. The tubes were shaken with a rotatory shaker with minimal speed for 1 h at RT. After incubation, the tubes were put into a magnetic particle concentrator (MPC-M, DYNAL) to separate the magnetic beads and the immune complexes to the tube side next to the magnet, and after 1 min the liquid part was aspirated from each tube. Then the tubes were released from the MPC-M and 1 ml casein buffer to each tube is added to wash excess or nonadsorbed reactants. Again the beads separated by the magnet and the liquid part discarded. The tubes were again released from the MPC and the magnetic beads were suspended into 200 µl casein buffer and the suspension was transferred to a top-yield-module (NUNC) blocked with 1 % BSA, which was put into a magnetic particle concentrator (MPC-96,

DYNAL). After 1 min, the liquid part was discarded, the module released from the MPC-96 and washed with 200 µl casein buffer. Washing was repeated four times, each time the beads were separated by the magnet and the liquid portion discarded. Streptavidin-HRP-80 (SDT), 1:2 in casein concentrate (5 %, SDT) was diluted to 1:10,000 in PBS (pH 7.3) and 200 µl were pipetted into each well. The module was sealed with a sealing tape (NUNC) and put into a horizontal shaker (HEIDOLPH INSTRUMENTS, Schwabach) for 1 h at RT. Afterwards; the plate was washed three times with 200 µl casein buffer as before. The substrate solution was prepared by dissolving one tablet of TMB (3,3´-5,5´-tetramethylbenzidine [SIGMA-ALDRICH CHEMICALS]) in 1 ml DMSO (dimethylsulfoxide [SIGMA] and then 9 ml phosphate citrate buffer (pH 5.0) and 2 µl H_2O_2 (30%) were added. In parallel, the magnetic beads in the top-yield module were washed with 200 µl phosphate citrate buffer as before. After washing, 200 µl of substrate solution were added to each well and the module was put onto a horizontal shaker for 10 min at RT. After incubation, the module was put into MPC-96 and then 150 µl of supernatant were pipetted from each well into an immuno-polysorp plate (NUNC, Wiesbaden). Then a first reading of the absorbance, including 150 µl of substrate as blank, was read at 655 nm by Digiscan plate reader (ASYS HITECH, Austria). A second reading after stopping the reaction by addition of 50 µl of H_2SO_4 (25 %), was made at 450 nm. The absorbance reading of a well containing all the reagents except the toxin was considered as the cut-off value. A schematic drawing for the steps of this procedure is shown in Fig. 7.

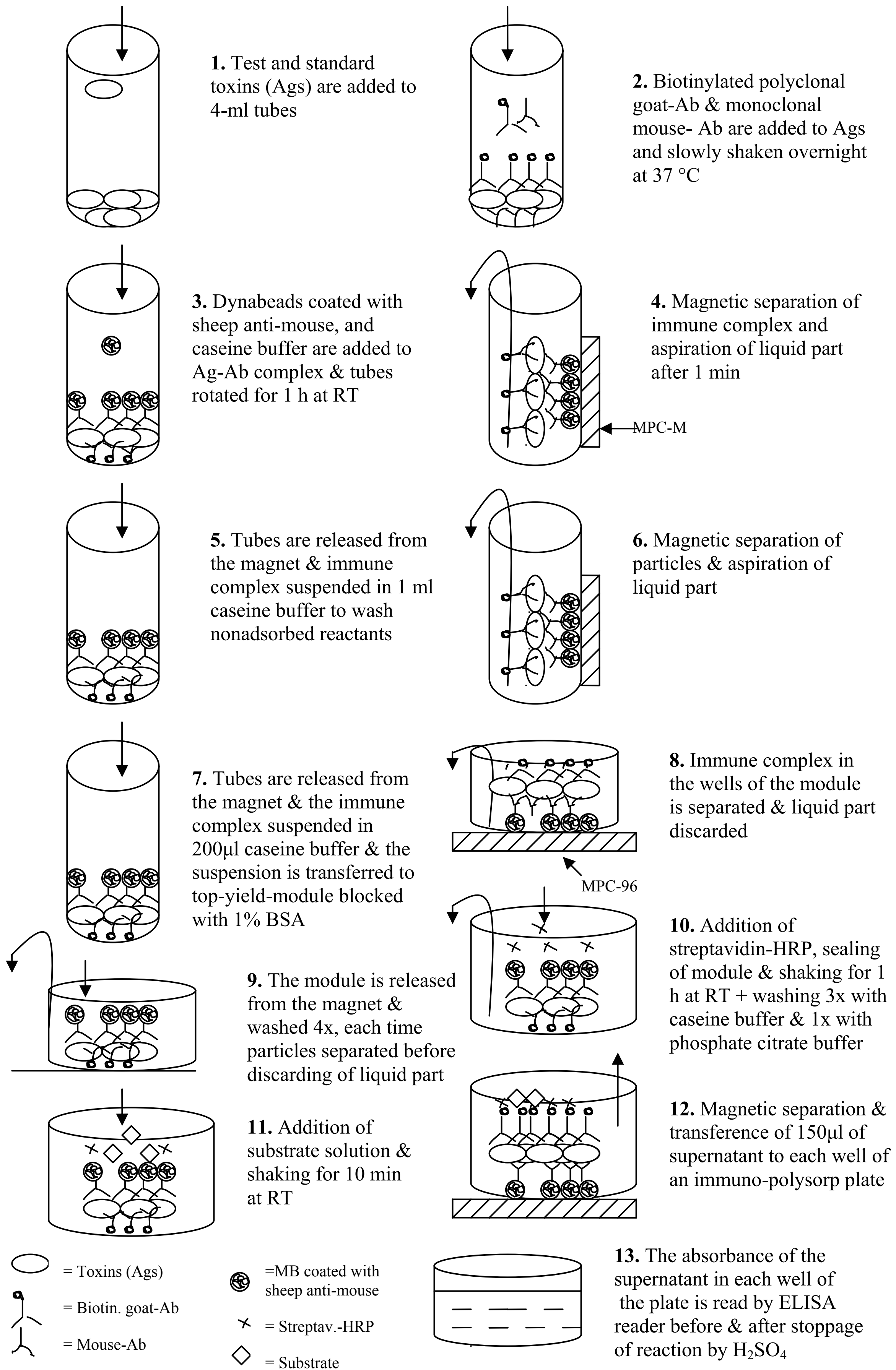


Fig. 7: Schematic drawing showing steps of MB-ELISA procedure

3.3 Results

3.3.1 Culture methods and isolation

According to colony and cellular morphology, toxin neutralization, and molecular and immunological typing, the confirmed *C. botulinum* isolates in the current study are only four. Two of them are type C and the other two are type D. The two type C isolates were from intestinal contents of two broiler chickens from a farm with botulism outbreak; one of type D isolates was from intestine of a case of bovine botulism and the other from faeces of a rabbit experimentally infected with *C. botulinum* type D together with other bacteria. The colonial and cellular morphology of the three field isolates is shown in Table 4. The two type C isolates are similar in cellular and differ in colonial morphology. Both are short to long slender Gram-positive rods with subterminal pulging spores, lipase-positive, β-haemolytic (human, but not horse blood) and both can swarm if the plates are not dry enough. The difference is that the colony of one of them is raised pyramidal with rhizoidal edge and the other is flat or slightly raised with rhizoidal edge (Fig. 15a and b). The colony of the bovine isolate type D is raised with uneven edge and cells are short to long slender rods with subterminal pulging spores (Fig. 15c). None of the four isolates was obtained after enrichment in liquid broth. Two of them were isolated after direct heat treatment, one after direct alcohol treatment and one by direct streaking.

A large number of lipase-, lecithinase-, lipase-/lecithinase-positive or lipase-/lecithinase-negative clostridia, and *E. coli*-like organisms were isolated. All the isolates were tested for toxicity and toxin neutralization by mouse bioassay and some of them by PCR and immunoassay (see below). Different clostridia-like colonies, as indicated by different colonial morphotypes and cellular morphology, were isolated.

According to colony and cellular morphology of clostridia in the literature, the following clostridial species were most likely isolated: *C. sporogenes*, *C. perfringens*, *C. tetani*, *C. novyi*, *C. bifermentans*, *C. septicum*, *C. tertium*, *C. ramosum* and *C. botulinum*. Inspite of many *C. botulinum*-like isolates, only four were confirmed to be *C. botulinum*. Colonies and smears of some lipase and lecithinase positive *Clostridium* spp. are shown in Figs. 16a-b and 17a-b. It was observed that the types and numbers of clostridia are more in GS cases samples than in control samples.

Table 4: Colonial and cellular morphology of confirmed field isolates of *C. botulinum*

Isolate	Source	Colonial characteristics					Cellular characteristics				
		Growth	Edge	Surface	Lipase	Haemolysis	Gram reaction	Width	Arrangement	Spore	Motility
C. botulinum type C	Chicken intestine	Localized (on dry plate), irregular raised pyramidal; swarming on less dried plate	Rhizoidal	Rough	+	β-haemolysis (human but not horse blood)	+	Slender	Occuring singly, short - long rods	Subterminal, oval, pulging	+
C. botulinum type C	Chicken intestine	Localized (on dry plate), irregular flat or slightly raised; swarming on less dried plate	Rhizoidal	Rough	+	β -haemolysis (human but not horse blood)	+	Slender	Occuring singly, short - long rods	Subterminal, oval, pulging	+
C. botulinum type D	Bovine liver	Localized (on dry plate), irregular raised, swarming on less dried plate	Uneven	Rough	+	β -haemolysis (human but not horse blood)	+	Slender	Occuring singly, short to long rods	Subterminal, oval, pulging	+

Why were lecithinase-positive and *E. coli*-like colonies isolated in this study? That because early in this study, an isolate of Gram-positive slender rods was picked from blood agar plate, i.e. at that time not known whether it is lipase-positive or negative, which was tested by bioassay and PCR and found positive for *C. botulinum* type D; and later was confirmed to be *C. perfringens* by 16S rRNA. Also, because *C. perfringens* may has a role in the causation of EGS (Ochoa and de Velandia, 1978). A supernatant of enrichment broth culture of first GS sample investigated was neutralized by type CD antitoxins and when this culture was thoroughly investigated, the only toxic isolate was Gram-negative non-sporulating short slender rods and no lipase-positive colony found in this sample. This isolate was neutralized by type CD and then type C antitoxin and later was confirmed to be *E. coli* by 16S rRNA. Then afterwards, a number of lecithinase- and *E. coli*-like isolates were isolated and tested by bioassay and some of them were found toxic and neutralizable by botulinum antitoxins (details are shown below). Identification of *E. coli* isolates was partly done by inoculation of Gassner agar medium (MERK), on which the growth of *E. coli* changes the colour of the medium from green to dark blue. It was observed that some of the *E. coli* isolates if incubated for more than two days, their cells appear filamentous or much longer than normal. Colonies and smears of some lecithinase positive and *E. coli* are shown in Figs. 17a-d and 18a-d).

From the initial study to select a medium to work with, fFAB and FAA media were found the best; depending on growth and toxicity of isolates. Also, the two media were compared with RCM (the standard medium used by the institute), BA (solid RCM with human blood) and EYA; inoculating reference strains of *C. botulinm* types A to F (Table 5). The growth was found better (larger colonies) on FAA than on BA and far better than on EYA; while the growth in fFAB at pH 6.8 and RCM was more or less the same. However, when FAB (without fortification, at pH 6.8 and 7.2) was compared with RCM, the growth in RCM was either better or the same as in FAB. The growth in FAB at pH 6.8 was better than at pH 7.2.

To facilitate the isolation of clostridial colonies, heat test was used. However, the test for most samples was done not perfect as indicated by the colony and cell types after incubation. Different facultative and anaerobic cocci and non-spore formers bacilli were observed. Also, *E. coli* isolates were able to grow at 60 °C for 4 h and at 80 °C

for up to 30 min. This was because the inoculum, whether from liquid culture or from the specimen was transferred not cautiously into the medium down to the bottom of the tube (Holdeman *et al*., 1977; CDC, 1998), but always part of it may touch the wall of the tube. This was confirmed by inoculating *E. coli* isolate into two liquid medium tubes; one of them in a way that part of the inoculum attached to the wall of the tube and the inoculum in the other tube was cautiously transferred into the medium. *E. coli* was able to grow at 80 °C for 10 min in the tube which was not properly inoculated and no growth in the other tube. This because the part of the inoculum attached to the tube wall above medium level was far less heated and later, due to water vapour or declining of the tube, it reached the medium and grew during the incubation period. To confirm this, two tubes containing liquid medium, one was put into a water bath with high level of water (near the top end of the tube) and the other in the water bath with lower level of water (just above the medium level in the tube). The set temperature was 80 °C. After enough time for the tubes to heat up, the temperature inside the tube at different distances above medium level was measured by introducing a calibrated thermometer inside each tube and the tubes were tightly closed with cotton wool (Fig. 8). The temperature was found decreasing from the medium level upwards in both cases of water level in the water bath. It was lesser in case of low level of water in the water bath and the temperature in this case near the top end of the tube was only 43 °C; a temperature at which *E. coli* can normally grow. Furthermore, this low temperature in this part of the tube is a dry heat which is less effective than moist heat to kill bacteria.

Table 5: Growth of reference strains of *C. botulinum** in FAB/FAA compared to RCM/BA and EYA

Medium		*C. botulinum* type					
Name	pH	A	B	C	D	E	F
RCM	6.8	+++	+++	+++	++	+	+++
FAB	6.8	+++	+++	++	+	+	+++
FAB	7.2	++	++	+	+	+	++
fFAB (+BH)	6.8	+++	+++	++	++	+	+++
FAA	6.8	+++	+++	+++	++	++	+++
BA	6.8	++	++	++	+	+	++
EYA	6.8	+	+	+	< +	< +	++

* The strains tested were: 62A (type A), Okra (type B), 003-9 (type C), C3-16 (type D), CB-S-21E (type E) and 83-4304 (type F).

Also, to select for spore-formers, alcohol test was performed for a large number of samples. However, after the initial mixing of equal volumes of broth culture and ethanol, the mixture was not mixed again every 15 min as described by CDC (1998). So, several types of non-spore-formers were encountered by this method. Mixing every 15 min was found important to kill all vegetative forms. It seems if the mixture mixed only once, large number of cells will adhere to the side of the tube and after some minutes the ethanol will evaporate from the side of the tube and the cells attached to the wall will be less affected. As the mixture is centrifuged and the pelleted material is suspended in a washing solution to remove the alcohol, the less affected cells will be mixed again.

