The role of *Clostridium botulinum* in the aetiology of equine grass sickness and other dysautonomias

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Contents

DECLARATION

The author performed the investigations and procedures described in this thesis unless stated otherwise.

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Abstract

Equine grass sickness is a primary dysautonomia of unproven aetiology that is mainly fatal, untreatable and unpreventable. Much circumstantial evidence has been demonstrated that supports the hypothesis that the disease is caused by a toxicoinfection with *Clostridium botulinum* type C/D. Further evidence supporting this hypothesis and an understanding of the immune response of the horse to this organism and its toxins will facilitate vaccine research and design.

Serum samples from 6-month old ponies (n = 26) were assayed for antibodies against botulinum neurotoxins type C and D (BoNT/C & D) and against surface antigens (SA). Statistically significant rises in specific IgG levels against all three antigens were shown across the sampling period, demonstrating specific antibody acquisition with age. Adult horses (n = 40) were sampled fortnightly for twelve months and assayed for specific IgG against the same antigens. In this group fluctuations in specific IgG levels were observed that corresponded with changes in management.

Seven acute cases of grass sickness that went to postmortem within 36h of onset of clinical signs and four control horses that went to post-mortem had both serum and gut samples taken. Specific IgA was assayed and cases demonstrated significantly more specific IgA against BoNT/C in the jejunum, ileum and caecum (P = 0.04, 0.02 and 0.006 respectively). Against BoNT/D, cases demonstrated significantly more IgA in the pharynx, duodenum and jejunum (P = 0.01, 0.01 and 0.02 respectively) and against surface antigens cases demonstrated significantly more specific IgA in the duodenum (P = 0.01). Serum samples from these acute cases were assayed for specific IgG and IgG subclasses against the same antigens and compared to co-grazing controls. Cases demonstrated lower specific IgG against surface antigens than co-grazing controls (P = 0.06).

Sera from twelve chronic cases of grass sickness were assayed for specific IgG. Six horses that were subsequently euthanased demonstrated significantly lower initial IgG levels against SA (P = 0.05) than those that survived and almost significantly lower IgG levels to BoNT/C (P = 0.06) indicating that immune status at disease onset may be important in survival / prognosis. Longitudinal analysis showed that rising or falling levels of specific IgG had no influence on disease outcome.

An outbreak of feline dysautonomia was investigated by bacteriological and serological techniques. BoNT/C was detected in faeces in seven of eight affected cats after enrichment and also detected in their food. No toxin was detected in the faeces of controls. Specific IgA was also present in significantly higher amounts in cases than in controls (P = <0.001) against BoNT/C and SA. In serological studies, associations were found between low antibody levels and disease in cats but not in dogs, which lacked an age-matched control group.

EGS and dysautonomias of other species appear to be associated with toxicoinfections with *C. botulinum* types C and/or type D. As such, a vaccine, consisting of both surface antigen and toxin components, would play a vital role in protecting animals against this distressing disease.

Publications

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Presented Papers

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An association between serological titres against botulinum neurotoxins and feline dysautonomia. BSAVA Congress 2004, Birmingham, April.

Posters

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Abbreviations

ADP	Adenosine diphosphate
AGS	Acute grass sickness
AHT	Animal Health Trust
ANOVA	Analysis of variance
AP	Alkaline phosphatase
BoNT	Botulinum neurotoxin
C2C1	C2 toxin: gene component 1
C2C2	C2 toxin: gene component 2
CD	Canine dysautonomia
CGS	Chronic grass sickness
CNS	Central nervous system
CMB	Cooked meat broth
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EGS	Equine grass sickness
ELISA	Enzyme linked immunosorbent assay
FAA	Fastidious anaerobe agar
FCS	Foetal calf serum
FD	Feline dysautonomia
GALT	Gut associated lymphoid tissue
GI	Gastrointestinal

GLC	Gas liquid chromatography
GUVS	Glasgow University Veterinary School
HA	Haemagglutinin
H+L	Heavy and light chains
HRP	Horse-radish peroxidase
IgA	Immunoglobulin A
ICC	Insterstitial cells of Cajal
IFN-α	Interferon gamma
IgG	Immunoglobulin gamma
IGHC	Immunoglobulin heavy chain constant gene
IL	Interleukin
IM	Intramuscular
IP	Intraperitoneal
IV	Intravenous
KCl	Potassium chloride
kDA	kilo Dalton
KGS	Key-Gaskell syndrome
LAH	Large animal hospital
MPRL	Microbial Pathogenicity Research Laboratory
NaCl	Sodium chloride
NTNH	Non-toxic non-haemagglutinin
OD	Optical density
PBS	Phosphate buffered saline
PBS-TG	Phosphate buffered saline with teleostean gelatine

PMBC	Peripheral blood mononuclear cells
PMSF	Phenylmethylsulphonyl flouride
RME	Rabbit mucoid enteropathy
RNA	Ribonucleic acid
SA	Surface antigens
SD	Standard deviation
SNAP	Soluble NSF fusion protein
TetNT	tetanus neurotoxin
TetSA	Surface antigens from *C. tetani*
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor-alpha
TNF-β	Tumour necrosis factor-beta
VAMP	Vesicle associated membrane protein

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Chapter 1

Introduction

1.1 *Equine Grass Sickness (EGS)*

Equine grass sickness is a primary dysautonomia of equids which is generally fatal and unpreventable. Although first recognised in 1909 at an army camp in Scotland the aetiology of the disease remains unproven. The disease has spread throughout Great Britain, with a higher incidence in Scotland (Milne et al, 1994), and also occurs in Scandinavia and other Northern European countries (Obel, 1955, Gilmour, 1987). An equine dysautonomia, known as mal seco (dry sickness), occurs in Argentina and Chile and is accepted as being the same as EGS (Uzal and Robles, 1993). Other documented cases include cases in the Falklands (Wood and Gilmour, 1991) and Australia (Stewart, 1977). Although previously rare in Southern Ireland, incidence there appears to be increasing (U. Fogarty, personal communication).

Primary dysautonomias are characterised by severe neuronal degeneration and neuronal loss in the enteric and autonomic nervous systems. The clinical, pathological and epidemiological features of the disease are now well defined and it is widely accepted that the disease is caused by a toxic insult (Scholes et al, 1993; Griffiths et al, 1994; Doxey et al, 1995) with severity of clinical signs reflecting the extent of neuronal damage (Doxey et al, 1992).

1.1.1 *Clinical signs and diagnosis*

Classified on the basis of clinical signs and duration of the disease, EGS can present in four overlapping forms-per acute, acute, sub acute and chronic (Doxey et al, 1991). Mortality rate approaches 90% (Pogson et al, 1992) with all but a few of the milder chronic cases dying or being euthanased. Per acute cases, the most severe form of EGS, demonstrate sudden onset of clinical signs with death or euthanasia within 24 hours, and acute cases within 48 hours. Subacutes can survive up to seven days with chronic cases lasting for several months before euthanasia or recovery.

The disease can present with a wide range of clinical signs which reflect the extent of neuronal damage. EGS is characterised by gastrointestinal (GI) dysfunction, the significance of which is thought to be responsible for the high mortality rate (Scholes et al, 1993). Depending on the form of EGS, animals present with varying states of GI stasis/dysfunction, dysphagia and colic. No one clinical sign is diagnostic of one form of EGS and many of the clinical signs can indicate other conditions/diseases, making diagnosis difficult for the inexperienced, particularly in acute/early stages of disease.

Some clinical signs typical of acute, subacute and chronic cases include GI stasis/reduced gut motility, dysphagia, ptosis, muscle tremors and patchy sweating (Doxey et al, 1992). Large fluxes of water and electrolytes occur across the walls of

the equine alimentary tract and stasis and the subsequent inability to regulate these fluxes can swiftly compromise the horse's health (Cottrell, 1999). Acute cases can demonstrate complete GI stasis with impaction in the large colon, fluid accumulation in the small intestine and stomach and gastric reflux is a characteristic of AGS. Acute cases can be depressed, tachycardic and exhibit hypersalivation although this may be attributable to the inability to swallow rather than true hypersalivation (Cottrell et al, 1999).

Dehydration is also demonstrated, brought about by the inability/inefficiency of swallowing. The inability of an animal requiring 10-15 litres of water daily to drink could lead to the preferential transfer of fluid from the rest of the body to the stomach and small intestine (Doxey et al, 1991Cottrell et al, 1999) resulting in severe dehydration.

Signs in the subacute form are less severe, though similar to those demonstrated in the acute form. Disease progression is accompanied by a rapid loss of weight and condition. A 'tucked up' abdomen which can be especially pronounced in chronic cases and which has been likened to that of an emaciated greyhound (Edwards, 1987; Gilmour, 1987; Pinsent, 1989). Chronic cases can also develop rhinitis sicca, base narrow stance-muscle tremors and patchy sweating can be observed in all forms of the disease.

A definitive, non-invasive diagnostic test for EGS is not yet available despite much research effort. Diagnosis is based on clinical signs with confirmation post-mortem

histopathological examination of autonomic ganglia. There has been some level of disagreement between research groups as to the accuracy of diagnosis based upon clinical signs. Scholes et al (1993) held the view that ileal biopsy is essential for accurate diagnosis and Doxey et al (2000) having the view that histological diagnosis is not essential. This latter view was supported by examination of ganglia of recovered chronic grass sickness cases that had later died or been euthanased due to unrelated causes. Not only had the initial diagnosis of GS been correct, it also demonstrated that these horses had suffered neuronal loss to such an extent, it cannot be understood how such cases were able to lead relatively normal lives (Doxey et al 2000).

Research into non-invasive diagnostic tests include the use of barium swallows (Greet and Whitwell, 1986); analysis of peritoneal fluid (Milne et al, 1990); clinical signs in conjunction with biochemical testing (Doxey et al, 1991); detection of acute phase proteins from serum (Milne et al, 1991); the use of phenylephrine to correct ptosis in GS cases (Hahn and Mayhew, 2000) and urinalysis (Fintl et al, 2002).

No pathogonomic outcome was identified in any area although most are useful in conjunction with clinical signs and case history. Of twenty-five suspected cases of EGS given a barium swallow to help detect abnormal oesophageal motility, eighteen that demonstrated impaired motility were histopathologically diagnosed as having EGS (Greet and Whitwell, 1986). Parallel studies in cats with feline dysautonomia (FD) have demonstrated abnormal motility in clinically normal cats, suggesting that

more work is required to identify what constitutes normal oesophageal movement (Cave et al, 2003).

Peritoneal fluid of a high specific gravity and total protein concentration but without blood-staining or marked alkaline phosphatase activity can help differentiate the acute or subacute GS case from a surgical colic. The high protein concentrations and specific gravity of GS peritoneal fluid may be accounted for in part by dehydration, which is common in GS cases although total serum protein does not show such a marked increase. The colour of peritoneal fluid in GS cases is often orange/dark yellow, thought to be caused by increased serum bilirubin levels demonstrated in GS cases (Milne et al, 1990). Doxey et al (1991) examined clinical signs and biochemical testing including tests to show if proposed possible vitamin or mineral deficiencies may be implicated in GS. Both magnesium and selenium deficiency was ruled out and levels of vitamins B_{12} and E were within normal ranges, supporting evidence that GS occurs in horses in good bodily condition rather than those that are run down.

The presence of certain acute phase proteins detected in the sera of GS cases was first reported by Johnson et al (1983) who demonstrated that GS cases had a two to three fold increase in serum haptoglobin, a protein whose role as an acute phase protein is thought to be involved in inhibition of lysosomal endopeptidase (Milne et al, 1991), compared to a four to five fold increase in horses with acute inflammatory conditions. However cases were grouped together with no consideration for differences in severity of clinical signs or time line of the disease. Milne et al (1991)

evaluated levels of four acute phase proteins - haptoglobin, orosomucoid, ceruloplasmin and α2-macroglobulin - in cases grouped into acute, subacute and chronic cases of grass sickness. Controls consisted of clinically normal animals, colic cases and those horses with acute inflammatory disorders such as cellulitis and post-surgery cases. Levels of all four proteins were elevated at different disease stages but ceruloplasmin and α2-macroglobulin levels may have been elevated due to dehydration. Further work was required to evaluate levels of the other proteins in colic cases and a new study is in the planning stage (E. Milne, personal communication).

Phenylephrine eye drops are used to diagnose Horner's Syndrome in small animals and humans. Horner's syndrome leads to ptosis of the upper eyelid due to paralysis of the cervical sympathetic nerve supply (Blood, 1999). Phenylephrine is an α-adrenergic stimulant used to induce mydriasis (Blood, 1999). In GS cases, low concentrations of phenylephrine drops can help correct ptosis for extended periods of time in GS cases as opposed to controls and demonstrates a sensitivity and specificity of 75% (Hahn and Mayhew, 2000).

Urinalysis showed that acute EGS cases demonstrated significantly higher protein and creatinine concentrations, higher blood and urine glucose concentrations, higher specific gravity and lower pH than controls (Fintl et al, 2002). However, none of these findings are pathogonomic for EGS, all occurring in other disorders and therefore are only useful in supporting a diagnosis of EGS used in conjunction with clinical signs.

1.1.2. *Treatment and recovery*

Per acute, acute and subacute cases of GS are invariably euthanased on humane grounds although certain selected cases of chronic grass sickness do recover and return to full work, something thought impossible previously (Greig,1942;Pinsent,1989) . Cases are selected on the basis of demonstration of comparatively milder symptoms, particularly in reference to appetite, dysphagia, colic, gut motility and rhinitis, those cases having a better chance of survival (Milne et al, 1994). The foundation of treatment is intensive nursing care with lots of human contact and certain relapsed cases that have been discharged and readmitted can be attributed to a lack of the intensive nursing required (Doxey et al, 1999). The feeding of moistened, palatable hard feed is also utilised to facilitate swallowing, stimulate appetite and help with nutrition. Cases usually take many months to recover and are hospitalised until their weight is stabilised. Often the animal will undergo periods of recovery followed by temporary periods of ill health (Doxey et al, 1995) and it is important not to work the animal too hard too soon. The nursing and management regime at Easter Bush Veterinary Centre has increased the survival rate to 70% of selected cases.

Cisapride is a prokinetic drug that did demonstrate some therapeutic benefit in the treatment of CGS cases (Milne et al, 1996). The drug worked by facilitating acetylcholine release from postganglionic nerves of the myenteric plexus in the gut. Unfortunately the drug was withdrawn from the market.

Certain supplementary treatments have been assessed (Fintl and McGorum, 2002) namely Brotizolam, a potential appetite stimulant; acetylcysteine, to correct the reduced levels of plasma cysteine found in cases and aloe vera, which has laxative, antioxidant and anti-inflammatory properties. The study was inconclusive mainly due to the mix of severity of the chronic GS cases used and the lack of a more stringent trial such as a blinded placebo-controlled trial which was not considered on humane grounds.

1.1.3. *Epidemiological studies*

The first recognised outbreak of GS occurred in a Scottish army camp in 1909 (Tocher et al, 1923) although it is possible that the disease occurred unrecognised and unreported before this (McKay, 1958). The lack of an identified aetiological agent has led to many epidemiological studies that have confirmed/disproved many observations about the disease.

After the outbreak described in the Barry Camp, the disease spread throughout North and South Eastern Scotland (McCarthy et al, 2001) and from 1911, the disease had been reported in the majority of counties in England and Wales (Greig, 1942). It was well reported in Northern Europe including Sweden and France (Bendixen 1946; Lhomme et al, 1996 referenced in McCarthy et al, 2001) and Denmark (Obel, 1955) among others. Apart from the pathologically identical disease, mal seco, in South America (Uzal et al, 1993) worldwide histopathologically confirmed cases have only

been reported in Australia (Stewart, 1977) and the Falkland Islands (Woods et al, 1991).

Epidemiological publications involving interviews/questionnaires date back to 1958 (McKay 1958) although only those from 1974 onwards use statistical analysis (McCarthy et al, 2001). Among those risk factors found to be relevant are younger age (Greig, 1942; Gilmour and Jolly, 1974; Doxey et al, 199; Barrett et al, 1992; Wood et al, 1998; Newton et al, 2004), horses kept outdoors (Doxey et al, 1991; Wood et al, 1998), time on pasture/premises (Greig,1942; McKay 1958; Gilmour and Jolly, 1974; Wood et al, 1998), association of the pasture with previous cases (Doxey et al, 1991; Wood et al, 1998; Newton et al, 2004), spring (Begg 1936; Guthrie, 1940; Doxey et al, 1991; Wood et al, 1998; Newton et al, 2004).

EGS was long believed to be a disease mainly of working horses and ponies and research suffered from a lack of funding due to this misconception. The prevalence of the disease in such animals is more related to the fact that these types of horses spend more time at grass than sports horses and thoroughbreds. Wood et al (1998) found that draught horses were seemingly more at risk from the disease when using a univariate analysis but that the relationship was no longer significant following adjustment for confounding variables, such as time spent at grass. Certainly another centre of high incidence in the UK is in the Eastern Counties of the UK, Newmarket in particular, due in part to the high concentration of horses and mares visiting studs and thus being subjected to change of pastures and conceivable stress. Certainly, some high profile cases such as the racehorses Mr Baileys, who recovered from

chronic grass sickness and Dubai Millenium, who was euthanased due to acute grass sickness, have demonstrated that this disease can affect any horse, even those with limited access to grass.

Certain weather conditions are thought to have been conducive to GS outbreaks, namely warm dry weather with ground frosts whereas wet weather was associated with a decrease in incidence (Tocher, 1924; Pool, 1928; Greig, 1942). Greig (1942) found no association of weather conditions with the disease although Doxey et al (1991), emphasising the local character of Scottish weather, found that the disease was associated with cooler, dry weather with intermittent ground frosts. In a UK-wide study Wood et al (1998) demonstrated that 66% of cases occurred following predominately dry weather.

More recent epidemiological studies have utilised case-control studies and have concentrated on the risks of certain premises (McCarthy et al, 2004a). The McCarthy (2004b) study will be further discussed in section **1.2.1**. McCarthy (2004a) identified two novel risk factors for increased EGS risk at certain premises with soil disturbance and increased soil nitrogen, as well as premises with previous cases of GS-previously reported by Wood et al (1998). McCarthy et al (2004) hypothesize that a high level of soil nitrogen influences the amount of herbage available to the horses present, which may be analogous to dietary change and may even be the long sought after dietary trigger.

Epidemiological findings indicate an infectious aetiology. The increased risk in younger animals, decreased risk in those horses having had prior contact with a GS case and those having spent longer periods of time on a certain premises/pasture and the increased incidence of the disease at certain times of year all imply that a protective immune response to the aetiological agent occurs.

1.1.4 *Aetiology*

Identification of the causal agent of EGS is imperative to enable treatment, intervention strategies and its prevention. Many possible putative agents have been investigated since the disease was first recognised including fungi, chemical toxins, poisonous plants and toxin-producing bacteria.

Plants

Due to the obvious association with grazing, toxic plants were investigated very early in the history of the disease. Tocher et al (1923) did not find any plant known to be toxic to horses on any GS associated premises. Alsike clover was thought to be involved but extracts of the plant were not toxic when fed to horses (Tocher, 1924; Greig, 1942). Botanical surveys found no association of any one plant common to all GS pastures (Greig 1942; Robb et al 1997). Uzal et al (1996) tried reproducing the disease by feeding *Festuca argentina*, a plant found in the diet of nine mal seco cases, but failed to reproduce clinical signs or pathology.

Fungi

Cultures of fungi found on GS pastures have also been tested and failed to reproduce the disease (Greig, 1942) and no one particular species of fungi has been isolated from GS cases (Doxey et al, 1990). It was thought that an endophytic fungus of *Festuca argentina* might be responsible but these fungi have not been found on all GS pastures and were identified in less that 20% of plants from GS and mal seco-associated pastures (Robb et al, 1997a).

Robb et al (1997a) identified *Fusarium* species on all GS associated pastures in the UK and in Patagonia that are capable of producing mycotoxins that were found to be cytotoxic to equine nerve cells *in vitro* (Robb et al, 1997b). Dosing horses in Patagonia with cultures of these fusaria isolates failed to produce clinical signs or pathological changes (Uzal and Robles, 1997). They hypothesised that exposure to fusarial toxins could be a predisposing factor to GS as they are known to alter GI flora, affect the immune system and affect vitamin metabolism. As they had also demonstrated that fusaria can out-compete other fungal species in dry weather conditions the evidence supporting their hypothesis was promising encompassing presence of the agent, cytotoxicity and a possible association with the weather patterns associated with GS outbreaks.

Abnormal plant biochemistry and oxidative stress.

The role of oxidative stress in GS cases has been under investigation for several years. Oxidative stress can result from an excess of free radicals or from a depletion of antioxidants and neurons are particularly sensitive (McGorum et al, 1998). McGorum et al (2000) hypothesised that EGS is associated with ingestion of plants under metabolic stress-due either to abnormal climatic conditions or fungal colonisation. Plants immediately collected from a pasture after an outbreak of GS demonstrated reduced antioxidant and weak pro-oxidant properties when compared to control plants which consisted of plants from GS pastures out of GS season and plants from GS pastures grown in optimal conditions. However, these biochemical changes could not be reproduced under experimental drought and chilling conditions.

Although the authors thought it unlikely that the levels of metabolites or the pro-oxidant levels detected could be responsible for neuronal damage directly, it was suggested that the stressed plants might play a part in onset of the disease by altering the equine intestinal environment thus allowing overgrowth of some pathogen; in conjunction with a neurotoxic metabolite not detected in the study; or if the metabolic stress is caused by a plant pathogen capable of producing a neurotoxin. A further study (McGorum et al, 2003) showed that EGS horses demonstrated no evidence of systemic macromolecular damage although the involvement of oxidative macromolecular damage local to neurons could not be precluded and further investigation is warranted.

Cyanogens

Grass sickness has been linked to ingestion of cyanogens either from white clover or linseed (Gordon, 1934; Greig, 1942; Lannek et al, 1961) but had not been proven (McGorum and Anderson, 2002). Following their previous work which demonstrated that plasma amino acid profiles in EGS cases resemble those found in of subacute and chronic cases of cyanide toxicity (McGorum and Kirk, 2001), a further study measuring whole blood cyanide and plasma and urinary thiocyanate showed that co-grazing controls demonstrated higher levels of these compounds than other controls, indicating increased exposure to cyanogens. These increased levels were not found in clinical cases although they could have been exposed before the onset of anorexia-thiocyanate is rapidly eliminated. The animals also may be unable to synthesise thiocyanate from cyanide. Further investigation to elucidate any causal relationship between cyanogens and GS is required.

Bacterial

Several potential bacterial causes of GS have been investigated following detection of specific organisms/toxins from clinical cases. Gaiger (1922, cited in Greig, 1942) isolated a diplostreptococcus from the CNS of an acute case. Attempts to reproduce the disease by injection of the organism failed. *Clostridium perfringens* type D was also investigated following detection of the toxin in several acute cases however, both a challenge and a protection study failed.

Ochoa and de Valendia (1978) demonstrated a positive relationship between

C. perfringens type A and sera from recovered CGS cases using seroneutralisation techniques. However, Gilmour et al (1981) did not reproduce these findings using sera from Scottish cases of GS indicating that the disease in Columbia was not the same due to certain clinical and pathological differences.

Tocher et al (1923) hypothesised a link between *C. botulinum* and GS, with botulinum neurotoxin produced *in vivo* and this hypothesis is further detailed in section **1.2.1**

Others

A review by Greig (1942) was able to disregard plant poisoning, anaphylaxis, toxic chemical production at pasture and poisonous products of intestinal origin as the cause of EGS. The same study also disregarded invasive and/or toxin producing bacteria and protozoal causes. Viruses have also been suggested as a possible cause of EGS-the nature of the initial spread of the disease was suggestive of some sort of insect vector-however investigations into an insect vector was negative (Greig, 1942) and a virus has not been isolated.

Selenium deficiency in cattle can be a problem in Scotland, leading to muscular dystrophy. It was suggested that selenium deficiency was indicated in GS when it was noted that cases often occurred on farms where selenium deficiency was a problem. Selenium levels were found to be low in cases and controls from the same premises (MacPherson, 1978).

1.1.5 *Evidence of a toxic aetiology*

It is now widely accepted that EGS is caused by a toxin that is probably produced enterically rather than ingested (Scholes et al, 1993; Griffiths et al, 1994; Doxey et al, 1995). Peritoneal injection of serum and serum fractions from acute GS cases have resulted in pathological changes in healthy horses although not in clinical disease (Gilmour, 1973; Gilmour and Mould, 1977).

Gel filtration and saturated ammonium sulphate precipitation were used to prepare plasma fractions of acute serum that was then injected intraperitoneally into experimental ponies (Gilmour and Mould, 1977). Another pony was given an oral dose of serum with known neurotoxicity and the same preparations were also administered to laboratory small animals. No clinical disease was demonstrated in any animal and histopathological changes were only observed in ponies, not in the small animals. No pathology was observed in the pony that was dosed orally. The lack of changes in this pony was attributed to inactivation/non-absorption in the alimentary tract, too small a dose or that the toxin is acquired by another route or is the result of the disease process. They established that the toxin is proteinaceous and/or bound to a protein in the high molecular weight (greater than 30 kDa) category. Every acute serum tested proved neurotoxic reproducing the histopathology typical of grass sickness.

Griffiths et al (1994) injected acute GS serum into the parotid salivary gland of ponies. The salivary gland was chosen due to ease of administration and because this gland receives its sympathetic innervation from the ipsilateral cranial cervical ganglion. The contralateral gland and ganglion was used as a control. Clinical disease was not reproduced but 4 out of 5 batches of acute sera reproduced chromatolytic changes in the ganglia with the most dramatic changes produced from sera from cases that were sampled within 12 hours of onset of clinical signs. One batch of the neurotoxic serum was fractionated and the filtrate and retentate (containing components of more that 30kDA) were injected into the same pony, into opposite glands. Only the retentate produced chomatolytic lesions. They hypothesised that the putative neurotoxin in GS gains access to the axon terminal and is then retrogradely transported to the neuronal perikaryon. An inflammatory reaction was observed in the ganglion of the pony that received sera from a chronic grass sickness case of three weeks duration. The lack of any similar lesions elsewhere in this pony was suggested to be due to some serum component - possibly due to antibodies produced to the toxin component by the donor animal. The authors go on to suggest that the majority of damage to neurones could happen within 1 to 2 days from exposure to toxin.