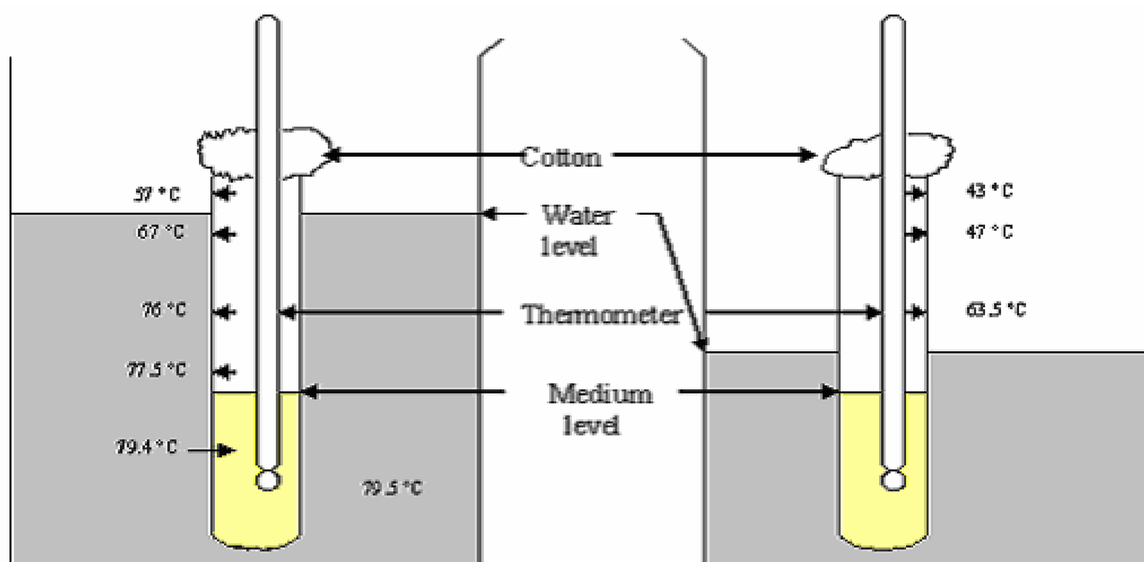


Fig. 8. Schematic drawing to show how the temperature inside the tube varies during heat test at 80 °C in the water bath

During the isolation process, swarming growth was frequently observed on primary or subsequent plates. It was frequently observed that the swarming growth may not appear on primary plates, but found in the subsequent purification process. More than one type of swarming cells were seen in Gram stain, but the majority of cases are *C. tetani*-like cells (racket-shaped). It was recommended to use phenyl ethanol at a concentration of 0.25 % in the plates to prevent the swarming character of some clostridia (Jousimies-Somer *et al.*, 2002). However, the swarming ability of the major type of swarming cells encountered (*C. tetani*-like) was not prevented by either 0.25

% or 0.375 % of 2-phenyl ethanol (PE) (SIGMA-ALDRICH CHEMICALS), but only by 0.5 % concentration. Reference strains of *C. botulinum* were able to grow on 0.25 % but not on 0.5 % concentration of PE.

Later to check for purity and toxicity, 14 of the isolates previously neutralized by botulinum antitoxins were found contaminated with *C. tetani*-like swarming growth, either on normal agar percentage (1.4 %) used for routine work or only on 1 % agar plates. 12 of them were lipase positive and 2 were *E. coli*-like isolates. The swarming growth was separated from each isolate and tested in mice together with the main isolates (results are shown below). The main isolates were apparently purified by using plates containing 0.5 % PE. The rest of the isolates, which were previously neutralized by botulinum antitoxins, were also investigated for contamination with *C. tetani* in moist and low agar percentage plates, but *C. tetani* was not detected in any of them. The isolates found contaminated with *C. tetani* are mainly from environmental samples (details are shown below). Swarming and discrete colonies and smears of *C. tetani* are shown in Fig. 19a-d.

To see if gentamicin has no inhibitory effect to growth of *C. botulinum*, so as to be used in isolation agar medium to reduce the number of accompanying flora to clostridia in test samples (Garrett *et al*., 2002), it was used at a concentration of 10 mg/l. Plates containing gentamicin together with control ones, were inoculated with reference strains of *C. botulinum* types A-F. The growth of all types, except F, was either completely or partially inhibited by gentamicin, compared to growth on control plates.

3.3.2 Mouse bioassay

Out of 288 samples investigated, toxins of 53 isolates from 49 samples (17 %) were neutralized by polyvalent botulinum antitoxins, either type ABE (19 isolates), CD (33 isolates) or by both ABE and CD (one isolate). Some of them were further splitted into single toxin types (Table 6). Distribution of these isolates according to their sample type has been shown in Tables 7 and 8. Out of the 53 neutralized isolates, 27 were from GS samples (24 from GS cases samples and 3 control samples, Table 7). The neutralized isolates are either lipase-positive clostridia (23), lecithinase -positive clostridia (23) or *E. coli* isolates (7).

Table 6: Results of toxin neutralization test of isolates

Toxin neutra-lization	EGS samples (British)				Non-EGS samples			Total
	Lipase +ve	Lecithinase +ve	*E. coli*-like	Total	Lipase +ve	Lecithinase +ve	Total	
A	0	0	0	0	1	0	1	1
B	3	0	0	3	2	0	2	5
ABE	1	6	0	7	3	3	6	13
C	0	0	3	3	2	1	3	6
D	4	0	0	4	2	2	4	8
C/D	1	1	0	2	3	1	4	6
CD	0	3	4	7	0	6	6	13
ABE/CD	1	0	0	1	0	0	0	1
Total	10	10	7	27	13	13	26	53

When toxin neutralization results of the isolates were compared with the results of their corresponding samples, which were obtained from the records of the routine standard diagnostic work of the institute (Table 3), the following was observed. The direct or after enrichment neutralization tests of samples showed that 77 (26.7 %) samples were neutralized by either polyvalent botulinum antitoxin type ABE (24), CD (38) or by both ABE and CD (19) (four of these samples showed different neutralization results by direct and after enrichment). Out of these neutralization results, 39 were from direct testing and 42 after enrichment (17 samples were heat-treated). British GS samples had only nine (4.8 %) results from direct testing, while non-EGS samples had 25 (32.9 %). Comparing this with the neutralization results of the isolates (Table 6), only 17 % of samples had neutralizable isolates and the agreement between the two results was shown by only 16 samples, 13 of which were neutralized by type CD in both cases and three by ABE antitoxin.

Table 7: Distribution of neutralized isolates of EGS samples according to sample type

Sample type*	GS samples				Control samples				Toxin neutralization results						
	Lipase +ve	Lecithinase +ve	*E. coli*	total	Lipase +ve	Lecithinase +ve	*E. coli*	Total	ABE	CD	C	D	C/D	B	ABE/CD
Stomach		1		1					1						
Jejunum		2		2					1	1					
Ileum		3	1	4			1	1	1	1	2[a]		1		
Caecum			1	1							1				
Colon						1		1	1						
Rectum							1	1		1					
Liver		2	1	3					1[b]	2					
Spleen		1		1					1						
Faeces	2			2										2	
Worm casts	4			4					1			2	1		
Unidentif-ied plant	1			1								1			
Soil	1			1										1	
Mud	1		2	3						2[a]		1			
Grass	1			1											1
Total	10	9	5	24	0	1	2	3	7	7	3	4	2	3	1

*The GI tract samples were tissue and/or contents, [a]*E. coli* isolate, [b]lecithinase-positive isolate

Table 8: Distribution of neutralized isolates of non-EGS samples according to sample type

Sample type	No. of isolates			Toxin neutralization results						
	Lipase +ve	Lecithinase +ve	Total	ABE	CD	C/D	A	B	C	D
Bovine small intestine	2	4	6	1*	2			1*		2
Bovine large intestine	2		2			1	1			
Bovine liver	1	3	4	2	1					1*
Bovine faeces	1	1	2	1	1					
Horse faeces	2	2	4	1	1	1		1		
Chicken intestine	2		2						2	
Rabbit faeces	1		1			1				
Soil	2	3	5	1	1	1			1	1
Total	13	13	26	6	6	4	1	2	3	4

*Lipase-positive isolates

Later to check purity and toxicity, 14 of the isolates previously neutralized by botulinum antitoxins were found contaminated with *C. tetani*-like swarming growth. 12 of them were lipase positive and 2 were *E. coli* isolates. *C. tetani*-like growth from nine isolates, after being separately isolated were tested and found toxic in mice (supernatant and filtrate [0.2 µm]) and all of them were neutralized by tetanus antitoxin (Tetagam®N, AVENTIS BEHRING). Six of them were tested by polyvalent botulinum antitoxins types ABE and CD and all were neutralized by both antitoxin types at different dilution rates. Furthermore, some of them were neutralized by other types of botulinum antitoxins (Table 9). A 15th isolate showed slight swarming or spreading characteristic and the cells are not typical for *C. tetani* (with subterminal spores), but also neutralized by tetanus antitoxin. Another three out of four new *C. tetani*-like isolates also were found positive by both tetanus and botulinum antitoxin (types ABE and CD) and the fourth one was not tested. Some of the isolates found contaminated with *C. tetani*, after being apparently purified by using plates containing 0.5 % PE, were found toxic and some not toxic. Most of the other neutralized isolates, which were found to be not contaminated with *C. tetani*, lost their toxicity.

Distribution of *C. tetani* isolates according to source and sample type is shown in Table 10. The tetanus antitoxin used, Tetagam®N, which is a human tetanus immunoglobulin was found potent enough to neutralize the *C. tetani* toxin even without dilution. It was observed that if mice were injected with diluted supernatant of tetanus toxin (1:100), the paralysis seen in hind limbs in the limb corresponding to the side of injection whether the left or the right side, but if not or less diluted, the paralysis is in both limbs.

Table 9: Cross-reactions shown by *C. tetani* isolates* against *C. botulinum* antitoxins

Type of *C. botulinum* antitoxin	Number of isolates			Source of isolates	
	Tested	Positive	Negative	EGS cases samples**	Non-EGS samples
CD (SA)	10	10	0	6	4
ABE	7	6	1[a]	5	2
CD (NL)	5	1[b]	4	4	1
A (NL)	6	3[c]	3	5	1
B (NL)	6	1[b]	5	5	1
E (NL)	5	1[b]	4	4	1
A (Russia)	2	2[b]	0	2	0
B (IBT)	7	0	7	6	1
E (IBT)	5	1[b]	4	4	1
F (USA)	6	2[b]	4	5	1

SA= South Africa, NL=Netherlands

*All the *C. tetani* isolates tested by tetanus antitoxin (12 isolates) were positive.

**No toxic *C. tetani* was isolated from control samples.

[a]One isolate (EGS) tested negative by ABE because the test carried at low dilution (1:4) and it was observed that if the test carried at low toxin dilution rate, either CD or ABE tests positive, but if more diluted then the toxin is neutralized by both CD and ABE antitoxins. [b]EGS isolate, [c]two of them were EGS isolates.

Table 10: Distribution of *C. tetani* isolates* according to source and sample type

Source	Sample type	No. of isolates
EGS cases samples	Faeces	2
	Mud	4
	Worm casts	5
	Soil	1
	Unidentified plant	1
Non-EGS samples	Bovine small intestine	2
	Bovine colon	1
	Bovine faeces	1
	Horse faeces	1
	Soil	1

*All isolates (19) were found toxic, but not all tested by botulinum and tetanus antitoxins (details in table 9)

Among the neutralized isolates, seven were E. coli isolates (all from GS samples) and 23 were lecithinase-positive isolates (Table 6). Their source and sample types are shown in Table 7. These isolates were checked for contamination with *C. tetani* or *C. botulinum* but only two of *E. coli* isolates were found contaminated with *C. tetani* that was found neutralizable by botulinum antitoxins. One of the lecithinase-positive isolates (from bovine intestine) was neutralized by two different types of botulinum antitoxin type D as well as it was also positive for type D by PCR. This isolate was identified by 16S rRNA as *C. perfringens*. Also, one of the *E. coli* isolates was neutralized by two different types of botulinum antitoxin type C. Colonies and smears of these two isolates are shown in Figs. 18a-d and 20).

To see if any of *C. botulinum* toxins can be neutralized by the tetanus antitoxin used, toxin of reference strains of *C. botulinum* types A, B and C were tested as diluted supernatants (1:00), but none of them was neutralized. Other types were not tested.

3.3.3 Polymerase chain reaction

All the isolates showed positive toxin neutralization by mouse bioassay were tested by PCR according to the methods shown above. Non-toxic isolates were not tested. The isolates neutralized by type CD antitoxin mixture were tested by PCR for presence of BoNT/C and BoNT/D genes. Likewise, the isolates neutralized by type ABE polyvalent antitoxin were tested by PCR for presence of BoNT/A, B, E and F genes. PCR results showed specific products for type C in only three isolates and

specific products for type D for other three isolates (Table 11). None of the isolates neutralized by ABE yielded a PCR product. To test for the inhibitory effect of other bacteria to *C. botulinum*, bovine, equine and human faecal samples were each inoculated into liquid medium together with a reference strain of *C. botulinum* type A, B or C. Only type A was isolated and gave positive result by PCR. Isolation as well as enriched broth culture for types B and C were negative in PCR. *C. tetani* isolates showed no product in PCR. Photos of gel electrophoresis for PCR products of positive isolates are shown in Figs. 9-13. Two of the three isolates positive for type C toxin gene were isolated from intestinal contents of broiler chickens from a farm of botulism outbreak and the third from intestinal contents of a case of suspected bovine botulism. Besides being positive by bioassay, the two chicken isolates were also confirmed by MB-ELISA and their phenotypic characteristics were similar to *C. botulinum*. However, the third isolate (faint band) was lipase-negative and lecithinase-positive and due to this phenotypic character, which is unlike *C. botulinum*, it was retested by PCR and found negative and also negative by MB-ELISA. Two of the three isolates positive for type D toxin gene were isolated from intestinal contents of two cases of bovine botulism and the third from faeces of a rabbit experimentally infected with *C. botulinum* type D together with other bacteria.

Table 11: Isolates positive by PCR

C. botulinum type	No. of positive isolates			Source of isolates	Remarks
	Lipase +ve	Lecithinase +ve	Total		
C	2	1	3	Chicken intestine (two, lipase +ve), bovine intestine (one)	The result of the lecithinase positive isolate was not reproduced
D	2	1	3	Bovine liver, bovine intestine (lecithinase +ve), rabbit faeces	The result of the lecithinase positive isolate was not reproduced

One of the two bovine isolates was lipase-negative and lecithinase-positive and due to this dissimilar character to *C. botulinum*, it was retested by PCR and found negative.

Later, DNA identification of this isolate was 100% complying with *C. perfringens*. Negative and positive controls in different PCR amplifications reacted accordingly.

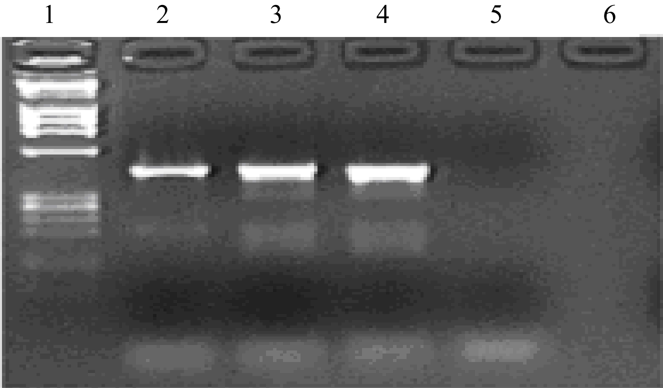


Fig. 9: PCR detection of *C. botulinum* type C. Lane 1, standard DNA marker; lane 2, positive control; lane 3 & 4, isolates from two intestines of broiler chickens; lane 6, negative control.