Johnson (1985) discovered a small molecular weight compound in sera of acute GS cases using solvent extractions and thin-layer chromatography. Extraction was carried out on serum samples that had known in vivo toxicity but no toxicity was demonstrated following treatment of the samples. A follow-up study using similar methods by Pemberton et al (1990) identified the compound as cortisol.

In vitro studies have also demonstrated the neurotoxicity of plasma from GS cases. John et al (1997a) used sera from previous in vivo studies to examine toxicity in equine-derived autonomic nerve cell lines. Concentration-dependent toxicity was found from all serum samples, even from those samples that had not produced in vivo changes, although this was much less pronounced in sera from this group. The authors suggested that this was due to immunity of the recipient horses in this group or due to a genetic resistance. The plasma also exhibited a more general toxic effect as demonstrated by toxic-mediated damage in liver and lung cells. Further work demonstrated differing susceptibility in different cell lines (John et al, 1997b). The results could not rule out possible differences caused by the presence of non-neuronal cells in the cultures; by cell lines derived from foetal, neonatal or mature animals; thoracic chain ganglia and cranial cervical ganglia; the presence of cell lines derived from GS cases that may have led to the most resistant cell lines being established in culture; the retroviral gene vector stimulating growth of other host genes, leading to the variable growth stimulation of the different cell lines by control plasma. They also raised the possibility that there could be a genetic susceptibility among different breeds.

Although this has long been suggested, specific breed susceptibility has never been demonstrated in epidemiological studies (Wood et al, 1998) There is anecdotal evidence that increased incidence can be linked to particular stallions (John et al, 1997b). Similar anecdotal evidence exists suggesting a genetic susceptibility in feline dysautonomia (Symonds et al, 1995).

1.1.6 *Pathology*

Early histopathological reports on the neuropathology of EGS failed to identify any lesion that would account for the clinical manifestation of the disease (Whitwell, 1997). Obel (1955) was the first to describe severity and distribution of neuronal lesions in affected animals and these lesions were later confirmed by various authors including Mahaffey (1959) and Gilmour (1973), among others.

Neuropathology

A characteristic neuronal lesion demonstrates a lack of Nissl substance resulting in a form of chromatolysis which is often accompanied by nuclear eccentricity, pyknosis and karyorrhexis (Whitwell, 1997). Neuronophagia follows cell death and neuronal damage in EGS is present in all autonomic ganglia and the enteric plexuses to different extents. Other frequent features include cytoplasmic vacuolation either in the form of multiple small vacuolations or single, larger ones and smooth, eosinophilic spheroids within/adjoining perikarya.

Historically there was some disagreement between researchers as to whether the degree of damage to the peripheral autonomic nervous system corresponded to the extent of clinical disease (Brownlee, 1959). A study that severed the nerve supply to

the large colon in ponies did not produce significant changes in colonic function (Sellers et al, 1979) and damage to the peripheral ganglia did not result in clinical signs (Gilmour, 1973a; Pogson et al, 1992). Attempts to correlate the degree of enteric neuron damage to level of intestinal dysfunction and/or severity of clinical signs did were not carried out until the 1990s (Doxey et al, 1995). Scholes et al (1993) demonstrated a relationship between neuronal damage and clinical signs. Chronic cases exhibited the majority of neuronal loss in the terminal small intestine (particularly the ileum) whereas acute cases showed severe neuronal degeneration and loss at most sample sites. These authors also suggested that discrepancies described previously i.e. when chronic cases were found to have more severe neuronal damage than acute cases where either attributable to a misdiagnosis of the form of GS seen or not using consistent GI sampling sites.

Doxey et al (1995) carried out an extensive study to build upon the data published by Scholes et al (1993). The study was designed to discover the sites of neuronal damage, its severity and distribution throughout the small and large intestine and to compare cases of differing clinical severity. They demonstrated that the ileum was subjected to a high level of neuronal damage in all presentations of GS. Acute cases had neuronal loss and damage to the jejunum and small colon as well as the ileum. However, not all cases showed the same pattern of neuronal damage with some acutes and subacutes demonstrating more damage in the jejunum than the ileum or comparative amounts of damage in both. The correlation therefore appears to lie in severity of neuronal damage and loss rather than where the lesions occur (Doxey et al, 1995).

The lack of GI motility observed in GS cases has also been investigated. When the cholinergic activity of equine intestinal muscle was measured *in vitro*, it was found that tissue from EGS cases released less endogenous acetylcholine than tissue from controls when stimulated with physostigmine (Murray et al, 1994). Following identification of interstitial cells of Cajal (ICC) in the equine GI tract, Hudson et al (1999) investigated numbers of in EGS and demonstrated diminished numbers of these cells in EGS cases (Hudson et al, 2001). ICC cells initiate slow wave activity in the GI tract that is central to the synchronization of GI motility. Similar findings were found in horses with large colon obstructive disorders (Fintl et al, 2004) leading to the question of whether the lack of ICC cells was the cause or the effect of severe colic.

Other pathology

Other non-neuronal lesions are generally secondary lesions that reflect the severity and/or duration of the disease (Whitwell, 1997). Oesophageal ulcers are often seen and probably the result of regurgitation due to oesophageal malfunction. The small intestine can be distended with fluid whereas the caecum and large intestine are generally impacted to various degrees. The nasal mucosa of longer duration cases can be dry and recent findings by Prince et al (2003) demonstrate that rhinitis sicca, often associated with chronic grass sickness, may have an underlying cause of a decrease in expression of sensory neuropeptides. Some inflammatory changes are sometimes observed in the livers of affected horses and spleens of longer duration

cases can be enlarged / engorged with blood (Whitwell, 1997). Lymphoid tissues are generally normal although lymph nodes at the chest entrance are frequently inflamed and /or haemorrhagic.

1.2.1 *Clostridium botulinum*

First isolated from an outbreak of botulism in Belgium in 1895 (Hathaway, 1989), the species *C. botulinum* encompasses a phenotypically and genotypically heterogenous group of organisms that are classified as one species due to production of immunologically distinct neurotoxins (BoNTs) that possess the same pharmacological action. The antigenicity of the neurotoxins has enabled the division of the species into seven types, designated A through to G in chronological order of discovery (Hunter and Poxton, 2002).

Nomenclature is further complicated due to isolation of strains capable of expressing combinations of toxins, with organisms demonstrating the production of both BoNT/A and BoNT/F (Hathaway and McCrosky, 1987; Barash and Arnon, 2004) and BoNT/A and BoNT/B (Collins and East, 1998; Kobayashi et al, 2003). Isolates of *C. barati* and *C. butyricum* have been found that produce BoNT/F and BoNT/E (Hall et al, 1985; McCrosky et al, 1986).

C. botulinum has been divided into four groups based on characteristics such as volatile fatty acid production. Group I contains type A and proteolytic strains of types B and F; Group II contains type E and non-proteolytic/saccharolytic strains of

B and F; Group III consists of types C and D; Group IV consists of type G (Hunter and Poxton, 2002) and this species has now been renamed *B. argentiense*. These groups also include phenotypically related, non-neurotoxigenic organisms such as *C. sporogenes* in Group I and *C. novyi* type A in Group III.

Nucleic acid hybridisation and 16S ribosomal RNA sequencing studies have supported the division of the species into the four groups with four distinct phylogenetic groups demonstrated (Hutson et al, 1993; Collins and East, 1998). There is evidence of lateral gene transfer as nucleotide sequences of the neurotoxin genes do not support the phylogenetic groupings and BoNT/E and G have been found on plasmids (Eklund et al, 1988; Hauser et al, 1992) and BoNT/C and D are bacteriophage encoded (Eklund et al, 1972; Eklund et al, 1974).

1.2.1 *Group III organisms*

C. botulinum type C and D and *C. novyi* type A are phenotypically similar and cannot be distinguished by culture methods, biochemical profiles or by gas liquid chromatography. Their surface antigens are immunologically cross-reactive (Poxton, 1984; Poxton and Byrne, 1984) and non-neurotoxigenic Group III organisms are effectively identical (Hunter and Poxton, 2002).

16S ribosomal RNA analysis demonstrates that these organisms can be grouped together as a separate phylogenetic lineage although a 1% sequence divergence suggests that types C and D are different species. *C. novyi* type A demonstrates a 2 %

sequence divergence again indicating that it is a different species (Hutson et al, 1993).

Group III organisms therefore can only be identified to the species level by detection of the major toxin produced with BoNT/C and BoNT/D produced by *C. botulinum* types C and D correspondingly and the novyi alpha toxin for *C. novyi* type A. Each of these toxins is encoded on separate pseudolysogenic bacteriophages with consequently unstable host-phage relationships. Cycles of phage loss and reinfection are thought to occur *in vivo* (Eklund et al, 1974) and phage loss can occur in the laboratory due to repeated subculture. Loss of the phage is associated with a loss of toxicity. When phage loss was induced, the organism could be re-infected with the original phage reinstating its original toxicity, or by phages encoding BoNT/D or the novyi alpha toxin (Inoue and Iida, 1971; Eklund et al, 1974; Eklund and Poysky, 1974).

1.2.2 *Group III toxins*

C2 and C3 exotoxins.

C. botulinum types C and D can produce up to three toxins including the neurotoxins and two ADP-ribosylating toxins, C2 and C3. C3, an exo-enzyme with ADP-ribosylating activity, is encoded on the same bacteriophage as the neurotoxins and is divided into two groups according to antigenicity (Moriishi et al, 1993) and there is evidence that suggests that the toxin is encoded on a transposon (Hauser et al, 1995). C2 is a binary ADP-ribosylating toxin that is chromosomally encoded and produced

by the majority of type C strains and by a number of type D strains although it has not been detected in other BoNT-producing strains. The C2 toxin is produced during sporulation (Nakimura et al, 1978) and is thought to be part of the structural proteins of the spore coat (Yamakawa et al, 1983). The genes for both C2 components have been detected by PCR in non-neurotoxigenic isolates (A. Heffron, unpublished data).

The C2 toxin consists of two components (Barth et al, 2000) an enzymatic component designated C2I, which is the ADP-ribosyltransferase which modifies G actin, and the binding component designated C2II that mediates entry of the toxin into the cell (Simpson, 1982; Ohishi and Miyake, 1985; Aktories et al, 1986). Both components are required for toxicity which also requires assembly of the two components at the target cell surface (Barth et al, 2000). Binding is via asparagine-linked glycans on the cell surface (Eckhardt et al, 2000) and following binding the toxin is taken up by receptor-mediated endocytosis (Simpson, 1989). Blocker et al (2000) demonstrated that receptor binding is mediated by the C terminus of C2II, in particular the last seven amino acid residues.

C2 ADP-ribosylates G – actin at arginine 177 (Aktories et al, 1986) causing the inhibition of actin-polymerisation (Vanderkerckhove et al, 1988). Certain amino acid residues are conserved among prokaryotic and eukaryotic arginine-modifying ADP-ribosylating toxins and Barth et al (1998) identified these amino acid residues in C2I and showed that they were fundamental for activity of the enzymatic component. The inhibition of actin polymerisation causes depolymersiation of actin filaments and the breakdown of the actin cytoskeleton (Barth et al, 2000).

C2 is able to cause substantial morbidity and mortality in experimental animals (Hunter and Poxton, 2002) although little is known of its pathophysiological role in naturally - occurring infection. It is a lethal toxin that demonstrates enterotoxicity by increasing vascular permeability (Ohishi et al, 1980), causing fluid accumulation in intestinal loops (Ohishi, 1983) with accompanying histological damage to the epithelial surfaces of the intestines (Ohishi and Odagiri, 1984). Visceral smooth muscle presents a potential target to the C2 toxin as they are high in γ-actin but relatively poor in α-actin (Mauss et al, 1990). This toxin also has effects on the immune system, demonstrating inhibitory effects on neutrophil migration (Aktories et al, 1992) and increasing the production of oxygen-free radicals (Aktories et al, 1992) and granule protein release in neutrophils (Norgauer et al, 1988) and such stimulation of a premature respiratory burst may assist immune response evasion (Hunter and Poxton, 2002).

Very few published reports exist of the identification of C2 toxin from clinical cases of botulism apart from in birds (Hunter and Poxton, 2002). It has been suggested that C2 was implicated in some outbreaks of type C botulism in broiler chickens, where cases presented with diarrhoea and enteritis, as well as the signs pathogonomic for botulism (Ohishi and Gupta, 1987). An outbreak of type C botulism in horses in the US led to detection of C2 in the GI tract of one animal, with intramuscular oedema found in five other animals in the outbreak (Kinde et al, 1991).

It is unknown if the exoenzyme C3 play any role in the pathogenesis of disease (Hunter and Poxton, 2002) although it has demonstrated some effects *in vitro* that could be important in disease such as the ability to depolymerise the actin cytoskeleton in the vicinity of tight junctions leading to increased permeability of the epithelium (Nusrat et al, 1995). This toxin has also demonstrated some neurocytotoxicity *in vitro* albeit non-specific to neurones and only at high concentrations (Williamson and Neale, 1998).

Type C and D neurotoxins.

BoNT/C and D have a sequence identity of 52.2% (Oguma et al, 1981; Oguma et al, 1982) and have both common and specific antigenic sites as well as strain-specific epitopes (Oguma et al, 1984; Moriishi et al, 1993). It is thought that the cycles of curing and reinfection that happen naturally in the environment may be responsible for the heterogeneity that is demonstrated in types C and D (Sunagawa et al, 1991).

Botulinum neurotoxins inhibit the release of acetylcholine from cholinergic synapses. They are synthesised as a single 150kDa polypeptide chain that is cleaved by host/bacterial proteases to form the active dichain which is covalently linked by a single disulphide bond. The dichain consists of a 100kDa heavy chain and a 50kDa light chain (Hunter and Poxton, 2002). Crystallographic structure of BoNT/A has determined the presence of three functional domains that were previously proposed by biochemical analysis (Lacy et al, 1988).

It has been proposed that botulinum neurotoxins act in a four step model involving cell binding, internalisation, membrane translocation and target modification in the cytosol (Montecucco and Schiavo, 1993; Montecucco et al, 1994) although the first three steps of this proposed model are less experimentally defined than the last (Hunter and Poxton, 2002).

Botulinum neurotoxins are produced as complexes called progenitor toxins which exist in three forms ranging from 300 to 900kDa, having sedimentation constants of 12S, 16S and 19S (Schiavo et al, 2000). BoNT/C and D can form the 12S and 16S complexes, the 12S consisting of the neurotoxin and non-toxic haemaglutinin component (NTNH) and the 16S consisting of the neurotoxin, NTNH component and a haemaglutinin component (HA) in the ratio 1:1:2 (Ohiishi et al, 1980; Fujinaga et al, 1997). The genes encoding the NTNH and HA components are immediately upstream of the neurotoxin gene (Hauser et al, 1995) although the NTNH components of *C. botulinum* type C and D do not share the same phylogeny as the neurotoxins- demonstrated by their identical antigenicity (Oguma et al, 1980) and nucleotide sequence (Inoue et al, 1999) compared to the antigenically distinct neurotoxins. The larger progenitor complexes are associated with higher oral toxicity (Fujinaga et al, 1997), and so it is thought that the non-toxic components are important in the pathogenesis of botulism, possibly protecting the neurotoxin from proteases and acidity in the GI tract (Ohiishi et al, 1980).

The HA component of the 16S type C progenitor toxin binds to glycolipids/glycoproteins in the small intestine of guinea pigs although the type C

neurotoxin or the NTNH component does not (Fujinaga et al, 1997). The 16S complex did not dissociate in the small intestine and it is thought that due to a higher binding affinity for intestinal epithelial cells, that the 16S progenitor toxin possesses a higher oral toxicity (Fujinaga et al, 1997). Inoue et al (1999) demonstrated that the 16S type C and D toxins do not bind to neutral glycolipids or asialoglycoproteins but do bind to sialylglycolipids and sialglycoproteins. Mahmut et al (2002) demonstrated that immunisation of the non-toxic component of the 16S complex could lead to a protective immune response in mice immunised nasally and then challenged orally with both BoNT/C and BoNT/D, indicating that mucosal antibodies may reduce absorption of the toxins from the small intestine.

BoNT/A and BoNT/B have been shown to be bound to human intestinal cells and the toxins transcytosed but BoNT/C was not transcytosed in the same study (Maksymowych and Simpson, 1998). Uncomplexed toxin was used so it is not known if the 16S progenitor toxin would be bound and transcytosed although the lack of an efficient mechanism for transcytosis in humans may explain why BoNT/C has not been implicated with human botulism (Maksymowych and Simpson, 1998). M cells may take up the toxin (Schiavo et al, 2000). BoNT/C is highly toxic in humans via inhalation (I.R. Poxton, personal communication).

BoNT/C has been shown to bind with higher affinity to mouse neuroblastoma cell lines than to human and to bind to both cholinergic and adrenergic cells *in vitro* (Yokosawa et al, 1989; Kurokowa et al, 1997).

The C terminal domain is composed of two subdomains, H_CN and H_CC the latter of which is concerned with binding and is poorly conserved between the different botulinum toxins (Schiavo et al, 2000). These authors have proposed a two-receptor model of binding of neurotoxins involving a polysialoganglioside receptor that is similar for the different botulinum toxins and a neurotoxin-specific glycoprotein receptor. Preincubation of BoNTs with gangliosides has been shown to inhibit binding - the extent of inhibition depending upon the type of ganglioside (Schiavo et al, 1993). Yokosawa et al (1989) demonstrated that BoNT/C was inhibited from binding by pre-incubation with neuraminidase, trypsin or pronase which points towards a glycoprotein containing sialic acid as a BoNT/C receptor.

Internalisation of BoNTs is thought to be by receptor-mediated endocytosis via clathrin coated pits (Niemann, 1991) and is temperature and energy dependent (Black and Dolly, 1986). The H_C domain is sufficient for internalisation (Schiavo et al, 2000) and neurotoxins of different strains of type B have demonstrated different binding affinities due to mutation of amino acid residues in this region (Ihara et al, 2003. Exit from the vesicle is though to be initiated by acidification of the compartment - the low pH induces a conformational change in the toxin's structure enabling both the heavy and light chains to penetrate the hydrocarbon core of the vesicular membrane (Donovan and Middlebrook, 1986) with the N terminal of the heavy chain forming the channels (Blaustein et al, 1987) and refolding of the toxin occurring in the cytosol following translocation of the light chain (Schiavo et al, 2000).

BoNTs are zinc-dependent metalloproteases which cleave the protein components involved in neuroexocytosis (Schiavo et al, 1992; Blasi et al, 1993a and 1993b; Schiavo et al, 1993a and 1993b). Zinc plays an essential role in peptide bond hydrolysis and the light chains of the neurotoxins possess a zinc-binding motif (Schiavo et al, 1993c). BoNT/C and BoNT/D cleave syntaxin and SNAP-25 and synaptobrevin/VAMP respectively (Blasi et al, 1993a; Schiavo et al, 1993b; Schiavo et al, 1995). The neurotoxins cleave a single site in their respective target proteins which results in a sustained blockage in neurotransmitter release (Hunter and Poxton, 2000).

Syntaxin and SNAP-25 proteins are both bound to the presynaptic membrane and synaptobrevin/VAMP is a synaptic vesicle membrane protein. These three proteins together form the synaptic SNARE complex, central to the neuroexocytosis process. The proteins interact with soluble factors to bring about membrane fusion and exocytosis (Sollner et al, 1994) and when assembled are highly stable and unaffected by BoNTs (Hayashi et al, 1994).

Peptides of the target proteins containing only the cleavage site are not cleaved by the neurotoxins (Montecucco and Schiavo, 1994) indicating that recognition of the substrate by the toxin is twofold; interaction of a region A which is a structural motif common to all three protein targets-common to all is the SNARE motif, a region of 9 amino acids (Schiavo et al, 2000) and region B that contains the peptide bond to be cleaved (Rossetto et al, 1994).

Uniquely among botulinum neurotoxins, BoNT/C has demonstrated cytotoxicity to murine neurones *in vitro* (Kurakawa et al, 1987; Williamson et al, 1995; Osen-Sand et al, 1996; Williamson and Neale, 1998) in both mature and developing neurones (Williamson and Neale, 1998) and it is thought that the effect of BoNT/C on syntaxin in the synapse mediates neuronal death. However, the use of BoNT/C in humans to treat dystonias did not result in neuronal death (Eleopra et al, 1997). No work has been published on the cytotoxic effects, if any, of BoNT/D.

1.2.3. *The involvement of C. botulinum type C/D in the aetiology of EGS*

C. botulinum was first implicated as the aetiological agent of EGS in 1919 (Tocher et al, 1923) following isolation of a large, anaerobic bacillus from clinical samples. By 1924, Tocher and his colleagues had collated a body of evidence that supported his hypothesis including recovery of the organism from the spleens of subacute GS and neutralisation of toxin from these isolates with antitoxin from known strains of *Bacillus botulinus*. The isolates and their toxin were toxic in experimental animals and apparently reproduced GS symptoms and antitoxin to BoNT/A was detected in chronic and recovered cases but not in acute cases (Tocher 1923; 1924). Tocher also hypothesised that the disease was caused by toxin production *in vivo* rather than ingestion of toxin as in classical botulism as evidenced by the detection of specific complement-binding antibodies against botulinum types A and B in subacute cases

and the lack of such antibodies in clinical normal animals or those vaccinated by toxin alone (Tocher et al, 1923).

In 1922-1923 a protection study was carried out in 1433 horses utilising a toxin-antitoxin mixture prepared from known strains. The mortality rate in vaccinated horses compared to unvaccinated controls was 2.8% versus 9.3% in 1923 and 1.5% compared to 8.2% of controls in 1923. Wood et al (1999) calculated the significance of the reduction of mortality as $P = <0.0001$ - highly statistically significant.

Tocher's work was strongly criticised at the time for not using a toxin-antitoxin mixture from equine isolates (human isolates were used) and because it was thought that the organism was not conclusively *C. botulinum* (Gaiger in Tocher et al, 1923). Toxicoinfectious botulism was not known at this time-infant botulism was not described until 1976 (Midura and Arnon) and toxin production within a living animal was not thought possible (Anon, 1927; Greig, 1942). Tocher thought that a predisposing factor, possibly a nutritional or metabolic disturbance that would lead to gastric irritation, would lead to toxin production within tissue as opposed to toxin production in the gut.

The botulinum hypothesis failed to gain support among the veterinary community and further experimental work, whereby type B toxin was stomach tubed into horses leading to classical botulism, was thought proof enough that GS could not be caused by *C. botulinum.*

In the early 1990s the botulinum hypothesis was revisited, namely that EGS is caused by a toxicoinfection by *C. botulinum* type C, with toxin production in the gut of susceptible animals. *C. botulinum* type C/D was suggested due to the cytoxicity of the type C neurotoxin and the pathology that can be caused by the C2 toxin - *C. botulinum* type C was not reported until 1922 (Bengston,1922; Seddon,1922) and so it is unlikely that this was the strain that Tocher used in his protection study. Pilot serological studies at Edinburgh University (JK Miller et al, unpublished data), implied that a serum IgG response was made against *C. novyi* type A, used as a surrogate marker for safety.

On the basis of the initial study, further work was carried out to determine the role of *C. botulinum* type C/D in the aetiology of EGS. Much circumstantial evidence has been found including toxin detection from clinical samples (Hunter et al, 1999); analysis of serum IgG and mucosal IgA from cases and controls (Hunter and Poxton, 2001; Hunter 2002); bacteriological studies of the equine GI tract in health and disease (Garrett et al, 2002); and epidemiological studies (McCarthy et al, 2004).

Using a mixture of direct detection and enrichment culture techniques, Hunter et al (1999) detected BoNT/C in 74% of acute cases, 67% of subacute cases and 67% of chronic cases. Using the same combination of methods toxin was also detected in 10% of controls, albeit in much lesser amounts. However, only 4% (3/77) controls demonstrated toxin via the direct method-one of these horses was a recovered chronic case from 16 months before and the other was a surgical colic. It is known that numbers of clostridia increase during ileus/colic (Garrett et al, 2002) and this

may explain the very low levels of toxin in non-EGS horses. The detection of toxin in healthy controls reinforces Tocher's view that spores of *C. botulinum* are transiently associated with the equine GI tract and that they can pass through without causing disease. The difficulty associated with isolation of the organism was also illustrated; not all samples positive for toxin via the direct method were positive after enrichment or vice versa, illustrating the instability of the toxin-encoding bacteriophage. Therefore the detection of toxin after enrichment was not necessarily producing the toxin and the opposite is also true. Isolates identified as Group III organisms by GLC and biochemical testing have been isolated from EGS cases (Hunter 2001; F.G. Nunn, unpublished data) but all have proved negative for type C toxin by ELISA and PCR methods and negative for other BoNTs using PCR methods. Enrichment methods could be responsible for loss of the prophage due to inhibition of organism growth/toxin production by faster growing organisms, bacteriophage instability or simply that the organism was not present in the enriched sample (Hunter et al, 1999). Based upon these results Hunter et al (1999) proposed that the toxin was produced locally, with axonal transport responsible for the varying degrees of neuronal damage in peripheral ganglia, preventing the clinical signs being consistent with classical botulism. However, although the circumstantial evidence was compelling, it is impossible to state whether *C. botulinum* type C/D was the causal agent of EGS, a cofactor or even that toxin production is a secondary event, facilitated by ileus and consequential overgrowth of *Clostridium* spp.