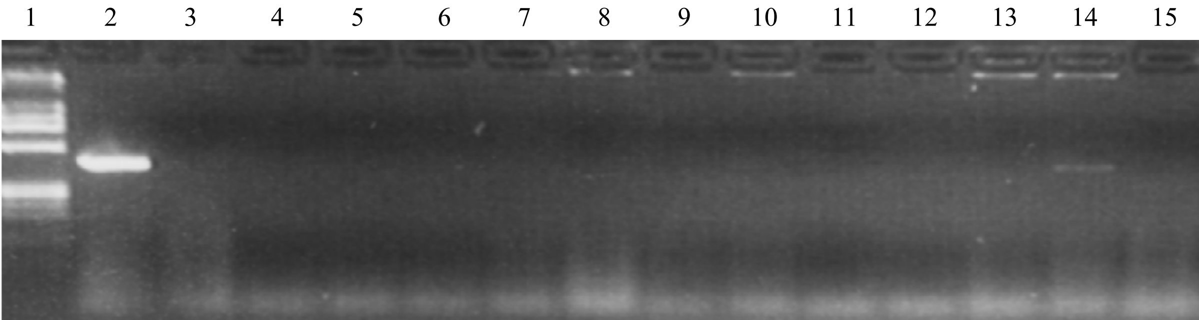


Fig. 10: PCR detection of *C. botulinum* type C. Lane 1, standard DNA marker; lane 2, positive control; lane 14, bovine intestines isolate (lecithinase +ve); lane 15, negative control.

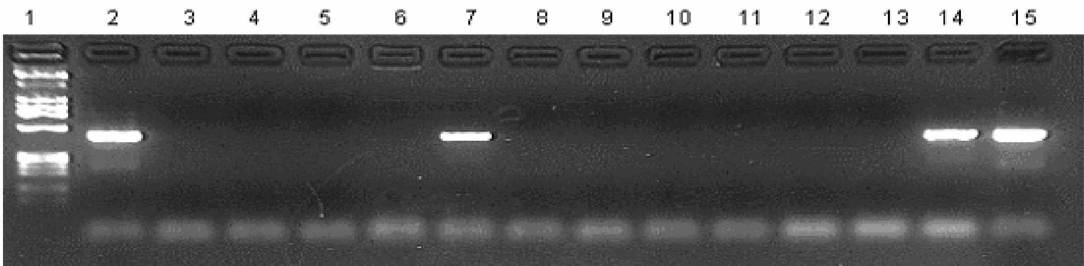


Fig. 11. PCR detection of *C. botulinum* type D. Lane 1, standard DNA marker; lanes 2 & 15, positive control; lane 4, negative control; lanes 7 & 14, isolates (bovine liver and rabbit faeces, respectively).

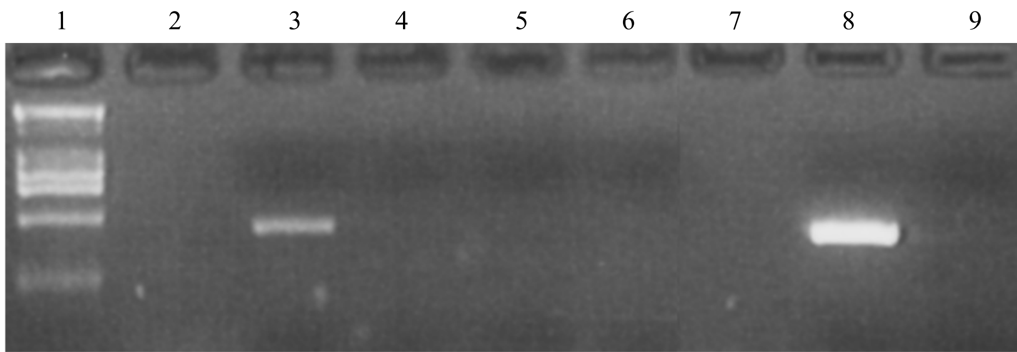


Fig. 12: PCR detection of *C. botulinum* type D. Lane 1, standard DNA marker; lane 3, bovine intestine isolate (identified by 16S rRNA as *C. perfringens*); lane 8, positive control; lane 9, negative control.

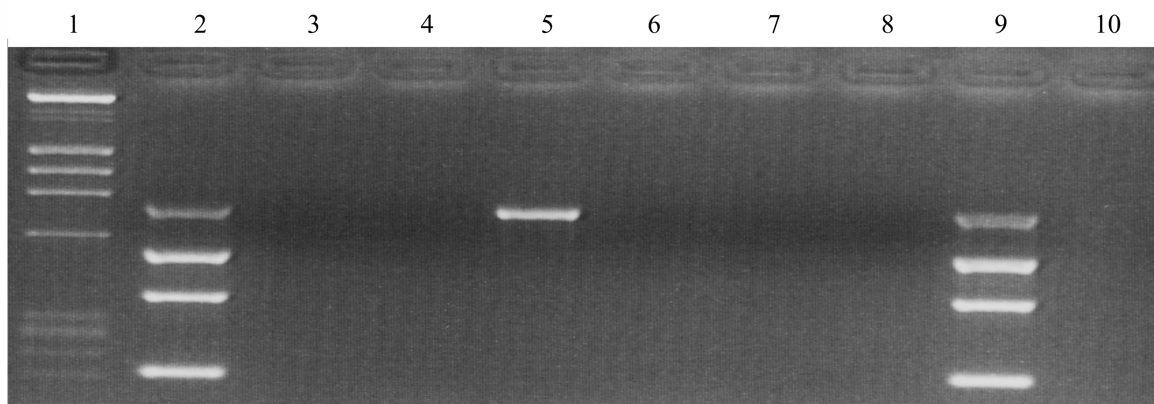


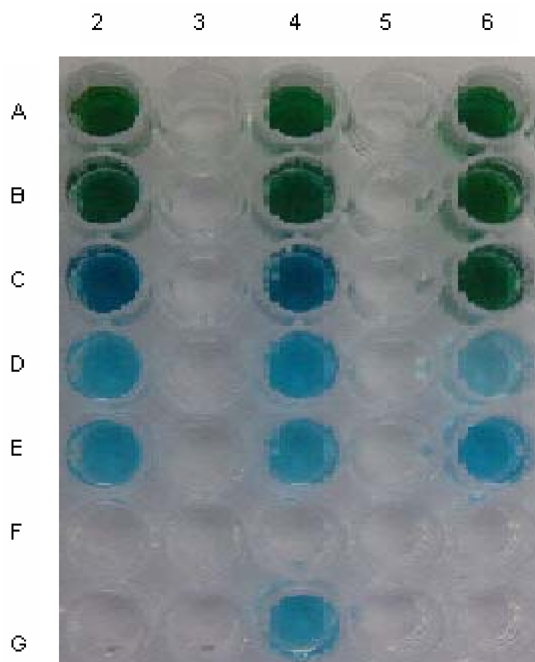
Fig. 13. Multiplex PCR detection of *C. botulinum*. Lane 1, standard DNA marker; lanes 2 & 9, positive control (types A, F, E & B resp.); lane 5, type A isolated from a sample artificially infected; lane 10, negative control.

3.3.4 Magnetic bead-ELISA

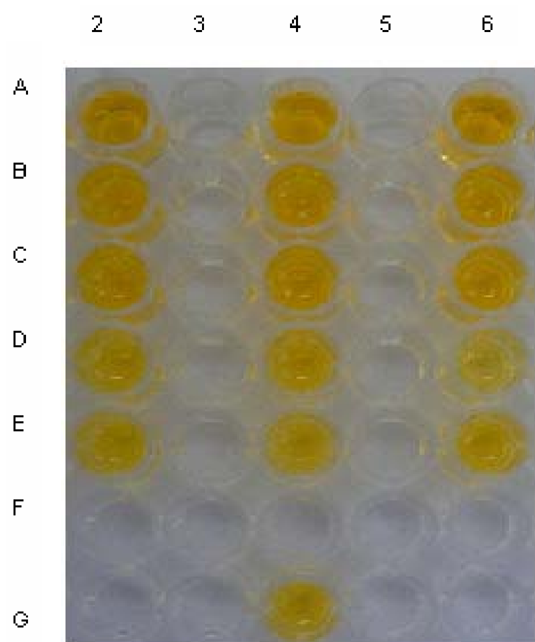
The isolates tested by MB-ELISA were only 28, from those neutralized by type CD botulinum antitoxin mixture by bioassay. Only three isolates had positive results (Fig. 14); two type C and one type D. The two type C isolates were chicken isolates and the type D isolate was from a rabbit artificially infected (Table 12).

Table 12: Isolates positive by MB-ELISA

Botulinum serotype	No. of positive isolates	Source of isolates	Remarks
C	2	Chicken intestine	Lipase positive, field samples
D	1	Rabbit faeces	Lipase positive, experimental infection



	1	2	3	4	5	6
A	0.043 0.002	10^{-2} 1.934 1.893	0.043 0.002	10^{-1} 2.062 2.021	0.042 0.001	1C 10^{-1} 2.062 2.021
B	0.044 0.003	10^{-3} 2.119 2.078	0.043 0.002	10^{-2} 2.134 2.093	0.045 0.004	2C 10^{-1} 2.025 1.984
C	0.048 0.007	10^{-4} 2.312 2.271	0.041 -0.000	10^{-3} 2.338 2.297	0.042 0.001	3C 10^{-1} 2.181 2.140
D	0.041 -0.000	10^{-5} 1.072 1.031	0.041 -0.000	10^{-4} 1.340 1.299	0.043 0.002	4D 10^{-1} 0.611 0.570
E	0.042 0.001	10^{-6} 1.036 0.995	0.042 0.001	10^{-5} 1.008 0.967	0.043 0.002	5D 10^{-1} 1.388 1.347
F	0.044 0.003	0.044 0.003	0.045 0.004	0.042 0.001	0.043 0.002	0.043 0.002
G	0.041 -0.000	0.042 0.001	0.041 -0.000	NW 0.986 0.945	0.042 0.001	0.043 0.002
H	0.041 0.041	0.042 0.042	0.041 0.041	0.040 -0.001	0.041 -0.000	0.042 0.001



	1	2	3	4	5	6
A	0.043 -0.004	10^{-2} 2.921 2.874	0.045 -0.002	10^{-1} 2.921 2.874	0.042 -0.005	1C 10^{-1} 2.824 2.777
B	0.042 -0.005	10^{-3} 2.880 2.833	0.046 -0.001	10^{-2} 3.102 3.055	0.049 0.002	2C 10^{-1} 2.977 2.930
C	0.050 0.003	10^{-4} 2.830 2.783	0.043 -0.004	10^{-3} 2.750 2.703	0.041 -0.006	3C 10^{-1} 2.750 2.703
D	0.041 -0.006	10^{-5} 2.192 2.145	0.043 -0.004	10^{-4} 2.618 2.571	0.046 -0.001	4D 10^{-1} 1.248 1.201
E	0.043 -0.004	10^{-6} 2.161 2.114	0.045 -0.002	10^{-5} 2.068 2.021	0.045 -0.002	5D 10^{-1} 2.605 2.558
F	0.046 -0.001	0.045 -0.002	0.047 -0.000	0.044 -0.003	0.043 -0.004	0.047 -0.000
G	0.042 -0.005	0.043 -0.004	0.043 -0.004	NW 2.036 1.989	0.044 -0.003	0.047 -0.000
H	0.048 0.048	0.048 0.048	0.046 0.046	0.042 -0.005	0.041 -0.006	0.044 -0.003

Fig 14: MB-ELISA result. The photos showing the picture of microtitre plate and its absorbance readings. Top, the plate and absorbance reading at 655 nm before stoppage of reaction; and down, the plate and absorbance reading at 450 nm after stoppage of reaction. Columns: 2, positive control type D (diluted 10^{-2}-10^{-6}); 4, positive control type C (diluted 10^{-1}-10^{-5}); 6, isolates (diluted 10^{-1}). Wells: A6 & B6, one of the two type C isolates (two broth cultures incubated for 2 & 5 days respect.); C6, the other type C isolate; D6, the bovine type D isolate (its reading is < negative control) ; E, rabbit isolate; and G4, negative control.

4 DISCUSSION

4.1 Culture methods and isolation

Inspite of the nearly ubiquitous distribution of *C. botulinum*, especially in soil samples (Whitlock and Buckly, 1997), the sound diagnosis of botulism (in case of some test samples in this work) and the several reports of evidences that associated *C. botulinum* with the EGS (Tocher *et al*., 1923; Hunter *et al*., 1999; Böhnel *et al*., 2003), in the current study only four isolates from 288 test samples were confirmed to be *C. botulinum*. Natural intestinal and environmental conditions are impossible to be simulated in vitro (Smith and Sugiyama, 1988; Seifert and Böhnel, 1995). In nature, the toxico-infectious form of botulism in humans and horses was reported to occur mainly in neonates and rarely in adults after a predisposing factor (Chia *et al*., 1986). This is because adults, unlike infants and foals, have mature intestinal microflora, which can compete with *C. botulinum* and prevent its colonization (Arnon *et al*., 1978; Bartlett, 1986). This fact may strongly support the belief that the main reason behind the difficulty to isolate *C. botulinum*, seems to be due to its inhibition by other bacteria (Graham, 1978; Szabo *et al*., 1994; Sandler *et al*., 1998; Böhnel and Lube, 2000). Some reports showed that *C. perfringens* and *C. tetani* are examples of these inhibitory bacteria (Smith, 1975: Smith, 1978). Also, in this study, *C. botulinum* types A, B and C were inoculated, as 1 ml 48 h broth culture, each together with faecal material, which was negative for *C. botulinum* and its toxin; in liquid medium and incubated anaerobically at 37 °C for two and five days. Both isolation and PCR for primary broth culture were negative for both types C and B and positive for type A. The sample inoculated with type C contained *C. tetani* (isolated and its toxin neutralized by tetanus antitoxin) and the sample inoculated with type B contained *C. perfringens*- and *C. tetani*-like cells. This confirmed the inhibitory effect of other bacteria. The inhibitory bacteria seem to be widely distributed in field samples investigated in this study. Also, the isolation results in this study clearly demonstrated the inhibitory effect of other microflora on growth of *C. botulinum*. Isolation of *C. botulinum* after enrichment in liquid medium was found hopeless. The four confirmed *C. botulinum* isolates were obtained either by direct streaking, after direct heat or alcohol treatment. Isolation of *C. botulinum* requires experience and proper selection and performance of technical methods; this was partly not fulfilled in this work. For example, direct streaking, direct heat or alcohol treatment, serial transfer of liquid

culture to increase few numbers of *C. botulinum* organisms (Segner *et al*., 1971), incubation for long time (5-7 days), picking of several lipase positive colonies because some isolates may be non-toxic (CDC, 1998), were either not done or improperly performed. *C. botulinum*, with exception of some strains of group III, is a strict anaerobe (Kriek and Odendaal, 1994). The samples of this study were transported, stored and processed in aerobic conditions; this exposure to oxygen may have adverse effect on viability and toxicity of *C. botulinum*. However, *C. botulinum* types A and B cultured in association with avian skin flora had similar growth patterns under both aerobic and anaerobic conditions (Dezfulian, 1999). This may be due to the reduced oxygen tension and low oxidation-reduction potential provided by the avian flora; but it is unlikely to say that strict anaerobic condition was achieved. Similarly, FIRSTENBERG *et al*. (1982) found that *C. botulinum* type E was able to grow and produce toxin in aerobic condition. The EGS samples were stored at -80 °C for long time; so, the viability of vegetative forms might be affected by deep freezing (CDC, 1998; Jousimies-Somer *et al*., 2002). This may partly be shown by the observation that most of the toxin neutralization test results in this study were obtained early after the beginning of the work. Moreover, *C. botulinum* can lose its toxicity during isolation process (Collins and East, 1998) or naturally it may not always be toxic (Galey, 2001), perhaps helping to explain the sporadic nature of the outbreaks and hence, some of non-toxic lipase-positive colonies could be *C. botulinum*. In addition, the presence of similar lipase positive colonies in a plate was sometimes represented by picking one colony, which may be not the target one, if present; however, this is unlikely to be always the case. Isolation of *C. botulinum*, including type C was found not impossible (Segner *et al*., 1971, Hatheway and McCroskey, 1987); however, in this study no single confirmed *C. botulinum* was isolated from EGS. This negative isolation result may also be contributed to the extreme sensitivity of horses to botulism (Galey *et al*., 2000), a case in which number of bacteria in the animal samples may be too low to be isolated. As *C. botulinum* is almost ubiquitous and can be found normally in soil and possibly in GI tract of horses (Ricketts and Greet, 1984), the isolation results of EGS samples were of many false-negatives, despite the possibility of isolation of some non-toxic *C. botulinum*. However, these results could show that *C. botulinum* is at least not prevailing in GS samples and its association with the disease remains doubtful.