Immunity at the mucosal level is likely to be more important than systemic immunity and specific IgA has been detected from clinical samples (Hunter, 2001). Detection

of specific IgA suggests recent exposure to the organism as the mucosal immune response is thought to be comparatively short-lived (Pierce and Cray, 1982). Hunter (2001) found that higher levels of specific IgA were demonstrated in subacute cases and proposed that this was due to subacute cases were exposed to an intermediate dose of toxin, sufficient to stimulate IgA production, but not so high as to lead to the onset of acute grass sickness. Detection of anti-BoNT/C IgA in acute cases gives strong evidence supporting the causal/co-causal role in EGS: if BoNT/C was produced after GI stasis it is thought unlikely that IgA to BoNT/C would be detected in AGS cases (Hunter, 2001).

At the time of the study, reagents for the detection and quantification of equine immunglobulins were not readily available and so only OD values were obtained for all specific IgG and IgA detection. Although large differences are easily perceived as being significant *in vivo*, the significance of small fluctuations are more difficult to ascertain and it was not possible to quantify specific IgA as a ratio of specific to total IgA recovered. Due to degradation of IgA by proteases in the GI contents, total IgA may be highly variable and a more robust study was required before definite conclusions could be drawn.

Further investigation of the systemic immune response (Hunter and Poxton, 2001) demonstrated that horses with EGS possessed lower levels of specific IgG (as measured by OD level) to BoNT/C and surface antigens than control horses but no difference was demonstrated between mean IgG levels between different EGS groups. However, a large range of values was observed for each data set and

although controls were grouped into contacts and non-contacts analysis could not account for ages of the animals, known to be a risk factor in development of EGS (Doxey et al, 1996). No information was available as to 'normal' levels of specific IgG at the time so only relative comparisons could be made. It is very interesting that those controls classed as contacts or at high risk in this study, had the highest median OD levels of all the groups, implying that these clinically healthy controls had mounted a possibly protective immune response. It is not known however, whether low antibody levels in cases is due to naivety or to IgG being bound to antigen.

Longitudinal analysis of CGS cases revealed that recovery from CGS did not follow an increase in specific IgG levels. This has possible implications for intervention strategies such as the use of hyperimmune sera as although serum IgG may be protective, levels of IgG do not appear to change the outcome of the disease. On the other hand, these results do support the use of a vaccine.

A large epidemiological study was carried out by Liverpool University (McCarthy et al, 2004a; 2004b) that built on Hunter and Poxton's initial study. The authors used a case-control study that was premises-matched and that utilised univariate and multivariate analysis that could account for ages of the horses and other confounding variables. Based upon their findings, the authors propose that low antibody levels to surface antigens enable proliferation of *C. botulinum* C/D in the gut leading to toxin production. Other interesting findings included the importance of age in assessing antibody levels, a recent change in diet of affected horses and the use of the antihelminthic, ivermectin, which requires further investigation.

1.2.4 *Dysautonomias of other species*

Primary dysautonomias, sharing clinical and pathological similarities to EGS have been reported in cats, dogs, hares and rabbits since the early 1980s (Key and Gaskell, 1982; Rochlitz and Bennet, 1983; Whitwell, 1991; Whitwell and Needham, 1996). The similarities suggest a common aetiological agent (Pollin and Griffiths, 1992) and although none has been definitively identified, similar circumstantial evidence exists for feline dysautonomia as in EGS (Hunter, 2001; Nunn et al, 2004). A full description of feline and canine dysautonomia can be found in Chapter 7.

Leporine dysautonomia has been reported on premises that have previously been associated with EGS (Whitwell, 1991). Histopathology demonstrates striking similarities in Golgi disappearance and endoplasmic reticulum distension as found in EGS cases although some changes, such as stacks of parallel smooth membranes and lectin-staining cytoplasmic inclusions are not seen in EGS but have been seen in feline dysautonomia (Sharp et al, 1984; Griffiths and Whitwell, 1993). Rabbit mucoid enteropathy (RME) was demonstrated to be a dysautonomia in 1996 (Whitwell and Needham, 1996). The rabbit therefore has the potential for use as a small animal model for EGS.

1.2.5 *Botulism in animals and humans.*

Classical botulism

Classical botulism, also called forage poisoning in animals, results from the ingestion of pre-formed toxin in contaminated foods. Whilst botulinum types A, B, E and F are mainly involved in human botulism, types C and D seem only to affect animals with only four poorly substantiated cases being attributed to type C in humans (Jensen and Price, 1987). It is thought that humans lack the receptors for BoNT/C (Maksymowych and Simpson, 1998) as BoNT/C can block neurotransmitter release in isolated human neuromuscular junctions (Coffield et al, 1997) although lack of exposure to the organism could also account for the lack of disease (Hunter, 2001).

Botulism has been reported in a wide range of species, both domestic and wild. Large outbreaks of botulism occur in birds (Lamanna,1987) with type C responsible for many cases in wild and farmed avian species. There are differing susceptibilities between species with horses being more susceptible than cattle, dogs and humans (Lewis and Metzger, 1980 cited in Whitlock and Buckley, 1997). Outbreaks of type C botulism have been reported in cats (Elad et al, 2004), mink and foxes (Lindstrom et al, 2004), cattle (Jean et al, 1995; Main and Gregory, 1996; Galey et al, 2000; Cobb et al, 2002), dogs (Darke et al, 1976; Barsanti et al, 1978; Richmond et al, 1978; Farrow et al, 1983), horses (Kinde et al, 1991;Mackay and Berkhoff, 1992; Schoenbaum et al, 2000), bighorn sheep (Swift et al, 2000), ferrets and pigs (Mitchell and Rosendal,1987). Botulism in cattle caused by type D has also been reported (Heider et al, 2001).

Botulism has been reported in horses in conjunction with feeding big bale silage (Ricketts et al, 1984; Broughton and Parsons, 1985). As the practice of feeding haylage and related products to horses increases, cases of botulism may also increase. It is thought that bales become contaminated with dead wildlife and that the bales can reproduce an environment favourable for the growth of anaerobes in microniches.

Further examples of changing husbandry practices causing disease in domestic animals can be found in the beef and dairy industry. It is increasingly difficult to dispose of increasing amounts of poultry litter. Poultry litter used as feed or bedding for cattle or used as fertiliser is known to be a source of botulism in cattle (Smart et al, 1987; Jean et al, 1995; Ortolani et al, 1997; McLoughlin et al, 1998; Neill et al, 1989; Livesey et al, 2004; D. Graham, personal communication).

Rotting carcases are the most common source of botulinum toxins for animals and birds. Maggots containing BoNT/C have been found in rotting carrion (Haagsma, 1973 cited in Haagsma 1987). Smith and Turner (1987) found high levels of BoNT/C for more than 28 days in carcases of mice that had been inoculated with spores before euthanasia demonstrating the risk of scavenging by other species or contamination of feedstuffs. It is thought that *C. botulinum* can invade the tissues from the gut post-mortem (Hunter, 2001). Landfill sites are another major source of botulism for avian species such as gulls and spores of *C. botulinum* types B, C and D

were found in the majority of sites examined (Ortiz and Smith, 1994) and it is thought that such contamination comes from the gulls themselves.

Toxicoinfectious botulism.

Intestinal colonisation of humans with *C. botulinum* spp. is rare, the most common form being infant botulism, which is also the most common form of human botulism in the US (Hatheway, 1990). It generally occurs in infants of less than 12 months, probably due to a lack of an established gut flora that facilitates growth of botulinum spores in the GI tract. It is very rare in adults and always associated with immunosuppression following surgery or after antibiotic use (Chia et al, 1986).
In horses, a toxicoinfection of foals With *C. botulinum* exists known as Shaker foal syndrome. It occurs in foals between two weeks and eight months of age (Whitlock and Buckley, 1997) again due to the lack of a mature gut flora - vaccination of the dam around one month prior to partuition is sufficient to give protection to the foal. Toxicoinfectious botulism in the GI tract of adult horses has not been reported (Whitlock and Buckley, 1997).

Toxicoinfection with *C. botulinum* type C can occur in broiler chickens with the high energy diet of these birds thought to enhance the rate of toxin produced (Eklund et al, 1987). Poultry used as bedding for cattle or used as fertiliser is known to be a source of botulism in cattle (Ortolani et al, 1997; McLoughlin et al, 1998; D. Graham, personal communication).

Wound botulism is rare in horses and associated with infection of injection sites (Mitten et al, 1994) or post surgical infection (Bernard et al, 1987) and has not been reported as a naturally - occurring disease.

1.3 *Equine serology / immunology*

The horse played a key role in the early years of immunological research (Marti et al, 2003) with the disparate immunoglobulin isotypes and subclasses being common fields of research for scientists interested in immunoglobulin structure (Sandor et al, 1964; Weir et al, 1966). Equine anti-lymphocyte serum was used in studies of cellular immunity (Mosedale et al, 1968; Stewart and Bell, 1970) but the introduction of monoclonal antibodies removed horses from the vanguard of immunological investigation (Marti et al, 2003).

Many immunological mechanisms are conserved within the evolution of the immune system but interspecies diversity is significant and systematic evaluation of mechanisms and reagents is required (Steinbach et al, 2002).

1.3.1 *Equine cytokines*

Given the importance of cytokines in an immune response, it is imperative to determine their functions. However, reagents such as antibodies for detection which are raised in other species only cross-react very rarely (Steinbach et al, 2002). Both

the cytokine and its receptor has to be conserved across species in order for bioactivity to occur and this is not the case for major cytokines such as IFN-γ and IL-4. Where cross-reactivity does occur, activity is often much reduced and antibody production is a risk during *in vivo* use (Steinbach et al, 2002a; 2002b).

Cloning and expression of the T helper cytokines, IL-4 and IL-2 was reported in 2000 (Dohman et al, 2000) and the homology of equine and human IL-4 was found to be just 65% and of horse and mouse just 55% (Steinbach et al, 2000a). IL-6 was also cloned, sequenced and expressed (Swiderski et al, 2000) as was IL-5 (Cunningham et al, 2003). This molecular research has greatly facilitated the understanding of immune responses in horses against both infectious agents such as parasites and viruses (Klei et al, 1989; Nelson et al, 1998), and in understanding recurrent airway obstruction (Foster et al, 1997 cited in Horohov (2000). Some work has been published on the detection of cytokine RNA in the equine gut in response to inflammatory conditions and parasites (Davidson et al, 2002; Davidson et al, 2005) none has yet to be published on cytokine production in response to enteric bacterial pathogens.

There is strong evidence of Th1 and Th2 responses in horses (Hooper-McGrevy et al, 2003) in response to the respiratory pathogen, *Rhodococcus equi* but again there is nothing published on immune responses to enteric pathogens.

1.3.2 *Equine immunoglobulins*

Although different immunoglobulin (Ig) classes viz. IgM, IgG, IgE, IgA and IgD, are conserved between mammalian species (Wagner et al, 2004) the number of Ig subclasses/ isotypes differs among mammals. As an example rabbits express 13 IgA isotypes whereas most other species express just one or two (Knight et al, 1985; Wagner et al, 2004). The horse possesses seven IgH chain constant (IGHC) genes, the most found in the IGH locus of any species (Wagner et al, 2004). A duplication event occurred leading to the IGHG4 and IGHG7 and these genes have been found in all individual animals and breeds examined, including those that have been bred in isolation such as Icelandic horses (Overesch et al, 1998; Wagner et al, 1998) and those used in production of a variety of more modern breeds such as the Thoroughbred. The authors conclude that due to the high sequence homologies and the lack of gene conversion between these two genes and the other IGHG genes, this duplication occurred most recently during the evolution of these genes in the horse.

In early immunological investigations, the equine Ig isotypes were studied using serological and biochemical analysis. More recently, monoclonal antibodies (Mabs) have been used (Lunn et al, 1995; Sheoran et al, 1998). These studies led to the identification of five IgG subclasses designated IgGa, IgGb, IgGc and IgG(T) and IgG(B) (Wagner et al, 2004) and so discovery of seven IGHG genes suggested that equine antibodies were more complicated although two subspecies of IgG(T) had been reported - demonstrated by their different binding properties to Proteins A and G (Sheoran and Holmes,1996). Wagner et al (2004) confirmed these two subspecies

by identification of the isotype genes IgG3 and IgG5. The genes of the subclasses have been identified as follows; IgG1 (IgGa), IgG3 (IgG(T)), IgG4 (IgGb), IgG5 (IgG(T)) (Wagner et al, 2004). IgG2 and IgG6 have been expressed in mammalian cells but not identified as yet. IgG7 has been detected at the mRNA level and the rate of its expression apparently suggests that it could be present in adult equine serum in high concentrations - in comparable levels to IgG4 (IgGb) and so it is possible that IgGb as detected by antibodies, consists of two similar isotypes. Sheoran et al (2000) used Mabs to quantify the levels of the different subclasses in serum, nasal secretions and colostrum in adult animals and in foals. IgGb was found to be significantly more abundant in serum, consisting of more than 60% of serum IgG and IgGc as the least abundant (less than 1%).

Studies of IgG subclass responses to infection have been reported to intracellular pathogens such as equine influenza virus (Nelson et al, 1998), equine herpesvirus (Mizukoshi et al, 2002) as well as *Rhodococcus equi* (Hooper – McGrevy et al, 2003).