According to colony and cellular morphology, several *Clostridium* species were isolated (see results). Their types and numbers in GS samples were obviously greater than in control samples. This was in agreement with the findings of GRIEß *et al*. (1996) and GARRETT *et al*. (2002). The high presence of clostridia in GS cases more than in controls may be due to the stasis or change in the ecology of GI tract of diseased animals or dropage of local immunity (Garrett *et al*., 2002). *C. perfringens*, *C. tetani* and *C. sporogenes* are possibly most isolated. *C. perfringens* was frequently isolated from GS samples, especially from animal samples. It was isolated from all samples tested from two cases of EGS; one of them is a case artificially infected by a suspected soil in Dubai. The samples of these two cases were thoroughly investigated for presence of *C. botulinum* and found negative. Also, *E. coli* and *C. tetani* were isolated from GS cases more than from control ones and some of their isolates were neutralized by botulinum antitoxins. So, a direct or indirect role to *C. perfringens*, *C. tetani* and/or *E. coli* is not unexpected.

The swarming growth frequently encountered in plates, especially *C. tetani*, was one of the problems that may contribute to failure of isolation of *C. botulinum*, either preventing its growth or found very difficult to be separated from the swarming bacteria. JOUSIMIES-SOMER *et al*. (2002) recommended the usage of phenyl ethanol to combat the swarming growth of some clostridia at a concentration of 0.25 %. However, in this study, the swarming character of *C. tetani* was not prevented by 0.25 % and 0.375 % and prevented by 0.5 %. Besides the growth of all types of *C. botulinum* was completely inhibited by PE at a concentration of 0.5 % and slightly affected at a concentration of 0.25 %. So, the usage of PE could be only in case of a lipase positive colony was picked from a plate with swarming growth or later found contaminated with other swarming bacteria, but not in the primary plates as it will prevent the growth of *C. botulinum* at the concentration required to inhibit *C. tetani* (0.5 %). The swarming growth may not appear in the primary plates; due to less moisture or effect of other bacteria, but appear in the subsequent plates during the purification process or only after an apparently pure colony being grown into liquid medium and then cultured on solid medium. Phenyl alcohol acts by inhibiting flagellation and thereby prevents swarming (Sharma and Anand, 2002). Also it can prevent growth of facultative Gram-negative rods. The critical factors that determine whether the cells swarm or form regular colonies are: the concentration of agar,

surface moisture and viscosity (Sharma and Anand, 2002). Some *Clostridium* species are able to form swarming colonies on the surface of 2 % agar plates (Sharma and Anand, 2002) and in this study *C. tetani* was able to swarm on 3 % agar plates if not well dried and sometimes not on 1.4 % agar plates if well dried. So, it seems the degree of dryness is more critical than percentage of agar. However, reduced surface moisture is not very reliable as the dryness required to inhibit swarming would completely suppress the growth of fastidious organisms (Sharma and Anand, 2002). This was in agreement with HERNÁNDEZ-CHAVARRÍA *et al*. (2001), who used plates with 4% agar to have discrete colonies of *C. tetani*. *C. tetani* is just second to *Proteus mirabilis* in the rate of surface translocation (Sharma and Anand, 2002). Other *Clostridium* species that can swarm are *C. sporogenes, C. novyi, C. bifermentans*, *C. septicum*, *C. butrificum*, *C. fallax*, *C. glicolicum*, *C. haemolyticum* and *C. beijerinkii* (Hernández-Chavarría *et al*., 2001; Sharma and Anand, 2002; Jousimies-Somer *et al*., 2002). It was not found reported that *C. botulinum* can swarm; however, in this study two type C and one type D *C. botulinum* isolates were able to swarm. *C. novyi* type A, the related *Clostridium* to group III *C. botulinum* (Eklund and Poysky, 1994), is known of its swarming character (Sharma and Anand, 2002). So, likely other members of group III (*C. botulinum* types C and D) or some of their strains, which are not yet isolated, could swarm. It is known that group III members can be converted to each other if infected by the specific phage in vitro, which was also thought to occur in nature (Eklund and Poysky, 1994); so, less likely, these type C and D isolates may be *C. novyi* type A strains naturally infected by type C and D specific phages. Thus, swarming growth should be tested for *C. botulinum*.

The agar medium used in this study (FAA) was found the best, compared to BA and EYA, to encourage growth of *Clostridium* spp., including *C. perfringens*, *C. tetani* and reference strains of *C. botulinum*. However, its broth, compared to RCM, was not better and the reason for that is difficult to show. One may say that isolation of *C. botulinum* is possible by direct streaking on FAA plates, and application of direct heat and alcohol treatment. However, the easy isolation of *C. botulinum* is still waiting for a highly selective medium for all groups of *C. botulinum*.

4.2 Mouse bioassay

A total of 53 isolates had positive toxin neutralization results. These isolates were either lipase-positive, lecithinase-positive clostridia or *E. coli*. Only four of them (lipase-positive) were confirmed to be *C. botulinum* by other diagnostic means. Later, some of them were found contaminated with *C. tetani*, which was proved to be the cause of their false-positive results; and no *C. tetani* contamination was seen with the other ones, which were tested again and most of them found not toxic. Why most isolates that showed positive neutralization results became non-toxic, may be attributed to the following. Mainly attributed to false-positive results due to non-botulinum lethalities, which their avoidance was not considered in this work; or to low level of contamination with *C. botulinum* which was later disappeared. This was partly confirmed in case of many isolates found contaminated with swarming *C. tetani*, which was isolated and found neutralizable by different botulinum antitoxins (Table 9) and one isolate (lecithinase-positive) found contaminated with swarming *C. botulinum*. In addition, the lipase-positive isolates or part of them may be *C. botulinum* but their toxicity was lost as it was reported that pure *C. botulinum* isolates may lose toxicity (Eklund *et al*., 1971). Seven *E. coli* isolates (all from GS samples) and 23 lecithinase-positive isolates (ten from GS samples) were found neutralizable by botulinum antitoxins. Five of the *E. coli* isolates were from GS cases samples and two from control samples and nine of the lecithinase-positive isolates (most of them look like *C. perfringens*) were from GS cases samples and one from control samples. When these isolates were investigated for contamination with *C. botulinum* or *C. tetani*, only two (*E. coli* isolates) were found contaminated with *C. tetani*. Interestingly, two of them (one lecithinase-positive and one *E. coli*-like isolate) were neutralized by two different types of botulinum antitoxin type D and C respectively, and the lecithinase-positive isolate was also positive for BoNT/D gene by PCR. They were thoroughly investigated for contamination by either *C. botulinum* or *C. tetani* but found pure. These two isolates were then confirmed to be *C. perfringens* and *E. coli* (16S rRNA). Such results could be attributed to a non-isolatable level of contamination by *C. botulinum* or *C. tetani* in case of the isolates other than the confirmed two isolates or to some gene transfer which was then lost. Nineteen *C. tetani* islolates (14 of them found as contaminant with other isolates) were found toxic and some of them were tested by tetanus and botulinum antitoxins and all found neutralizable. Thirteen of them were from GS cases samples and no toxic isolate

from control samples. It was observed that *E. coli* and *C. perfringens*-like isolates were mostly from animal and not environmental samples and *C. tetani* isolates were from environmental samples. These findings may strongly suggest a role to one or more of these three species in the causation of EGS either directly or indirectly.

The neutralization results of non-botulinum isolates or at least part of them indicate that mouse bioassay is non-specific and questionable. Many false-positive results were confirmed to be due to cross-reactions between botulinum and tetanus toxins. *C. tetani* is a normal inhabitant of soil (Smith, 1978), so results from soil samples should be checked for tetanus toxin. Other non-botulinum lethalities such as endotoxins of Gram-negative bacteria also may have a role in these results. In general, the mouse bioassay was claimed to be not suitable for examination of test samples containing other lethal substances (Dezfulian and Bartlett, 1985). To avoid or reduce the effect of lethalities other than botulism in the bioassay, high centrifugation and/or filtration of liquid culture (Solomon and Lilly, 1998), treatment of supernatants by overnight deep freezing (Smith and Sugiyama, 1988), dilution of supernatants (Hatheway and McCroskey, 1987), addition of BSA (Solberg *et al*., 1985), use of antibiotics (Sandler *et al*., 1993), and preinjection of antitetanus (Smith and Sugiyama, 1988), were indicated. It was observed that a sample may be neutralized by both ABE and CD antitoxins; this was shown possible in case the sample containing tetanus toxin, but not possible in case of a botulinum toxin, as group I or II toxins were not reported to cross-react with group III toxins. Also, not possible in case of existence of more than one toxin type from these groups, as the neutralization in this case will happen only if ABE and CD antitoxins were mixed together. Tetanus toxin in this study was shown to cross-react with different botulinum antitoxins (Table 9). Toxins of all *C. tetani* isolates tested were neutralized by both ABE and CD at different dilutions. However, results of other antitoxins were not repeated to see whether the results are reproducible or not. Tetanus toxin may cross-react with BoNT/B and A (Dolimbeck *et al*., 2002), but cross-reactions with other BoNTs may not be reported. However, at least part of these results could be attributed to the quality of the antitoxin used. For example, all supernatants tested by CD (SA) or ABE (AVENTIS BEHRING) had positive results, while none of seven supernatants tested by type B antitoxin of IBT (raised in chickens) had positive result. So, type and specificity of the proteins of the toxoid and/or the animal species from

which these antisera were obtained may have an effect. To see if BoNTs can be neutralized by tetanus antitoxin, toxins of the reference strains types A, B, and C were tested; but none of them was neutralized. However, cross-reaction between botulinum toxins types A and B with tetanus toxin was reported by DOLIMBECK *et al*. (2002).

The samples of this work were all tested for presence of BoNTs by the standard mouse bioassay either directly or after enrichment by the institute (Table 3). When the neutralization result of the institute were compared with the neutralization result of the isolates in this study to see how the results agree with each other, the following was observed. Evidence of BoNT was shown in 26.7 % (77/288) of all samples and in 19.3 % (36/187) of GS samples compared to neutralizable isolates from 17 % (49/288) of all samples and 14.4 % (27/288) of GS samples. The apparent agreement between the two results was shown by only 16 samples, 13 of which were neutralized by type CD in both cases and three by ABE antitoxin. This low agreement could be partly attributed to the low isolation rate (from 49 samples compared to 77 samples showed evidence of BoNTs) and to non-specific results especially due to tetanus toxin which was proved in this study that it can be neutralized by ABE, CD or both according to its concentration in the test supernatant. This may be supported by the observation that 19 of the 77 samples were neutralized by both ABE and CD, which is not expected in case of BoNT as none of types A, B or E was reported to cross-react with either type C or D. One interesting observation is that only 4.8 % (9/187) of GS samples had evidence of BoNT from direct testing compared to 32.9 % (25/76) of non-EGS samples. This may indicate that the presence of BoNT is not prevailing in these samples or may be due to the extreme sensitivity of the horse it exists but in a non-detectable level.

4.3 Polymerase chain reaction

PCR assays were performed to test for BoNT genes (A-F) in all the isolates which gave positive results by the mouse bioassay (Table 6). Only six isolates yielded PCR products. The products of three of them were specific for type C and the products of the other three were specific for type D. Two type C and two type D isolates were culturally similar to *C. botulinum*. However, the third type C and type D isolates were culturally dissimilar to *C. botulinum*; both were lipase-negative and lecithinase-

positive. The type D isolate was phenotypically and genotypically (16S rRNA study) identified as *C. perfringens*. This isolate was neutralized by two different type D antitoxins by the bioassay. Both type C and type D isolates were thoroughly investigated for contamination with *C. botulinum*, but not detected. Later, both of them lost their toxicity and their PCR result was not reproduced. These unexpected two results can be attributed mainly to the following. 1) Very low level of contamination with *C. botulinum* from the sample of the isolate; however, this may be possible in case of type C isolate but not type D isolate as it was 100% identified as *C. perfringens* by 16S rRNA study. *C. botulinum*, in this study, was found can swarm and its swarming growth can be in a very thin layer that could not be detected by naked eye. So, if a plate with apparently only one type of colony, after subculture in a liquid broth, the contaminant could appear. This was confirmed by one of type D isolates, which was a lipase-negative and found positive for type D by PCR. This isolate was investigated for contamination with *C. botulinum*, which was found as a very thin layer of swarming growth. 2) Detection of *C. botulinum* type B gene in two of *C. subterminale* cultures was reported (Franciosa *et al*., 1994); *C. baratii*, *C. butyricum* and *C. argentinense* are phenotypically different from all *C. botulinum* strains, but are known to produce BoNTs (Collins and East, 1998); organisms of group III *C. botulinum* (type C, D, and *C. novyi* type A) can be converted to each other, both in nature and in vitro, if infected by the specific phage (Eklund and Poysky, 1994). These reports may indicate that these two results or one of them could be due to some gene transfer from *C. botulinum* or a mutation happened in nature to these isolates. 3) Other factors such as molecular contamination with DNA from the reagents used or during preparation of template or afterwards, are not totally excluded. However, to avoid contamination, sample preparation, PCR amplification, and electrophoresis were done in three different rooms.