In addition to the seven IgGHG genes, IGHC region in horses consists of one of each of *IGHA, IGHE, IGHM* and *IGHD* genes. Although previously found only in primates and rodents, genes encoding IgD have been described in cattle, sheep and pigs (Zhao et al, 2002) indicating that evolution of this gene occurred early on in evolution of the mammalian immune system.

IgA is the most abundant immunoglobulin class in saliva, milk and colostrum (Vaerman et al, 1970; Sheoran et al, 2000). Once again, studies of production of specific IgA are limited to the same pathogens as mentioned previously with no published work found on enteric bacterial pathogens. Until recently, quantification of equine IgA was difficult due to the lack of commercial reagents. Hunter (2001) could not investigate the possible differences in IgA recovery from different sample sites or between horses due to this problem.

Aims of thesis

Although a substantial body of evidence has been collated supporting the hypothesis that equine grass sickness is caused by a toxicoinfection with *Clostridium botulinum* type C/D, the aetiology of equine grass sickness is still unproven. Serological evidence strongly suggested that a systemic immune response to toxins and surface antigens of the organism may indicate protection from the disease and possibly demonstrate and/or influence recovery from the disease. Initial studies of the mucosal immune response have also been encouraging, with recent exposure to the organism leading to higher specific IgA levels in AGS cases. However, controls were often not appropriate to enable useful comparisons between groups and little is known of specific antibody fluctuations in clinically normal animals. In addition, equine specific reagents are now commercially available that facilitate the quantification of IgG levels, allowing researchers to judge if fluctuations in OD levels are significant in vivo. The aims of this thesis were as follows:

1) **To examine IgG levels against extracellular and surface antigens of *C. botulinum* Group III organisms in clinically healthy animals, in order to establish 'normal' levels or to examine differences between groups, i.e. age or geographic location.**

2) **To compare specific systemic IgG levels in affected animals; both acute cases and premises-matched controls and also in chronic cases. This would enable comparisons of specific IgG levels in cases as well as those seemingly immunologically protected. To establish whether fluctuations in IgG were significant and to look at immune response in chronic cases.**

3) **To study the mucosal immune response in the gut by examining IgA levels in affected and non-affected animals and to improve on qualitative studies carried out previously**

4) **To investigate outbreaks of dysautonomia in other species.**

Chapter II

Materials and Methods

2.1 *Animals investigated:*

Full descriptions of the animals studied and the type of samples used in each study can be found at the beginning of the relevant chapters. Briefly;

Horses

Healthy horses and ponies used were from E & O Laboratories, Bonnybridge and the Animal Health Trust, Newmarket respectively. Serum from the adult horses was excess sample from routine bleeds under the premises' own Home Office Licence. Samples from the ponies were likewise excess serum used in conjunction with a study run under an AHT-held licence.

Cases of grass sickness and those animals referred to as non-GI controls (Chapter IV) were all patients at the Easter Bush Large Animal Hospital, Edinburgh and samples were taken by clinicians employed there with the owner's consent.

Cats and Dogs

Sera used from cases of canine dysautonomia were all retrospective samples. Serum samples from healthy cats were library samples used in routine screening by the FELV and FIV unit at Glasgow University Veterinary School (GUVS). Faecal samples from healthy cats were donated by cat owners, all of which were staff of

GUVS. Clinical samples from cases of feline dysautonomia were all taken by clinicians at GUVS.

The feline work undertaken during my PhD made up a substantial part of my first eighteen months of study. I therefore decided that the feline and canine dysautonomia studies would be treated as a separate chapter, Chapter VII.

2.2 *Samples.*

Samples consisted of whole blood or serum; GI contents which could include tissue and/or contents.

2.2.1 *Treatment of blood and serum samples*

Samples received were either blood or serum. Sera were stored at –20°C until assayed. Blood samples from clinical cases were collected in sterile vacutainers with no additives and allowed to clot overnight at 4°C. Serum was collected after centrifuging at 1000 g for 15 min and stored at –20°C until assayed.

2.2.2 *Treatment of equine GI contents for IgA extraction*

All reagents were stored at –20°C and kept on ice during the procedure. Two parts protease inhibitor solution (soybean trypsin inhibitor 1mg/ml in PBS, 50mM EDTA containing 0.05% Tween 20) were added to one part weighed sample (~1g). Phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich, 0.1M in ethanol) was added to a final concentration of 1mM. The mixture was vortex mixed for 30-60 sec and

then centrifuged at 3800g for 10min.The supernate was removed and PMSF was added to a final concentration of 1% (v/v). After mixing well it was allowed to stand for 15min on ice. Heat inactivated foetal calf serum (FCS) was added to a final concentration of 4% (v/v) and centrifuged at 15700g for 5min. The supernatant was removed and stored at –70°C.

2.2.3 *Treatment of equine GI tissue for IgA extraction*

Antibody extraction from tissue was achieved with saponin as described by Berquist (2000) with some modifications. One to three grams of frozen tissue was aseptically removed and thawed for 8 h at 4°C in PBS containing 2% saponin (w/v), soybean protease trypsin inhibitor (1mg/ml), EDTA; 0.05M, Tween 20 (0.05%), PMSF (2mM), sodium azide (0.2mg/ml) and 4% FCS (v/v). A 2ml volume was added per gram of tissue. Samples were vortexed for 30s. After thawing, samples were agitated using sterile forceps, vortexed for 30s and then placed in a sonicating water bath for 2min. After further vortexing, samples were centrifuged at 15,000g for 5 min and the supernatants collected and stored at –70°C until used. All reagents and samples were kept on ice between steps.

2.3 ELISA

ELISAs were optimised by chequerboard titrations of sample and conjugate. Standard curve of IgG can be seen in Chapter 3 and IgA ELISA optimisation can be found in Chapter 4.

2.3.1 *Preparation of EDTA extracts of surface antigens*

Lyophilised cultures of strains were inoculated into pre-reduced cook meat broth and incubated for 24-48h in an anaereobic cabinet to prepare stock cultures. These were subcultured into 3ml of medium and overnight cultures of these were used to inoculate 20ml cultures of the same medium. These 20ml cultures were incubated overnight before cell harvesting.

EDTA extracts were prepared as by Poxton (1984). Cells were harvested by centrifugation at 3,800g for 20 min and the pellet then resuspended in 10ml cell washing buffer (0.05M sodium phosphate buffer pH7.4 with 0.15M NaCl). The cells were then washed twice by centrifugation as above. Cells were then resuspended in 1ml EDTA buffer (0.05M sodium phosphate buffer pH7.4 with 0.15M NaCl and 0.01M EDTA) and incubated for 90 min in a 45^0C water bath before treating in an ultrasonic water bath for 60sec. Cell suspensions were then transferred to microcentrifuge tubes and centrifuged at 13000g for 2 min. Supernatants were collected and centrifuged as before and the surface extracted antigens collected, assayed for protein content by the method of Lowry (1951) before being stored at – 20^oC.

2.3.2 *Coating of plates*

Equine IgG standard curve

A standard curve of equine IgG was constructed using Bethyl Laboratories reagents. Plates were coated with affinity purified goat-anti equine IgG (A70 – 121A) diluted

at 1 in 100 in 0.05M sodium carbonate buffer (0.05M sodium carbonate buffer, pH9.6, 0.02% w/v sodium azide) (100 µl/well) and incubated for 90 min at room temperature.

ELISA to detect total specific IgG

Surface antigens were diluted to 30µg/ml in 0.05M sodium carbonate buffer (0.05M sodium carbonate buffer, pH9.6, 0.02% w/v sodium azide), C1 and type D toxoid complexes (Metabiologicals Inc,) were diluted to 5µg/ml, TetNT toxoid (List Biologicals) diluted to 0.5µg/ml and added to Nunc Polysorb plates (100µl/well). Plates were incubated overnight at room temperature and 4°C respectively.

ELISA for the detection of specific IgGa, IgGb and IgG(T)

Coating was as described for total specific IgG ELISA (as above).

ELISA to detect IgA

Standard curve of IgA and total IgA detection

A standard curve of equine IgA was constructed using a Bethyl Laboratories Equine IgA Quantification kit (E70 – 116). Plates were coated with affinity purified goat-anti equine IgA diluted at 1/100 in 0.05M sodium carbonate buffer (0.05M sodium carbonate buffer, pH9.6, 0.02% w/v sodium azide) (100 µl/well) and incubated for 90 min at room temperature.

ELISA to detect specific IgA

Coating was as described for total specific IgG ELISA (as above).

2.3.3 *Blocking of plates*

All plates were washed four times between each stage with ELISA wash buffer (1 x PBS BR14a 5mg tablet/litre Oxoid, Basingstoke, U.K. 15mM/l NaCl, 2mM/l KCL, Tween 20 0.05%) pH7.3 between each stage.

All plates were blocked with PBS-TG for 90 min with PBS-TG (0.05M phosphate buffer pH7.4 with 2% teleostean gelatine and 0.1% Tween 20, final pH7.4) (200µl/well) at 37°C with the exception of both surface antigen ELISA, where blocking was found to be unnecessary.

Plates were either used straight away or stored in sealed plastic bags at -20°C for up to one month.

2.3.4 *Addition of samples*

Equine IgG standard curve

Reference serum was diluted in PBS-TG to concentrations recommended by the manufacturer (1000, 500, 250, 125, 62.5, 31.25 ng/ml) and added (100µl/well).

ELISA to detect total specific IgG

Samples were diluted 1 in 300 in PBS-TG and added in duplicate (100 µl/well). Samples were repeated at 1 in 600 or 1 in 100 if necessary to fall within the linear portion of the standard curve of total IgG. Plates were incubated for 90 min at 37°C with shaking. OD values were converted into concentrations of ng/ml by Prism. These were then multiplied by the appropriate dilution factor and converted to µg/ml.

ELISA for the detection of specific IgGa, IgGb and IgG(T)

Samples were diluted 1 in 50 in PBS-TG and added in duplicate (100 µl/well). Plates were incubated for 120 min at 37°C with shaking.

ELISA to detect total IgA

The reference serum was diluted according to the manufacturer's instructions and added (100µl/well). Samples were diluted 1 in 500 for faecal extractions and 1 in 1000 for saponin extractions. Samples and reference sera were assayed in quadruplicate at 100µl/well and incubated overnight at room temperature. Plates were washed six times between steps.

ELISA to detect specific IgA

Samples were assayed in quadruplicate and averages of these OD values used for IgA quantitation. Optimal dilution was at 1 in 8 for the majority of samples although some were repeated at 1 in 4 and 1 in 16.

2.3.5 *Conjugates*

Equine IgG standard curve

Goat anti-equine IgG (H+L) chain HRP conjugate was diluted 1 in 10000 in PBS-TG and added 100µl/well. Plates were incubated for 90 min at 37°C with shaking.

ELISA to detect total specific IgG

Conjugate was as for IgG standard curve.

ELISA for the detection of specific IgGa, IgGb and IgG(T)

Goat anti equine IgGa, IgGb or IgG(T) was diluted 1 in 2000 in PBS-TG. Plates were incubated for 120 min at 37°C with shaking.

ELISA to detect total IgA

Goat anti-equine IgA alkaline phosphate conjugate was diluted 1 in 10000 in PBS-TG and added 100µl/well. Plates were incubated for 4h at 37°C with shaking.

ELISA to detect specific IgA

2.3.6 *Substrates*

Horse radish peroxidase (HRP): One tablet (1mg) 3'3',5'5' – tetramethylbenzidine (TMB) (Sigma – Aldrich) was dissolved in 0.05M phosphate-citrate buffer pH5.0 with 0.006% hydrogen peroxide and added at 100 µl/well. The colour was allowed to develop for 30 min for total and specific IgG and for 60 min for IgG subclasses. Development was stopped by addition of 100 µl/well of 2M H_2SO_4. Plates were read on an Anthos 2001 plate reader at 450nm, referenced at 620nm.

Alkaline phosphatase: alkaline phosphatase substrate tablets (Sigma 104-105 phosphatase tablets, p-nitrophenyl phosphate 5mg) were diluted 1 in 10000 with substrate solvent (0.05 mol/l sodium carbonate solution, pH 9.8, with 1mmol/l magnesium chloride) to give a concentration of 1mg/ml. Plates were then incubated at room temperature for 60min and read in an Anthos 2001 plate reader at 405 nm, referenced at 620nm.

2.3.7 *Controls*

Blank wells, consisting of coating buffer were included on each plate. Negative controls, consisting of antigen diluent in place of sample, to control for nonspecific binding of conjugate to the antigen, were included on plates in initial assays but were found to be negligible and were not included on further plates.

The Bethyl Laboratories standard reference serum was found to be positive against all antigens in IgG assays. This was included on all subsequent plates and used to normalise all readings between plates.

2.3.8 *Statistical analysis*

IgG and IgA quantitation was carried out using non-linear regression (curve-fit) using a 4 parameter logistic equation on GraphPad Prism version 3.0 software. Concentration of IgG or IgA from clinical samples was an estimation obtained by comparing OD values of samples with OD values of known concentrations of IgG or IgA which were assayed under the same conditions.

Correlation and all other statistical analyses were also carried out with this software.

IgG subclasses were not quantified and are expressed in OD units.