Compared to the results of immunoassay, PCR is considered more sensitive. The immunoassay did not detect a low level of contamination by *C. botulinum* in one isolate, which was detected by both PCR and bioassay. Also, when *C. botulinum* was purified from this isolate, it was found positive by both bioassay and PCR and not by immunoassay. In addition, PCR had two more results for dissimilar *C. botulinum* isolates, at least one of them may be due to very low level of contamination with *C. botulinum*. However, due to these last two results, it may be less specific. Compared

to isolation and bioassay, PCR is considered less sensitive. It showed at least one false-negative result, compared to the isolation; and many, compared to the bioassay. A type D isolate from a faecal sample of a rabbit experimentally infected with type D together with other bacteria, was isolated from a broth culture found negative by PCR. This isolate was then tested positive by both PCR and immunoassay. Most of the mouse bioassay results were found false-positive and no confirmation that bioassay was more sensitive than PCR. False-negative results by PCR could be attributed to factors such as inhibitory substances in the media (Ferreira *et al.*, 1993). Within the type D strains neurotoxins differ in molecular structure and antigenicity (Moriishi *et al*., 1989), so, the primers may have an effect (Campbell *et al*., 1993), template preparation method which can affect DNA isolation in good quality (Szabo *et al*., 1994a), and DNA concentration (Fach *et al*., 2002) and purification (Williamson *et al*., 1999) may also have an effect.

4.4 Magnetic bead-ELISA

Out of 28 isolates, which were positive by bioassay for type C, D or both botulinum toxins, only three had positive results by MB-ELISA. False-positive results by ELISA were reported (Potter *et al*., 1993) due to the reaction of capture antibodies with non-toxigenic haemagglutinin molecules produced along with the toxin, or capture antibodies reacted with a somatic antigen or other protein produced by the organism. However, this immunoassay is considered 100% specific, compared to both bioassay and PCR performance. Sensitive immunoassays, unlike the mouse bioassay can detect the toxin either in active or inactive form, or in a level below mouse sensitivity. However, this MB-ELISA assay showed at least one false-negative result, compared to both bioassay and PCR. Three isolates (lecithinase-positive) were positive by both bioassay and PCR and none of them was positive by MB-ELISA, but only one was proved to contain *C. botulinum*. When *C. botulinum* was separated from this isolate, it was also found positive type D by both bioassay and PCR and negative by MB-ELISA. This isolate is also culturally similar to *C. botulinum* and showed the classic botulism signs in mice. This false-negative result may be because the antigenecity of the toxin of the strain used to raise the antibodies used in this assay is different from that of this isolate. It was found that within the type D strains, neurotoxins differ in molecular structure and antigenicity (Moriishi *et al*., 1989). Also, the absorbance reading of this isolate was much higher than the expected readings for negative

isolates; thus the cut-off value for this assay may be too high. None of the rest isolates positive by mouse bioassay was confirmed to be *C. botulinum*, so, no confirmation that mouse the bioassay is more sensitive than the MB-ELISA except in case of this one isolate.

4.5 Discussion (general)

Botulism is diagnosed by detecting BoNT and/or *C. botulinum* cells in the test sample. The sample may contain only detectable toxin or cells. So, diagnostics should include both toxin and cell detecting methods. In this study mouse bioassay, MB-ELISA and PCR were used to test for BoNTs or their genes of the isolates obtained by culture methods. Of 288 samples tested, only four isolates were confirmed (culture, bioassay, PCR and only three by immunoassay) to be *C. botulinum* and all from samples other than EGS samples. From this apparent final result, one may say that prevalence of *C. botulinum* in the test samples is extremely low, especially in GS samples, and the proposed linkage of EGS with *C. botulinum* is weakened. However, *C. botulinum* is deemed to be widely distributed in nature and may be normally present in the GI tract of horses (Ricketts and Greet, 1984), and hence expected to be found in both the disease and control samples (Szabo *et al*., 1994b) with significantly more prevalence in the former. In addition, *C. botulinum*, its toxins, especially type C, and antibodies were detected in GS cases (Tocher *et al*., 1923; Miller, 1994; Poxton *et al*., 1997; Hunter and Poxton, 1998; Poxton *et al*., 1998; Hunter *et al*., 1999; Hunter and Poxton, 2001; Garrett *et al*., 2002; Böhnel *et al*., 2003; McCarthy *et al*., 2004b); however, isolation of the organism was reported only once (Tocher *et al*., 1923). *C. botulinum* isolation is known to be difficult (Smith and Sugiyama, 1988) due to its strict anaerobic character, high fastidiousness, needs good experience and special laboratory facilities, the bacteria can lose its toxicity during subcultures or before that, and above all, its isolation is specially difficult because of the inhibitory effect of other microflora (Graham, 1978; Szabo *et al*., 1994; Sandler *et al*., 1998; Böhnel and Lube, 2000). So, this negative result of isolation is not totally unexpected; and it may be more sound to relate the negative result found in this study to difficulty in isolating *C. botulinum* rather than its degree of prevalence in the samples tested. However, the *C. botulinum* isolates in the current study were from small number of botulism samples compared to the large number of GS

samples examined. Thus, one can say *C. botulinum* is at least not prevailing in these GS samples and its linkage with the disease remains doubtful. The neutralization results showed by some *E. coli*, *C. perfringens*-like and *C. tetani* isolates and their prevalence in GS cases samples and not in control samples may strongly suggest some association with the EGS either directly or indirectly.

It is reported that the only reliable means of detecting, identifying, and typing BoNTs is the mouse bioassay (CDC, 1998). However, mouse bioassay was claimed to be not suitable for examination of test samples containing other lethal substances (Dezfulian and Bartlett, 1985). In the present study out of 53 neutralizations, only four were confirmed to be specific. Many of these non-specific results were confirmed to be due to cross-reactions with tetanus toxin and this can be avoided only by inclusion of antitetanus in the bioassay, especially in case of soil samples. Tetanus toxin was neutralized by different botulinum antitoxins (A-F) (Table 9), but mainly by polyvalent type ABE (AVENTIS BEHRING) and type CD mixture (Onderstepoort). Cross-reaction between TeNT and BoNTs is mainly expected to happen with type B (Dolimbeck *et al*., 2002); however, none of the isolates tested cross-reacted with type B antitoxin raised in chicken by IBT. So, specificity of the proteins of the toxoid and/or the animal species from which these antisera were obtained may have an effect. The toxin neutralization results shown by some *E. coli* and *C. perfringens* isolates (Table 6), which were investigated for contamination with *C. tetani* or *C. botulinum*, but not found, were strange. Such results were attributed to a non-isolatable level of contamination by *C. tetani* or *C. botulinum* or to some gene transfer which was then lost.

The inhibitory effect of other microflora to *C. botulinum* was clearly demonstrated in the present work. None of the four confirmed *C. botulinum* isolates was isolated after enrichment in liquid medium. They were isolated either after direct heat or alcohol treatment or by direct streaking. The improper performance of heat or alcohol treatment also failed to demonstrate the bacterium. Furthermore, the author tried the isolation of *C. botulinum* from artificially contaminated samples. *C. botulinum* type A, B or C was added to liquid medium as a heavy inoculum together with other naturally contaminated faecal material and incubated for two and five days. Both types B and C were not recovered and the enrichment broth was negative by PCR, while type A

was recovered. The inhibition of *C. botulinum* by other microflora in vitro was similarly suspected to happen in vivo (Arnon *et al*., 1978; Bartlett, 1986) and hence types of organisms in the GI tract will determine if this animal is susceptible to *C. botulinum* infection or not.

Among the confirmed *C. botulinum* isolates, one type D isolate was from the liver of a case of bovine botulism. Another isolate, dissimilar to *C. botulinum*, was isolated from the small intestine of the same animal which was positive for type D by the mouse bioassay and this result was attributed to contamination with *C. botulinum*. Botulism in this bovine case was diagnosed by the direct detection of the same toxin type during the routine diagnostic work in the institute. Also, from contents of small intestine of another bovine case, a lecithinase-positive clostridial isolate was positive for type CD in mice and type C by PCR; this was also attributed to low level of contamination by *C. botulinum* type C organisms (this was found possible due to non-detectable swarming growth and character of *C. botulinum* demonstrated in this study). Again, the same toxin type was directly detected in the sample by the institute. These results may indicate that the bovine botulism in Germany could be mainly due to types D and C. This is in accordance with the findings of BÖHNEL (1999). Besides the classical form of botulism in cattle, a toxico-infectious form (visceral) was recently described (Böhnel *et al*., 2001). The isolation of confirmed two strains of *C. botulinum* type C from intestines of two broiler chickens from a farm with botulism outbreak may indicate that chicken botulism in this country is due to type C. Botulism in these two chicken cases was also confirmed by direct detection of the toxin by the institute. This result is in consistence with the fact that avian botulism worldwide is mainly due to type C (Graham and Smith, 1978; Smith and Sugiyama, 1988). In addition to classical form of avian botulism, TI form was also reported in broiler chickens (Eklund *et al*., 1987, cited by Hunter and Poxton, 2002) due to high energy diet. Due to the increasing incidence of TI botulism and presence of different names for this form, one may classify botulism in humans and animals into two forms: 1) intoxication (the toxin produced ex vivo), which includes food-borne and inadvertent types; 2) toxico-infection (the toxin produced in vivo), which includes wound and visceral types (Böhnel *et al*., 2001); the visceral type includes the toxico-infectious forms of young and adult humans and animals.

It was observed that the colonial morphology of the two type C isolates from broiler chickens and type D from a cow is different. The colony of one type C isolate is raised pyramidal with rhizoidal edge and the other one is flat or slightly raised with rhizoidal spreading edge; while that of type D is raised with uneven edge. So, these isolates may be different strains and because of their swarming character, which was not found reported, they may require further study to see if they were isolated before or not.

The current study recommends the usage of Fastidious Anaerobic Agar (FAA) for isolation of *C. botulinum* and other clostridial species, especially *C. perfringens* and *C. tetani*.

5 SUMMARY

Isolation and identification of *Clostridium botulinum* from field samples obtained from: i) equine grass sickness (EGS) cases (animal and environmental samples) together with control animal samples; and ii) classical botulism-suspected cases, was an objective. Using mouse bioassay, MB-ELISA and PCR to identify the isolates, out of 288 samples investigated, only four isolates were confirmed to be *C. botulinum* and all from samples other than EGS samples. This result could show that *C. botulinum* is at least not prevailing in GS samples and its association with the disease remains doubtful. Two of the confirmed *C. botulinum* isolates were type C and the other two were type D. The two type C isolates were from intestinal contents of two broiler chickens from a farm with botulism outbreak; one of the type D isolates was from intestine of a case of bovine botulism and the other from faeces of a rabbit experimentally infected with *C. botulinum* type D together with other bacteria. The colonies of the two type C and the bovine type D isolate are not the same and the cells can swarm if the plates are not dry enough. So, these isolates may be different strains and because of their swarming character, which was not found reported, they may require further study to see if they were isolated before or not.

Toxins of 53 isolates were neutralized by botulinum antitoxins by mouse bioassay, 27 were from GS samples (24 from GS cases samples and three from control samples). The neutralized isolates were lipase-positive clostridia (23), lecithinase-positive clostridia (23) and *E. coli* isolates (7). Only four of them (from non-EGS samples) were confirmed to be specific. Ten of the lipase-positive isolates were from GS samples (all from case samples), ten of the lecithinase-positive isolates were from GS samples (nine from case samples and one from control samples) and all the seven *E. coli* isolates were from GS samples (five from case samples). Fourteen of the 53 isolates were found contaminated with *C. tetani*, which was proved to be the cause of their false-positive results. Tetanus toxin from several *C. tetani* isolates was neutralized by different botulinum antitoxins (A-F), but mainly by polyvalent type ABE (AVENTIS BEHRING) and type CD mixture (OVI). The neutralization results showed by *E. coli*, *C. perfringens*-like and *C. tetani* and the association of these species with the GS case samples and not with the control samples was considered to have a direct or indirect role in the aetiology of EGS. Also, it was observed that types and numbers

of some other *Clostridium* spp. are more prevalent in GS case samples than in control samples.

The isolates with a positive result in the mouse bioassay were tested by PCR. Only six of them were positive by PCR, three type C and three type D. One type C and one type D isolate were lipase-negative and their result was not reproduced.

The isolates tested by MB-ELISA were only 28, from those neutralized by type CD botulinum antitoxin mixture in the bioassay. Only three isolates had positive results, which were also positive by PCR. Two of them type C and one type D. MB-ELISA was specific, but less sensitive than other diagnostic means.

The isolation and identification results showed that bovine botulism in Germany could be due to types D and C and avian botulism due to type C.

The current study recommends the usage of Fastidious Anaerobic Agar medium for isolation of *Clostridium* species, including *C. botulinum*, *C. perfringens* and *C. tetani*. The inhibitory effect of other bacteria was confirmed by the finding that none of the four *C. botulinum* isolates in this study was obtained after enrichment in liquid medium; they were isolated either by direct plating, direct heat or alcohol treatment.

5 ZUSAMMENFASSUNG

Das Ziel war die Isolierung und Identifizierung von *Clostridium botulinum* aus Feldproben von: i) Equine Grass Sickness (EGS)-Fällen (Tier- und Umweltproben) zusammen mit Kontrollproben von Tieren; und ii) Verdachtsfälle auf klassischen Botulismus. Mit Hilfe des Maus Bioassay, MB-ELISA und PCR wurden die Isolate identifiziert. Dabei konnten von 288 untersuchten Proben nur vier Isolate sicher als *C. botulinum* identifiziert werden, die alle nicht aus EGS-Proben stammten. Dieses Ergebnis konnte zeigen, daß *C. botulinum* zumindest nicht ausschlaggebend in GS-Proben ist und seine Verbindung mit dieser Krankheit zweifelhaft bleibt. Zwei der vier bestätigten *C.-botulinum*-Isolate waren vom Typ C, die anderen beiden vom Typ D. Die beiden Typ-C-Isolate stammten aus dem Darminhalt zweier Masthähnchen eines Betriebes, auf dem Botulismus ausgebrochen war; eines der Typ D-Isolate war aus dem Darm eines Rindes mit Botulismus und das andere aus dem Kot eines experimentell mit *C. botulinum* Typ D und anderen Bakterien infizierten Kaninchens. Die Kolonien der zwei Typ-C-Isolate und des Typ-D-Isolates vom Rind sahen unterschiedlich aus und die Zellen können schwärmen, wenn die Agar-Platten nicht ausreichend trocken sind. Deshalb handelt es sich bei diesen Isolaten möglischerweise um unterschiedliche Stämme und wegen des schwärmenden Verhaltens, über das in der Literatur keine Angaben gefunden werden konnten, müssten sie weiter untersucht werden, um festzustellen, ob sie schon früher isoliert wurden.

Toxine von 53 Isolaten wurden mit Botulinum-Antitoxinen im Mäuse-Bioassay neutralisiert. Davon waren 27 von GS-Proben (24 von GS-Fällen und 3 von Kontrollproben). Die neutralisierten Isolate waren Lipase-positive Clostridien (23), Lecithinase-positive Clostridien (23) und *E. coli* (7). Nur vier von diesen (keine Isolate der EGS-Proben) wurden sicher als *C. botulinum* identifiziert. Zehn der Lipase-positiven Isolate waren von GS-Proben (alle von kranken Tieren), zehn der Lecithinase-positiven Isolate waren von GS-Proben (9 von kranken Tieren und eines von Kontrollproben) und alle sieben *E. coli*-Isolate von GS-Proben (5 von kranken Tieren). Vierzehn der 53 Isolate waren mit *C. tetani* kontaminiert, was als Ursache der falsch-positiven Ergebnisse nachgewiesen wurde. Tetanustoxin von verschiedenen *C.-tetani*-Isolaten wurde mit verschiedenen Botulinum-Antitoxinen (A-F) neutralisiert, hauptsächlich jedoch mit polyvalenten Typ ABE (AVENTIS BEHRING)

und Typ CD-Mischungen (OVI). Die Ergebnisse der Neutralisationen von *E. coli*, *C. perfringens*-ähnlichen Bakterien und *C. tetani* sowie die Verbindung dieser Species mit Proben von GS-Fällen, jedoch nicht mit Kontrollproben, wurden als Hinweis auf eine direkte oder indirekte Rolle bei der Entstehung der EGS betrachtet. Ebenso wurde beobachtet, daß Typen und Anzahl einiger anderer *Clostridium* Species häufiger in GS-Proben als in Kontrollproben verkommen.

Die Isolate, die im Mäuse-Bioassay positiv waren, wurden mit Hilfe der PCR untersucht. Nur sechs von diesen waren dabei positiv, drei waren Typ C und drei Typ D. Ein Typ C-Isolat und ein Typ D-Isolat waren Lipase-negativ; dieses Ergebnis var nicht reproduzierbar. 28 Isolate, die im Mäuse-Bioassay mit der Botulinum CD-Antitoxin-Mischung neutralisiert wurden, wurden im MB-ELISA untersucht. Nur drei waren hier positiv dieselben, die auch in der PCR positiv waren. Von diesen waren zwei Typ C, eines Typ D. Der MB-ELISA war spezifisch, aber weniger sensitiv als andere Arten des Nachweises.

Die Ergebnisse der Isolierung und Identifizierung zeigten, daß Botulismus bei Rindern in Deutschland durch die Typen C und D, bei Geflügel durch Typ C hervorgerufen werden kann.

Die vorliegende Arbeit empfiehlt den Gebrauch von Fastidious Anaerobic Agar für die Isolierung von Clostridian, einschließlich *C. botulinum*, *C. perfringens* und *C. tetani*. Der hemmende Einfluß anderer Bakterien wurde dadurch bestätigt, dass keines der vier *C.-botulinum*-Isolate dieser Arbeit nach Anreicherung in flüssigem Medium nachgewiesen werden konnte; sie wurden nur entweder nach Ausstrich, Hitze- oder Alkoholbehandlung isoliert.

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7 APPENDIX

7.1 Media

Fastidious Anaerobic Agar (FAA) (QUELAB, Montreal) (With some modifications)

Formula (g/l):

Peptone from meat	10.0
Peptone from casein	10.0
Yeast extract	3.0
Glucose	1.0
Starch	1.0
Sodium chloride	5.0
Sodium pyruvate	1.0
$NaHCO_3$	0.4
L-Arginine	1.0
L-Alanine	1.0
Cysteine HCl	0.5
Trizma base	0.25
Sodium succinate	0.5
Hemin	0.01
Vitamin K1	0.001
Agar	14.0
Horse blood or egg-yolk emulsion (50%)	25 ml

Fastidious Anaerobic Broth (FAB) is the same as FAA above except that it is without agar and blood or egg yolk.

Fortified FAB (fFAB) is FAB plus either EM pellets (0.5 g/5 ml), CM pellets (0.625 g/5 ml) or BH granules (37.0 g/l).

Reinforced Clostridial Medium (RCM) (MERCK)

	g/l
Yeast extract	3.0
Peptone from meat	10.0
Meat extract	10.0
D-Glucose monohydrate	5.5
Starch	1.0
Sodium chloride	5.0
Sodium acetate	4.93
L-Cysteine HCl	0.55
Agar-agar	0.5

Blood Agar (BA) is the same as RCM above, except that the agar is 15 g/l and plus 5% (v/v) human blood.

Cooked Meat (CM): (DIFCO, Detroit)

CM pellets composition:

	g/l
Beef heart from 454 g	98.0
Proteose peptone	20.0
Dextrose	2.0
Sodium chloride	5.0

Further enriched with:

L-Arginine	1.0
Hemin	0.01
Vitamin K1	0.001
L-Cysteine HCl	0.5

0.625 g of CM pellets were added to 5 ml liquid medium.

Cooked Meat Glucose Starch (CMGS) (Holdeman *et al*., 1977)

CM plus glucose (0.3%), starch (0.1%).

Egg Meat (EM) (DIFCO)

EM pellets composition per litre:

Beef muscle, from	454 g
Egg white, from	6 eggs
Calcium carbonate	5.0 g

The following are further added to it:

Yeast extract	2.0 g
Ammonium sulphate	2.0 g
Glucose	2.0 g
L-Arginine	1.0 g
Haemin	0.01 g
Vitamin K1	0.001 g

0.5 g EM pellets were added to 5 ml liquid medium.

Brain Heart (BH) (MERCK)

BH granules composition:

	g/l
Extracts of brain and heart, and peptose	27.5
D (+) Glucose	2.0
Sodium chloride	5.0
di-Sodium hydrogen phosphate	2.5

37.0 g BH granules per 1l demineralised water.

Further supplemented with:

Yeast extract	5.0

L-Arginine	1.0
L-Cysteine HCl	0.5
Haemin	0.01
Vitamin K1	0.001

Chopped Meat: (Holdeman *et al.*, 1977)

Ground beef (fat free)	500 g
Demineralised water	1000 ml
1 N NaOH	25 ml

Fat and connective tissue have been removed from 500 g lean beef before grinding. Meat, water and NaOH are mixed and brought to boiling with stirring. Mixture is cooled down to RT and fat is skimmed off surface and filtered. To filtrate, sufficient water is added to make 1 litre. To this filtrate, trypticase (30 g), yeast extract (5 g), K_2HPO_4 (5 g) and resazurin solution (4 ml) are added. Then the mixture is boiled to dissolution and cooled. Then cysteine HCl (0.5 g), haemin (0.01g) and vitamin K1 (0.001 g) are added. Meat particles are added to tubes containing 5 ml liquid medium (as 1 part to 4 parts fluid).

7.2 Solutions and buffers

Resazurin solution

25 mg resazurin were dissolved in 100 ml demineralised water. Kept at 4 °C and discarded after 1 month.

Hemin solution

10 mg hemin were vortexed with 1 ml 1 N NaOH in a small plastic tube and added to 1l medium.

Vitamin K1

10 µl vitamin K1 (MERCK) were dissolved into 1 ml 99% ethanol and used as 100 µl per litre of medium.

Lysozyme solution

10 mg lysozyme were dissolved into 10 ml demineralised water and filtered through 0.45 µm filter. The solution added to the sterile liquid medium as 25 µl/5 ml.

Gelatine phosphate buffer

Solution A	
Gelatine	2%
$Na_2HPO_4 \times 2\ H_2O$	50 mM

Solution B	
Gelatine	2%
$NaH_2PO_4 \times H_2O$	50 mM

Solution A and B were mixed together, heated to dissolve gelatine and cooled at RT. The pH was adjusted to 6.2 and the solution sterilized at 121 °C for 15 min.

Phosphate buffered saline

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

pH 7.3

Phosphate citrate buffer

Solution A

$Na_2HPO_4 \times 2\ H_2O$	200 mM

Solution B

$C_6H_8O_7 \times H_2O$	100 mM

Solution A	25.7 ml
Solution B	24.3 ml
Demineralised water	50 ml

pH 5.0

7.3 **Modified Gram stain** (Hucker's modification)

Solutions

1. a. 20.0 g crystal violet
   200 ml ethanol (96-99%)

   b. 8 g ammonium oxalate
   800 ml demineralised water

2. 5 g iodine
   10 g KI
   1l demineralised water

3. 200 ml acetone
   250 ml ethanol

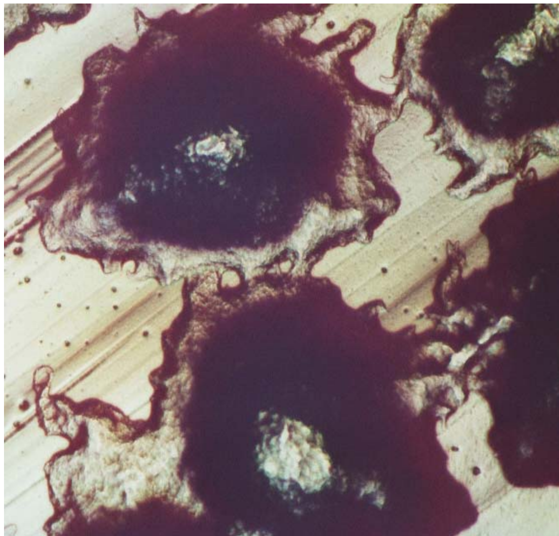
5. 20 ml Ziel-Neelsen carbol fuchsin
   180 ml demineralised water

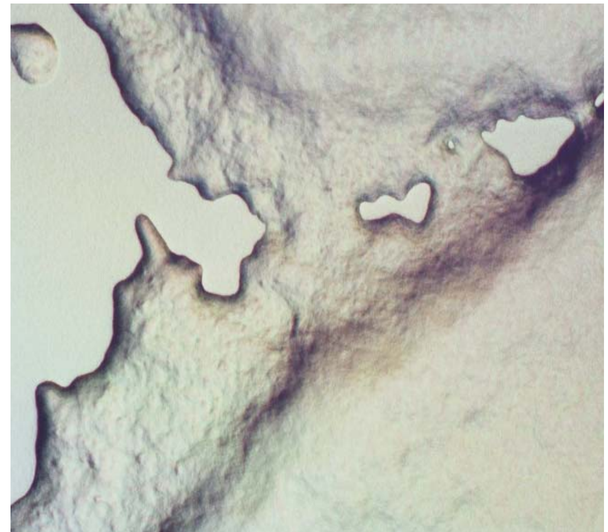
Method

Air-dried and heat-fixed smears are stained by solution 1 (primary stain) for 30-90 s and then washed. Lugole's iodine (solution 2) is added as a mordant for 60-120 s and then washed. Solution 3 is added as a decolourizing agent and the smear is washed. Then solutin 4 is added as a counter stain. The smear is washed, air-dried and examined under oil emmersion lens. Gram positive bacteria stain blue instead of violet (in case of non-modified stain) and the Gram negative bacteria stain red.

7.4 Colonies and Gram-stained smears of some important isolates in the study (colonies magnification is 40x and smears' is 1000x)

7.4.1 Swarming and discrete colonies and smears of the three confirmed *C. botulinum* isolates (Fig. 15a-c)





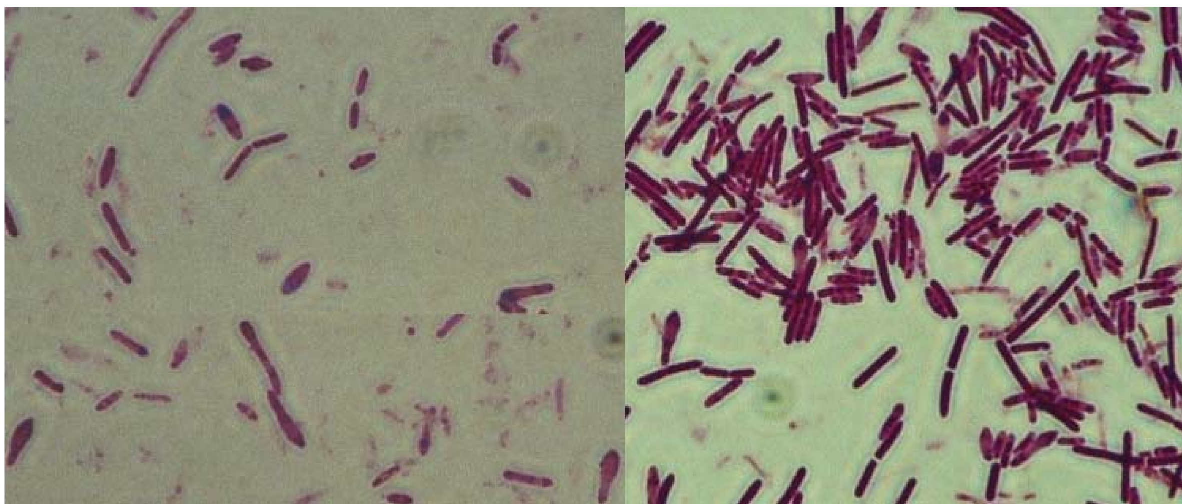
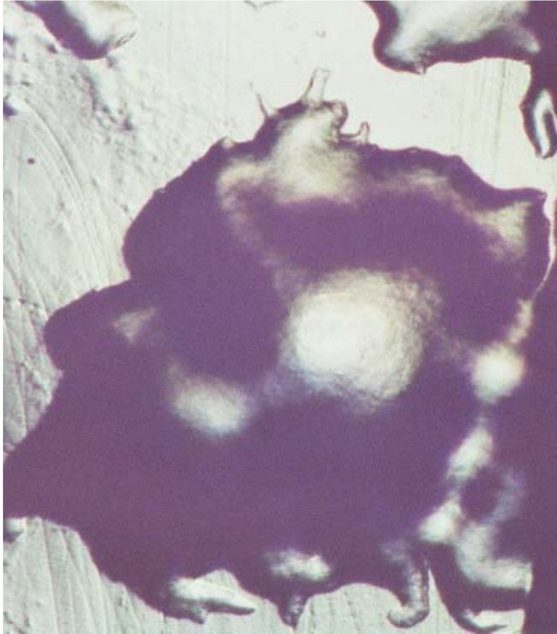
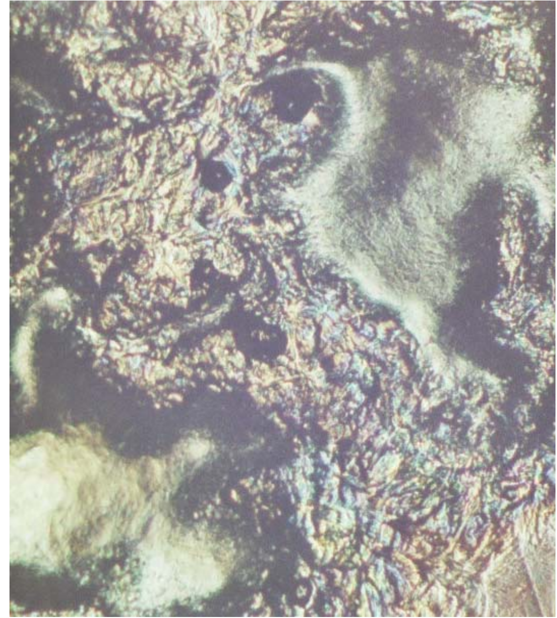