Prism software was used to check for Gaussian distribution of data within groups. Where a Gaussian distribution was demonstrated, parametric tests such as unpaired t-tests for means comparison and Pearson's correlation and One-Way ANOVA with Tukey's comparisons were used (Motulsky 1996). Where a Gaussian distribution was not observed, data was either transformed (X = LogX) so that it did or if that were not successful, non-parametric tests were used such as Mann-Whitney U test

for comparison of medians or Spearman's correlation and Kruskal-Wallis test for multiple comparisons. However, no P value became significant due to the use of one test or another unless stated.

2.4 *Culture of type strains and identification of toxin genes using PCR*

2.4.1 *Strains used*

MPRL* number	NCTC** number	description
2778	5413	neurotoxin negative
2779	9569	neurotoxin positive
4564	8548	neurotoxin positive, C2 positive
2510	3732	neurotoxin negative, C2 positive
3923	8265	neurotoxin positive, C2 positive

*Microbial Pathogenicity Research Laboratory, University of Edinburgh.

** National Collection of Type Cultures, Colindale Ave., London.

2.4.2 *DNA extraction*

DNA extraction was carried out using the chelex method of de Lamballerie *et al* (1992).

Pre-reduced media was inoculated with 1ml of stock culture and incubated anaerobically at 37°C overnight. Cultures were checked for purity by plating on blood agar aerobically and anaerobically (48h) and also by Gram film and wet film. 1.5ml of culture was centrifuged (5min at 13.000 g) and the pellet resuspended using 100 µl of 5% Chelex suspension (suspended in sterile, pyrogen free water, Bioconnections- BC2090). The suspension was then boiled for 10 min before centrifugation at 13,000 g for 2 min. Supernatant was collected and frozen at -20°C in aliquots of 20µl.

2.4.3 *PCR primers*

Primers

BoNT/C

ToxC-384 5'-AAACCTCCTCGAGTTACAAGCCC-3'

ToxC-850 5'-GAAAATCTACCCTCTCCTACATCA-

Williamson et al (1999)

C2 Component1

C2C1-F 5'-AAGGAAGATAAAACAAAAAT-3'

C2CI-R 5'-CCTAATGATACAAATGAAAA-3'

Fujii et al, (1996)

C2 Component 2

C2CII-F 5'-GCAGAAGTTTCAGGTAGTTTACAAC-3'

C2CII-R 5'-CGCATTCTATAACGACCTTCTGGA-3'

Kimura et al (1998)

BoNT/D

ToxD-F 5'-GTGATCCTGTTAATGACAATG-3'

ToxD-R 5'-TCCTTGCAATGTAAGGGATGC-3'

Takeshi et al, (1996)

TetNT

TetC-F 5'-CGCGTCGACTCAACACCAATTCCATTTTCTTATTC-3'
TetC-R 5'-GCGCTGCAGTCATGAACATATCAATCTGTTTAT-3'

Halpern et al (1990)

2.4.4 *PCR reaction mixtures and cycling conditions*

Reaction mixes (all 50µl) with 5 µl DNA :

C2I, C2II and BoNT/D

Buffer	1X (50mM KCL, 10mM Tris-HCL)
$MgCl_2$	1.5mM
dNTP	12.5mM
Primer	10pmol/ul
Taq	2.5U

BoNT/C

Buffer	1X (50mM KCL, 10mM Tris-HCL)
$MgCl_2$	3.75mM
dNTP	0.2mM
Primer	1µM
Taq	1.25U

TetNT

Buffer	1X (50mM KCL, 10mM Tris-HCL)
$MgCl_2$	3.75mM
dNTP	0.2mM
Primer	50 nM
Taq	1.25U

2.4.5 Cycling conditions

	Initial denaturation	cycles	cycling conditions	final extension
BoNT/C	5min at 80°C	30	1min at 95°C 1min at 55°C 2min at 72°C	10min at 72°C
C2CI	3min at 94°C	35	45s at 94°C 2min at 45°C 1min at 72°C	5min at 72 °C
C2CII	3min at 94°C	40	45s at 94°C 1min at 53°C 3min at 72°C	5 min at 72 °C
BoNT/D	10min at 94°C	25	1min at 94°C 1min at 55°C 1min at 72°C	3 min at 72 °C
TetNT	5min at 94°C	25	2 min at 94°C 3 min at 30°C 10 min at 67°C	5 min at 67°C

2.4.6 *Controls*

Positive controls were the DNA extractions from toxin positive strains. Negative controls consisted of 5µl H_2O in place of the template DNA.

Visualisation of PCR products

10µl portions of amplification products are run on 1% ethidium bromide stained agarose gels. All products from PCR were run on 1.5% gels.

Chapter III

Immunity to Group III protein antigens: longitudinal studies of clinically healthy horses and ponies.

Results

3.1 Longitudinal study of healthy ponies

Specific antibody acquisition against *C. botulinum* type C toxoid and surface antigens have been shown to plateau at seven years of age (HE McCarthy, personal communication) which correlates with the physical maturity that horses reach around the same age. An opportunity arose to assay a herd of young ponies that had recently been moved from a premises not associated with GS to one that had. IgG levels to BoNT/C and BoNT/D toxoids, EDTA extracted type C surface antigens were assayed as well as levels of specific IgG subclasses (IgGa, IgGb and IgGT). Ratios of specific IgG subclasses were also examined to see if younger animals demonstrated any bias towards a Th1 or Th2 response to these antigens.

3.1.1 *Samples and study*

Twenty six ponies from the Animal Health Trust (AHT) were sampled on three occasions in a 10-month period between October 2001 and August 2002. All of the ponies were 6 months old at the time of first sampling and therefore ~16 months old by end of the study. The animals were all clinically healthy and samples donated were surplus from a study carried out under an AHT held licence.

The ponies had previously been barn kept and fed large-baled hay with no concentrate. They were moved to Newmarket 2 weeks before date of first sampling and subsequently kept on land previously associated with grass sickness cases.
All data had a Gaussian distribution with unequal SD. One-way ANOVA was used with Tukey's post-test for multiple comparisons. Un-paired t-tests with Welsh's correction were also used to test for significant differences in antibody level between sampling dates. Correlation between specific antibody levels and specific IgG subclass levels were calculated using Pearson's correlation coefficients.

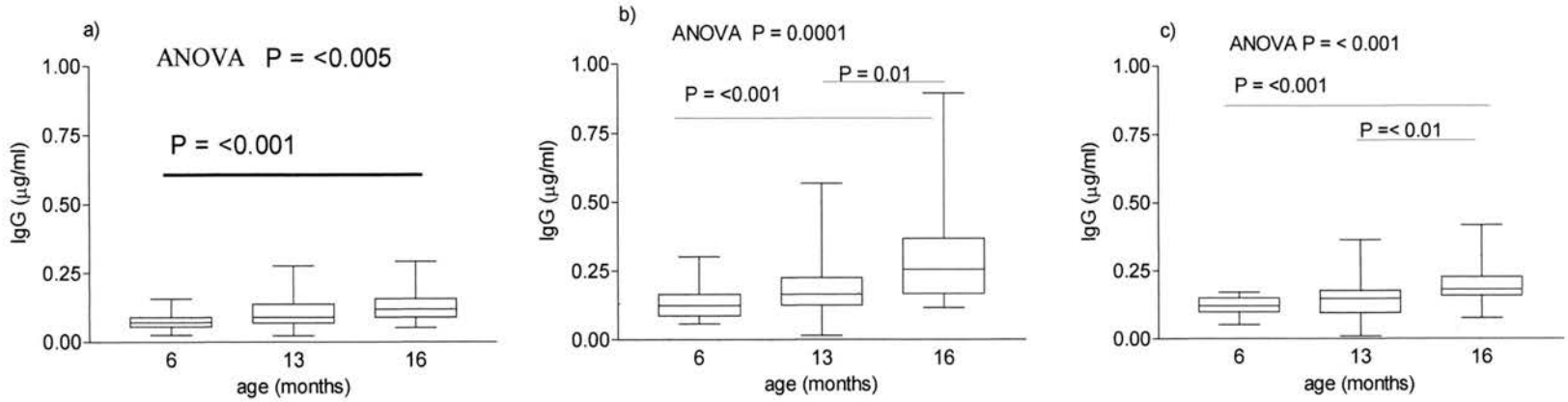
3.1.2 *Specific IgG to neurotoxins and surface antigens (SA).*

Levels of antibodies to all three antigens were examined and all rose over the sampling period. Each is described separately:

BoNT/C

Boxplots of the three sampling times are shown in Fig. 3.1a with the P values resulting from one way ANOVA. Significant differences were seen between levels at 6 and 16 months (P=0.0001) and almost significant differences between 6 and 13 months and 13 and 16 months (P=0.06 and P = 0.05 respectively). ANOVA results demonstrated an overall P value of 0.005 while the Tukey's post-test only showed a significant difference between levels at 6 months and 16 months (P=<0.001).

Fig. 3.1



AHT ponies: total specific IgG against a) BoNT/C b) BoNT/D c) SA

Boxplots showing total specific IgG against antigens. There was a significant increase in titre against all antigens.

The P values were calculated with one - way ANOVA with Tukey's post-tests to compare groups of data.

BoNT/D

Similar results were observed for this neurotoxin. Unpaired t-tests demonstrated significant differences between all data points and these are illustrated of Fig.3.1b. ANOVA results demonstrated an overall P value of 0.0001 while the Tukey's post-test showed a significant difference between levels at 6 months and 16months (P=<0.001) and between 13 and 16months (P = 0.01). The P value between 6 and 13 months was >0.05.

SA

Results of this analysis are shown in Fig.3.1c. Unpaired t-tests demonstrated significance between 6 and 16 months (P=<0.0001) and 13 and 16 months (P = 0.006) but none between 6 and 13 months (P = 0.13). ANOVA results demonstrated an overall P value of <0.001 while the Tukey's post-test only showed a significant difference between levels at 6 and 16 months (P=<0.001) and between levels at 13 and 16 months (P = <0.01). Using this test P= >0.05 between 6 and 13 months.

3.1.3 *Correlation of specific IgG to different antigens*

Results from this analysis can be seen in Fig. 3.2.

Anti-SA, anti-BoNT/C (Fig. 3.2a)

Using Pearson's correlation a positive correlation was observed between anti-SA and anti-BoNT/C antibodies at 6 and at 16 months (Pearson's $r = 0.41$, $r^2 = 0.17$, $P = 0.03$: Pearson's 0.4, $r^2 = 0.16$, $P = 0.04$ respectively) but not at 13 months (Pearson's $r = -0.34$, $r^2 = 0.11$, $P = 0.1$).

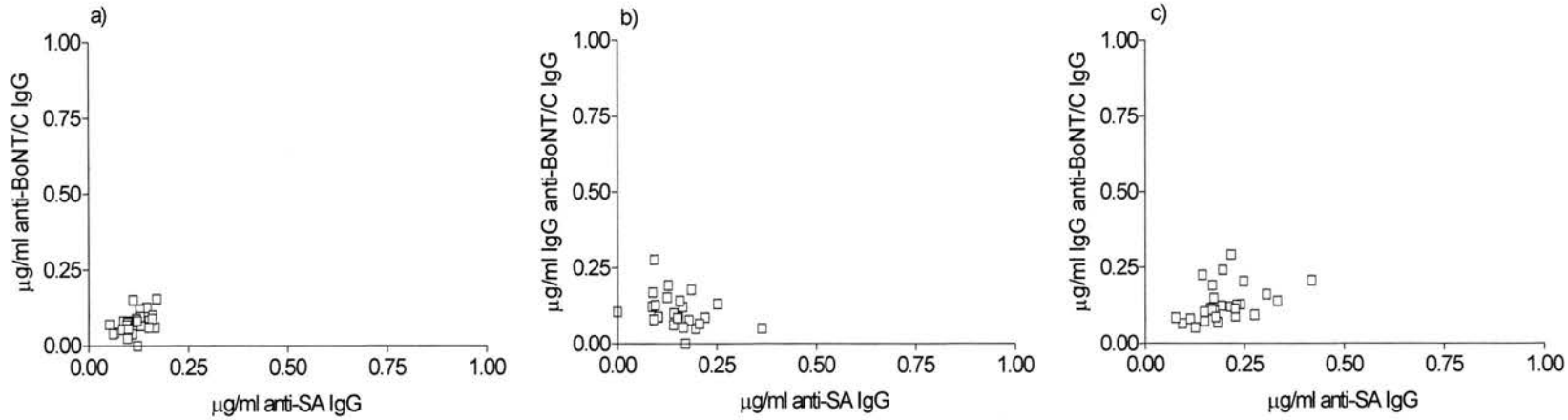
Anti-SA, anti-BoNT/D (results not shown)

No correlation was observed between anti-SA antibodies and anti-BoNT/D antibodies with Pearson's $r = 0.24$, $r^2 = 0.06$, $P = 0.21$: Pearson's 0.2, $r^2 = 0.04$, $P = 0.3$: Pearson's $r = 0.41$, $r^2 = 0.017$, $P = 0.03$: Pearson's 0.05, $r^2 = 0.002$, $P = 0.8$ at 6, 13 and 16 months respectively.