Fig 15a: Colony and smear of one of the two type C isolates from intestines of two broiler chickens

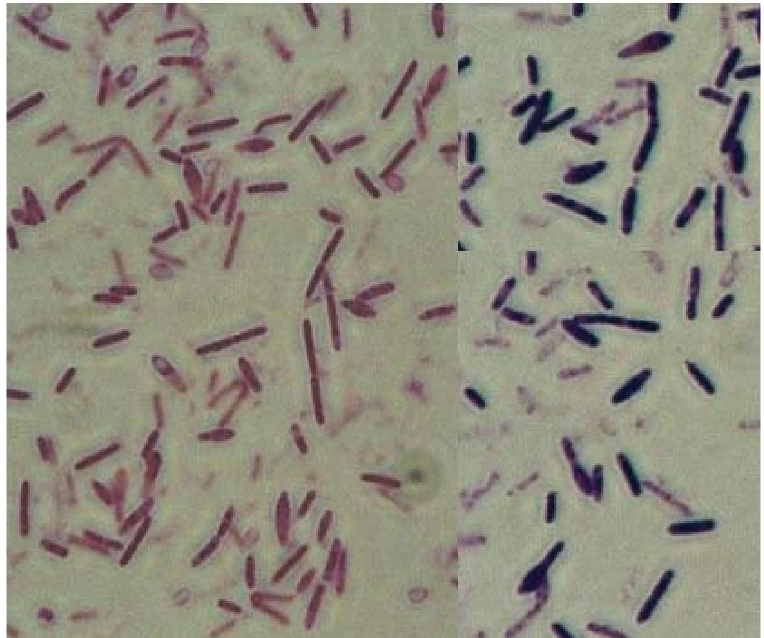
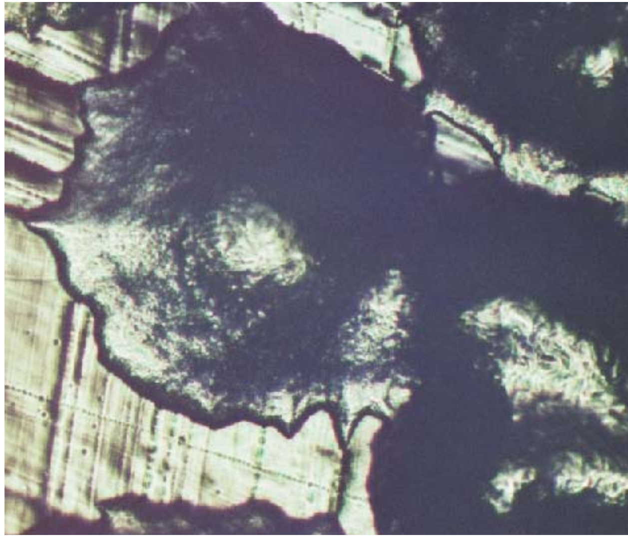
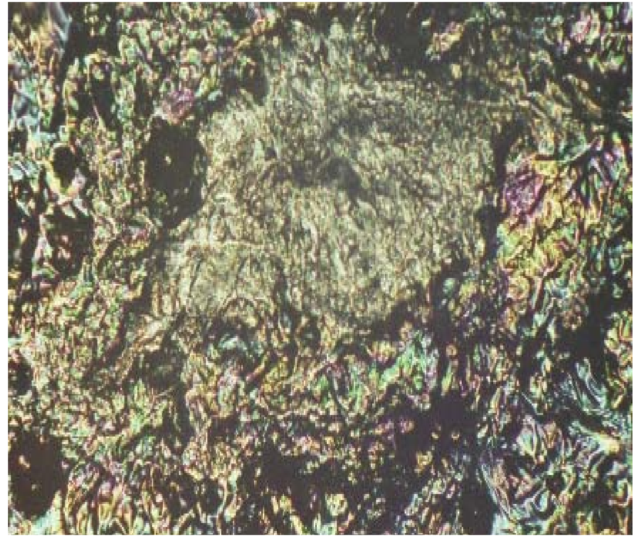
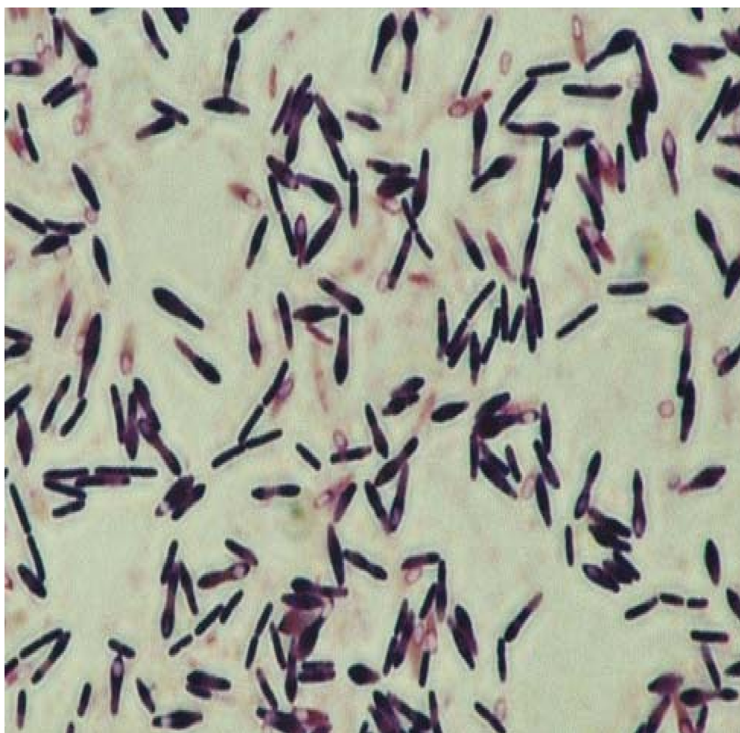


Fig 15b: Colony and smear of one of the two type C isolates from intestines of two broiler chickens







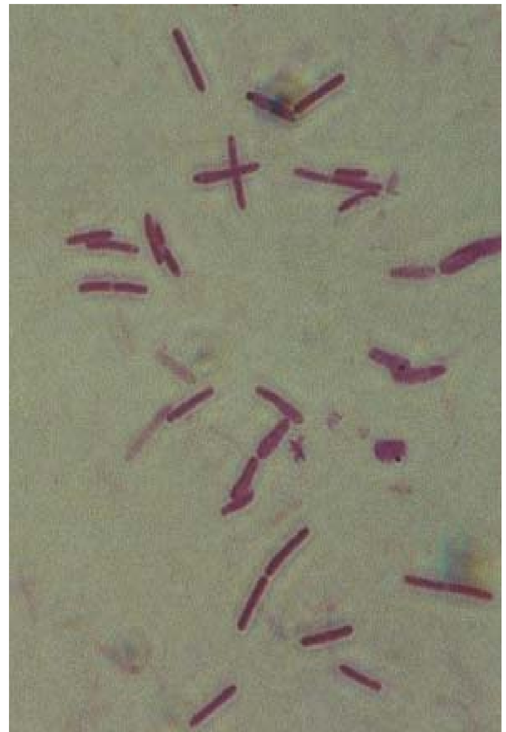
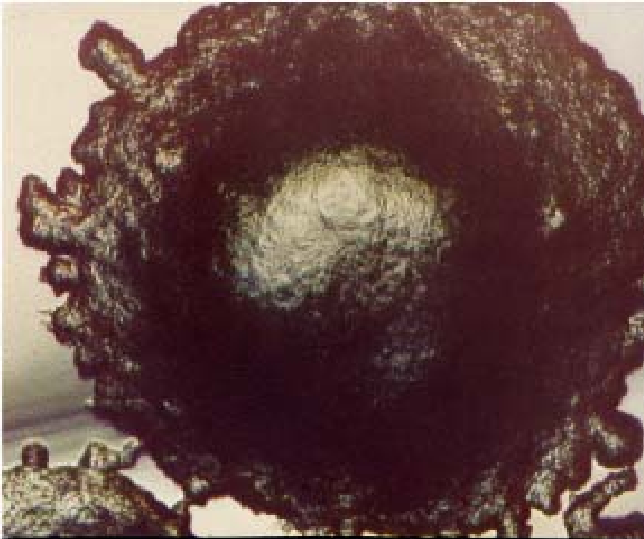
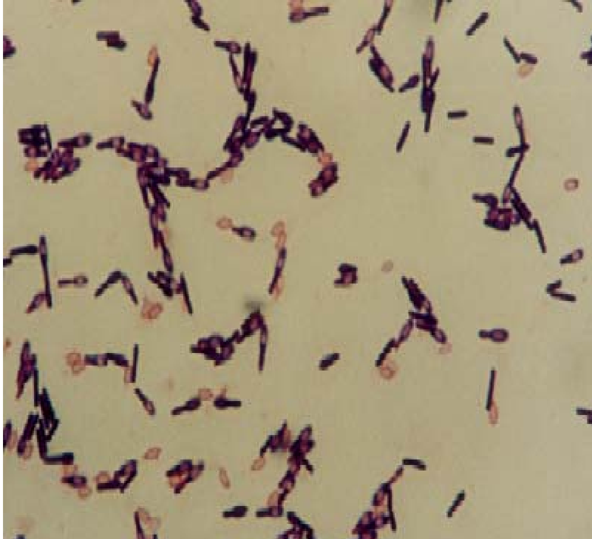


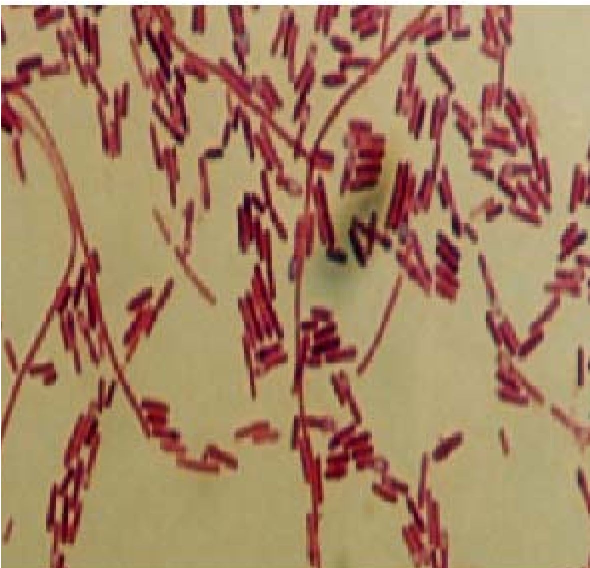
Fig. 15c: Colony and smear of the type D isolate from bovine liver

7.4.2 Colonies and smears of some lipase-positive, lecithinase-positive, *C. tetani* and *E. coli* isolates from EGS samples which had positive neutralization results (Figs. 16-18)



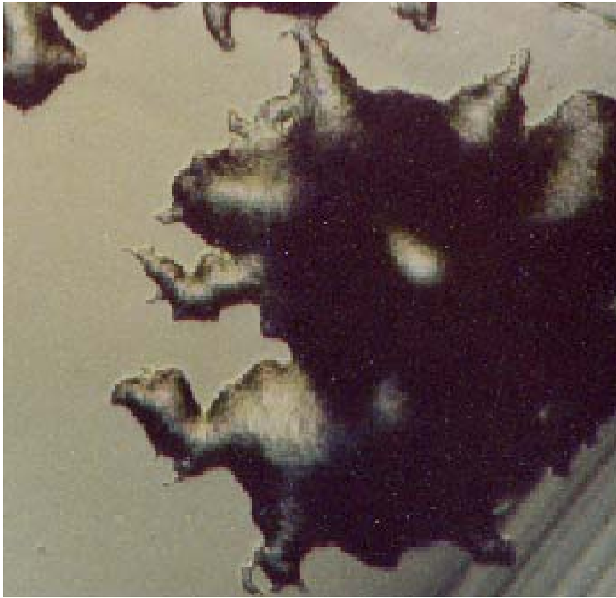


a.

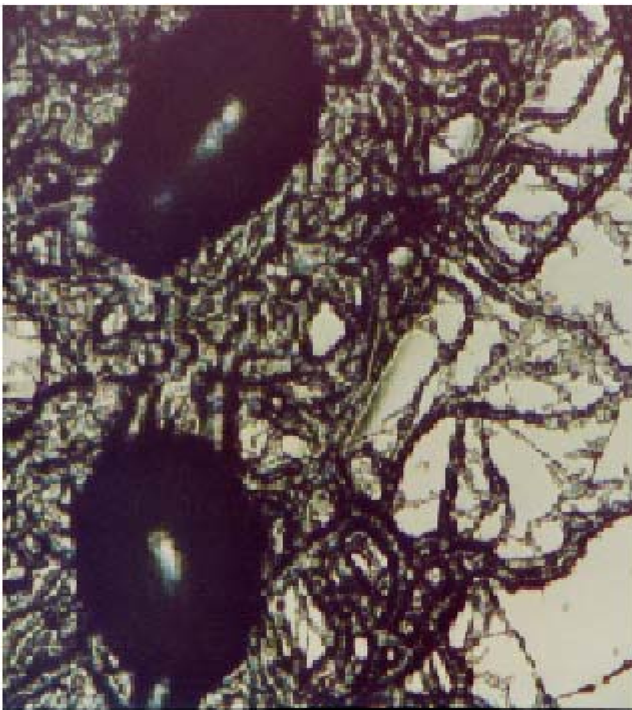


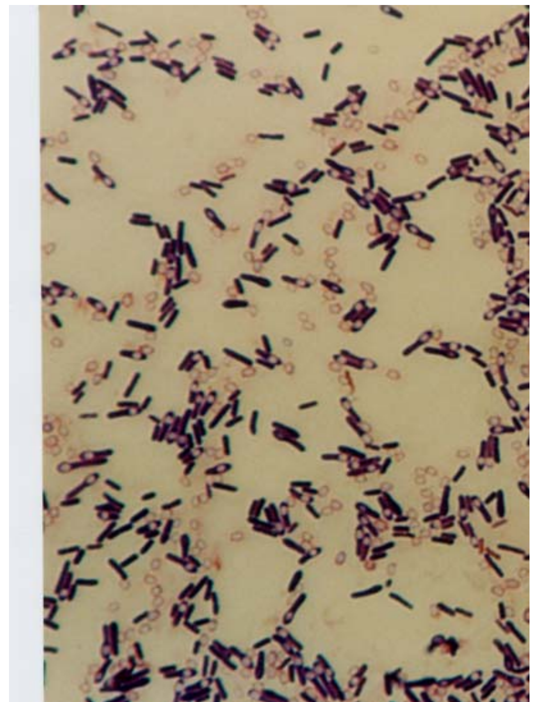
b.

Fig. 16a &b: Colonies and smears of some lipase-positive isolates from GS samples



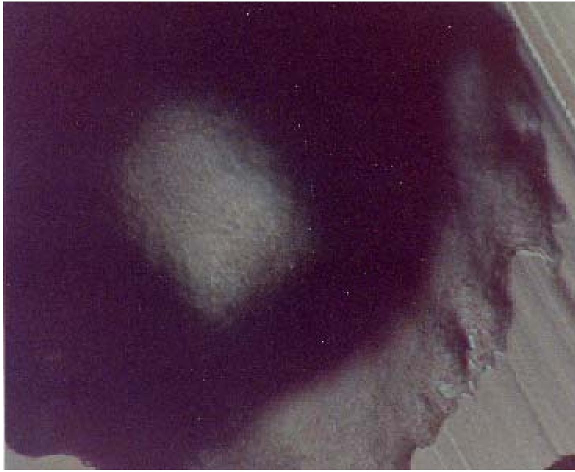
c.

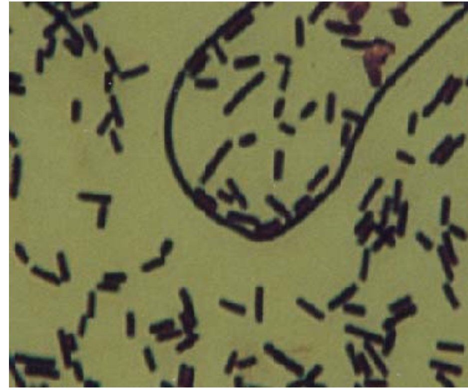




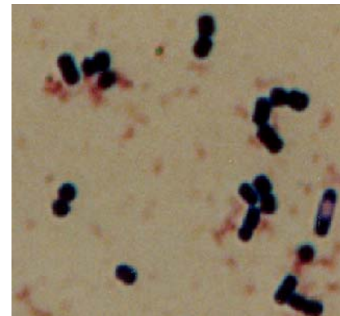
d.

Fig. 16c & d: Colonies and smears of some lipase-positive isolates from GS samples (cont.)

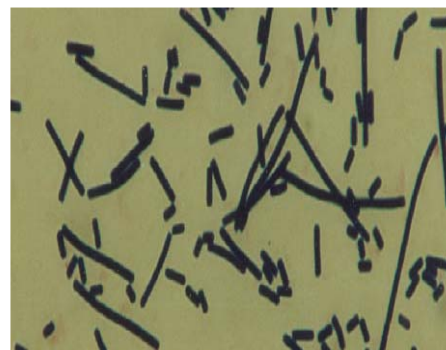




a.



b.



c.

Fig. 17a-c: Colonies and smears of some lecithinase-positive isolates from GS samples

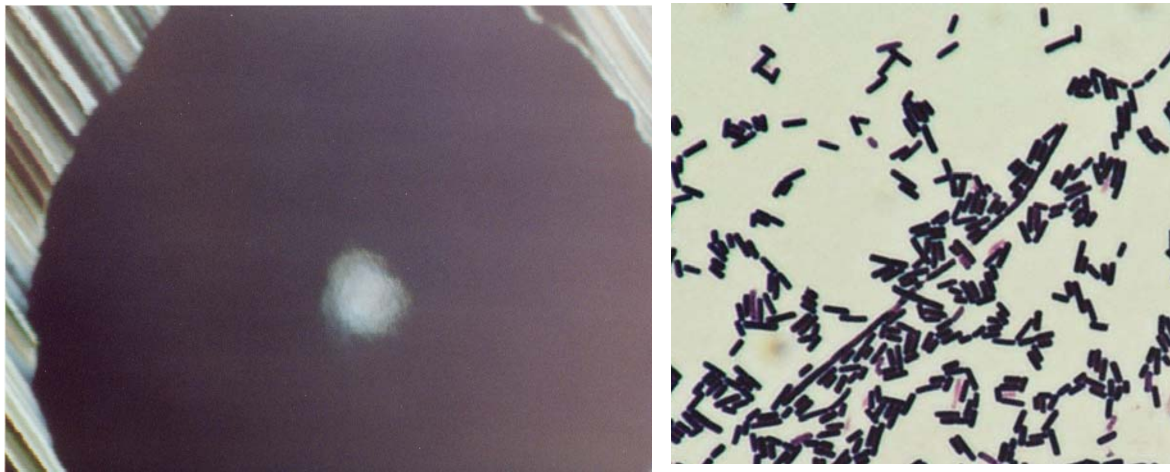
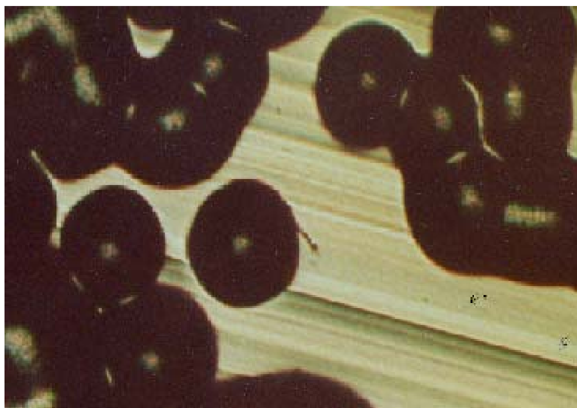
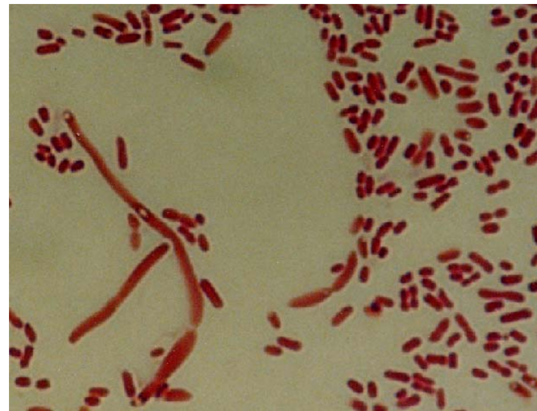


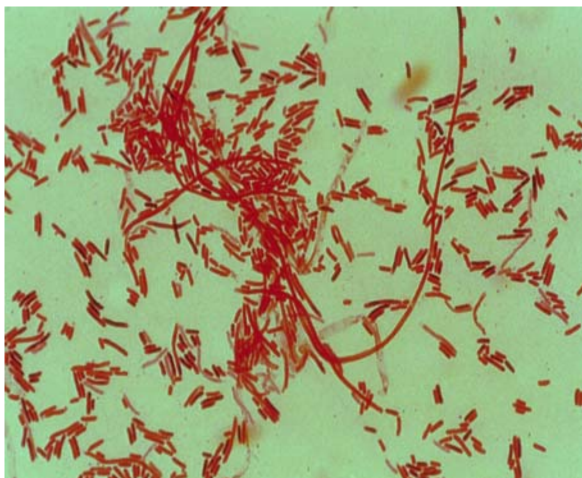
Fig. 17d: Colonies and smears of some lecithinase-positive isolates from GS samples (cont.)



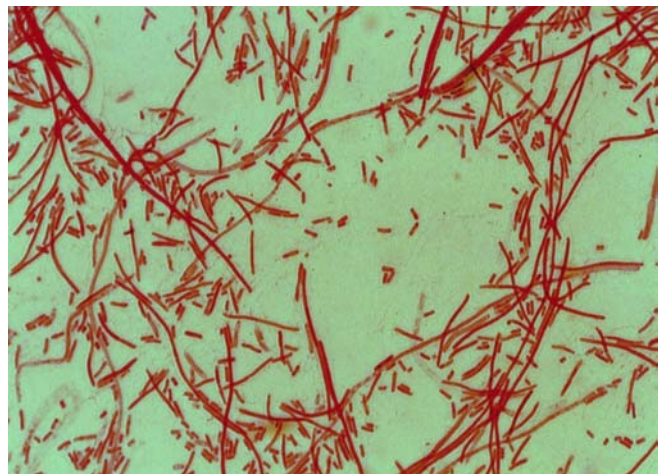
a. Colony



b. Smear from FAA medium (anaerobic)



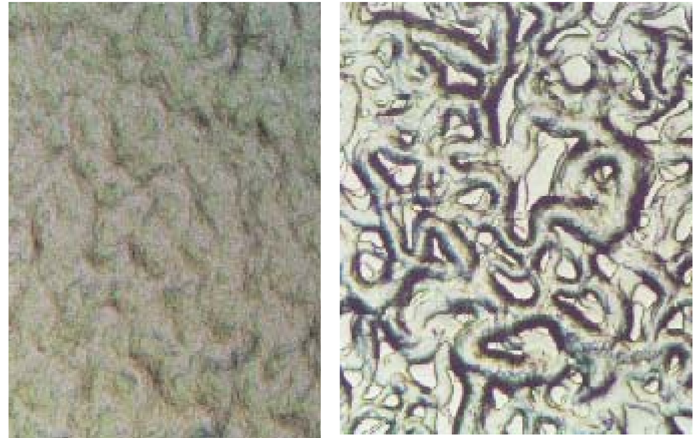
c. Smear from BA medium (anaerobic)



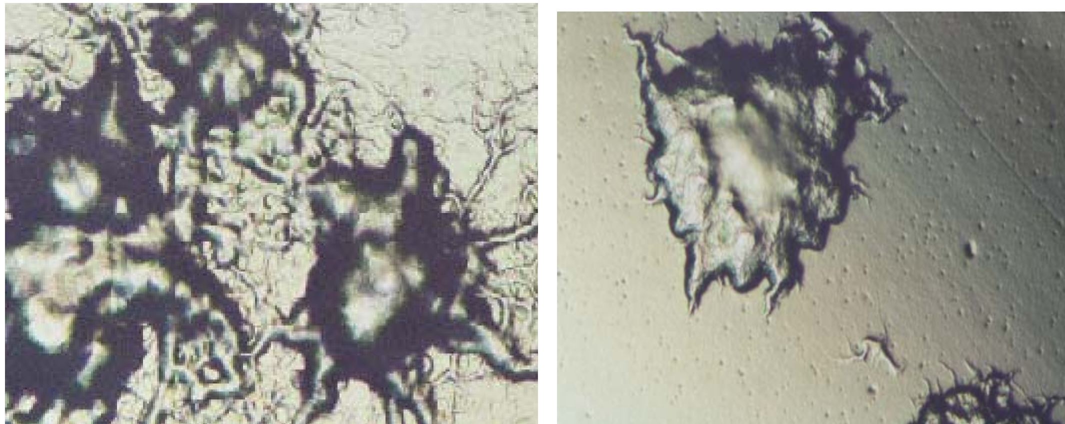
d. Smear from Gassner medium (aerobic)

Fig. 18a-d: Colonies and smears of *E. coli*

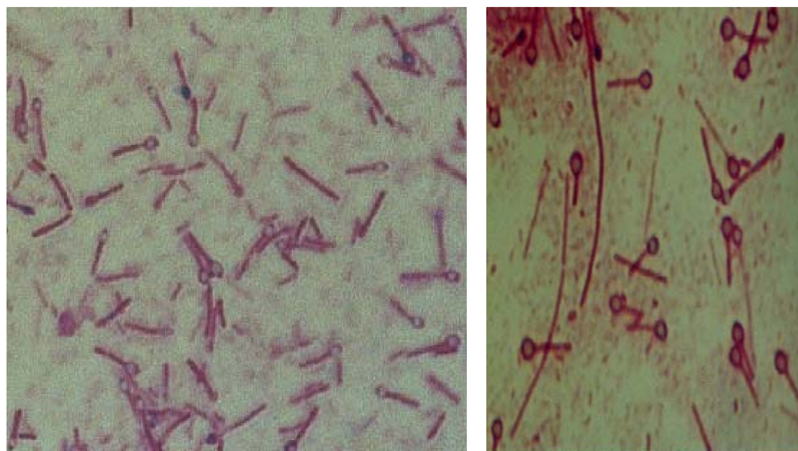
a. Swarming growth (thin film)



b. Swarming growth (thick film)



c. Discrete colonies (0.5 % PE-containing plates)



d. Gram-stained smears

Fig. 19a-d: Colonies and smears of *C. tetani*

7.4.3 Colony and smear of *C. perfringens* isolate from bovine intestine which neutralized by two types of botulinum antitoxin type D and also type D by PCR

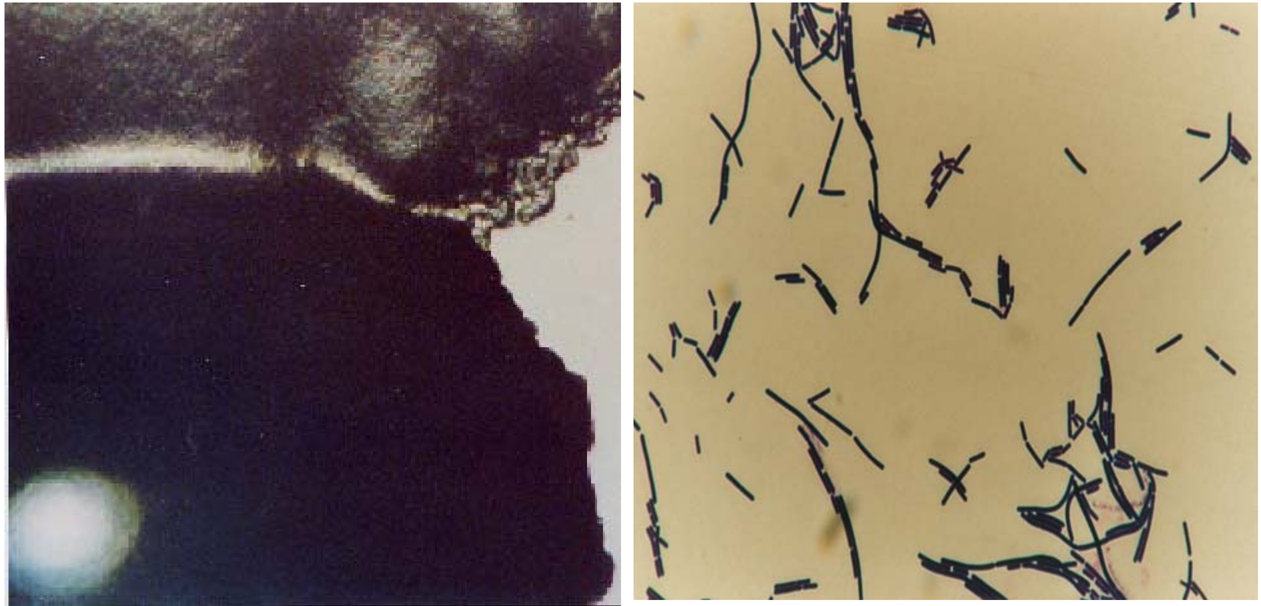


Fig. 20: Colony and smear of *C. perfringens* isolate from bovine intestine

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1980–1984	High secondary school (Gezira, Sudan)

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1998–1999	Master of Veterinary Science (M.V.Sc.), Graduate College, University of Khartoum
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