Anti-BoNT/C and anti-BoNT/D (Fig. 3.2b)

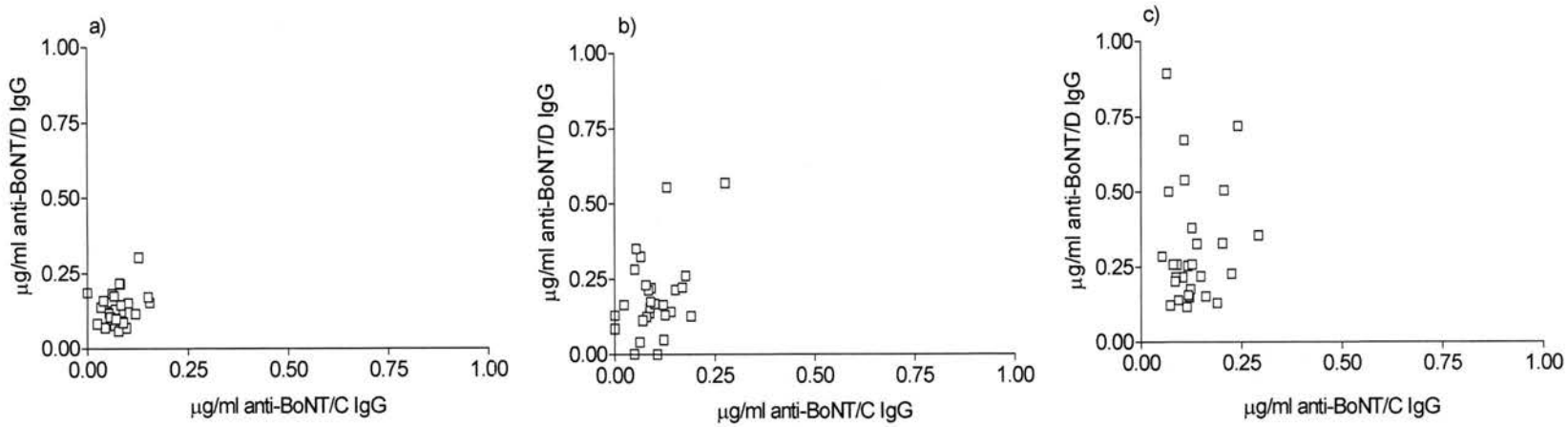
No correlation was demonstrated between anti-BoNT/C and anti-BoNT/D levels at 6 or 16 months (Pearson's $r = 0.21$, $r^2 = 0.04$, $P = 0.3$: Pearson's $r = 0.08$, $r^2 = 0.18$, $P = 0.69$ respectively). A positive correlation was seen at 13 months (Pearson's $r = 0.43$, $r^2 = 0.18$, $P = 0.02$).

Fig. 3.2a



Correlation between anti-SA IgG acquisition and anti-BoNT/C aquisition at a) 6 months b) 15 months and c) 18 months. All data was normally distributed and Pearson's correlation used. Positive correlation seen in a) Pearson's r = 0.41, r2= 0.17 and P = 0.03 and in c) Pearson's r = 0.4, r2 = 0.16 and P = 0.04. However, positive correlation not observed at b) Pearson's r = -0.34, r2= 0.11 and P =0.1

Fig. 3.2b



Correlation between anti-BoNT/C and anti-BoNT/D IgG levels at a) 6 months b) 13 months and c) 16 months

No correlation observed at 6 or 18 months with a) r2 = 0.04, Pearson's r = 0.21 and P = 0.3 and c) r2 = 0.006, P = 0.69 and Pearson's r = 0.08. A positive correlation was observed at 15 months: b) r2 = 0.18, P = 0.02 and Pearson's r = 0.43

3.1.4 *IgG subclasses to antigens*

These results are graphically represented as boxplots in Fig.3.3a-c.

BoNT/C

IgGa

Overall a significant increase in specific IgGa was demonstrated over the sampling period with ANOVA P value =0.004. Tukey's post test showed significant differences between levels at 6 and 16 months (P = <0.05) but not between 6 and 13 months or 13 and 16 months (P =>0.05 for both).Unpaired t-tests showed significance between 6 and 16 months between (P=0.02) and approaching sigificance between 13 and 16 months (P = 0.08).

IgGb

Specific IgGb showed an overall increase in level with ANOVA P value at P = 0.005. Tukey's post tests showed significant differences between 6 and 13 months only (P = <0.01). Between 6 and 16 months and 13 and 16 months P = <0.05. Unpaired t-tests with Welsh's correction demonstrated P values of P = 0.002 between 6 and 13 months and P = 0.06 between 6 and 16 months and P = 0.13 between 13 and 16 months.

IgGT

Overall, an increase in specific IgGT was observed (P = 0.006) with the post tests showing P=>0.05, P =<0.01 and P = <0.05 between 6 and 13 months, 6 and 16 months and 13 and 16 months respectively. Unpaired t-tests demonstrated P values

of P = 0.43 between 6 and 13 months. The P value for levels between 6 and 16 months and 13 and 16 months were P = 0.003 and P=0.03.

BoNT/D

IgGa

A significant increase in specific IgGa was observed over the sampling period with ANOVA P value of 0.01. Post tests demonstrated significant differences between 6 and 13 months and 6 and 13 months (P = <0.05) but not between 13 and 16 months (P => 0.05). Unpaired t-tests showed P values of <0.002, 0.006 and 0.3 respectively.

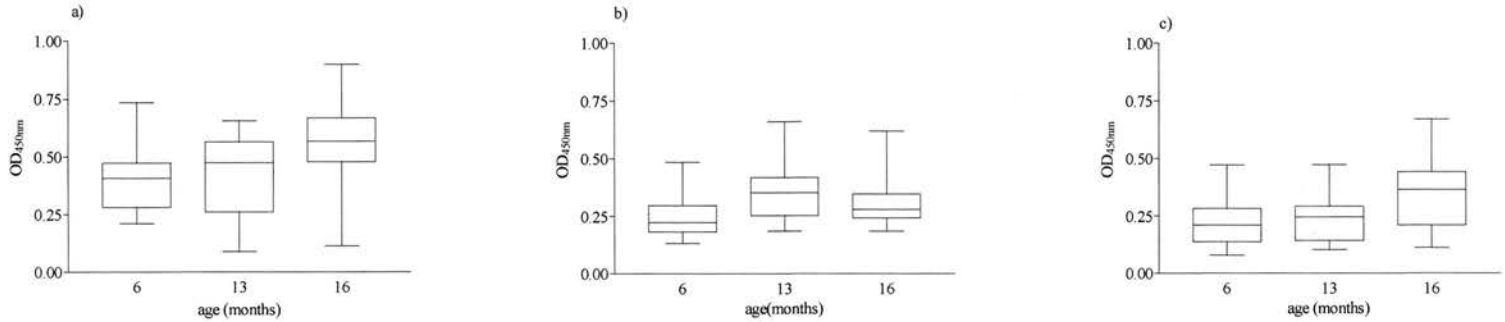
IgGb

No significant increase was seen in specific IgGb using ANOVA (P = 0.1, multiple comparisons all >0.05). Using unpaired t-tests a significant difference was observed between 6 and 16 months (P = 0.03) but not between 6 and 13 months (P= 0.2) and 13 and 16 months (P = 0.4).

IgGT

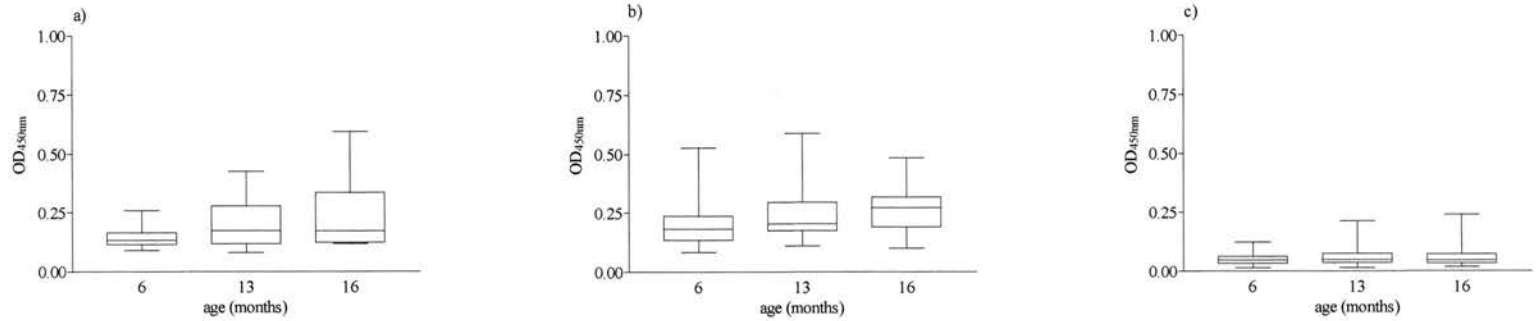
No significant increase was seen overall in specific IgGT with an overall ANOVA P value of 0.56 and all comparisons at P = >0.05. Unpaired t-tests gave P values of 0.28, 0.38 and 0.87 between 6 and 13 months, 6 and 16 months and 13 and 16months respectively.

Fig. 3.3a



Box plots showing specific IgG subclass levels against BoNT/C a) IgGa b) IgGb c) IgGT

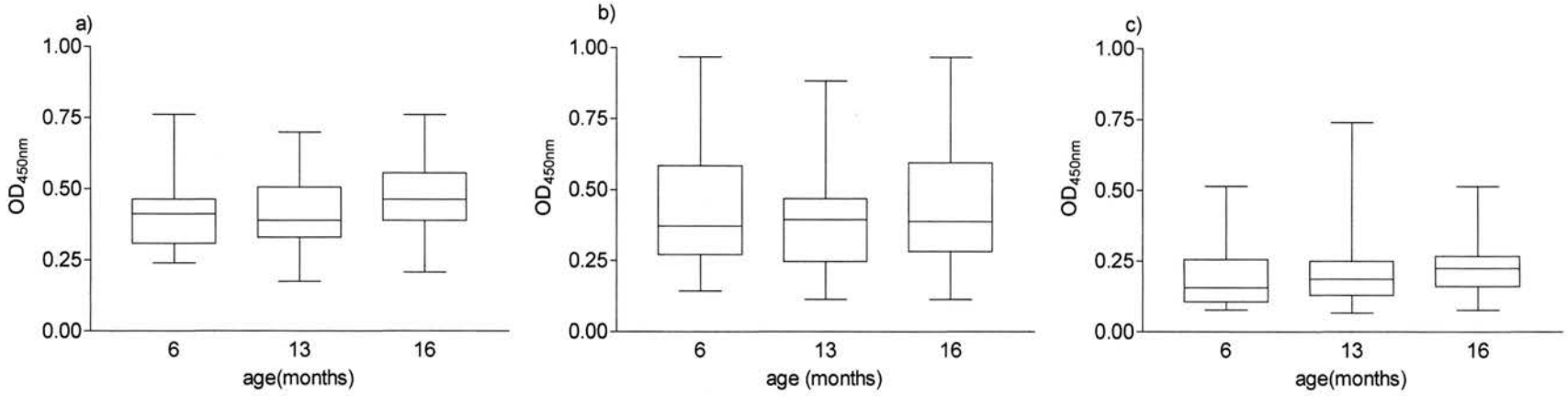
Fig. 3.3b



Specific IgG subclass levels against BoNT/D: a) IgGa b) IgGb c) IgGT

Boxplots showing differences in specific IgG subclass titre with age. Significant increases of IgGa (P= 0.006) and IgGb (P = 0.03) but not IgGT were demonstrated between 6 and 16 months.

Fig. 3.3c



IgG subclasses against SA: a) IgGa b) IgGb and c) IgGT

Increases in mean specific IgG subclass titres were observed over the sampling period although the only statistical significant increase was demonstrated in IgGa between 6 and 16 months (P = 0.05).

SA

IgGa

No significant increase in specific IgGa was seen using ANOVA (P = >0.05) although a significant difference was seen between levels at 6months and 16months using unpaired t-tests (P = 0.05). P values between levels at 6 and 13 months and 13 and 16 months were P =0.62 and 0.15 respectively.

IgGb

No significant increase between data points of specific IgGb was seen with an overall ANOVA P=0.77 and post-tests all P = >0.05. Unpaired t-tests were insignificant at P = 0.65, P = 0.79 and P = 0.47 at 6 and 13 months, 6 and 16 months and 13 and 16 months respectively.

IgGT

No significant increase between data points of specific IgGb was seen with an overall ANOVA P=0.61 and post-tests all P = >0.05. Unpaired t-tests were insignificant at P = 0.6, P = 0.27 and P = 0.7 at 6 and 13 months, 6 and 16 months and 13 and 16 months respectively.

3.1.5 *Correlation between IgG subclasses*

No relationship was demonstrated between specific IgG subclass levels at any data point (using Pearson's correlation).

3.1.6 *IgG subclass ratios against neurotoxins*

Ratios between IgG subclass ratios were analysed singly (i.e. IgGa/IgGb or IgGT) or with IgGb and IgGT combined. These results can be seen in Fig. 3.4a-c.

BoNT/C

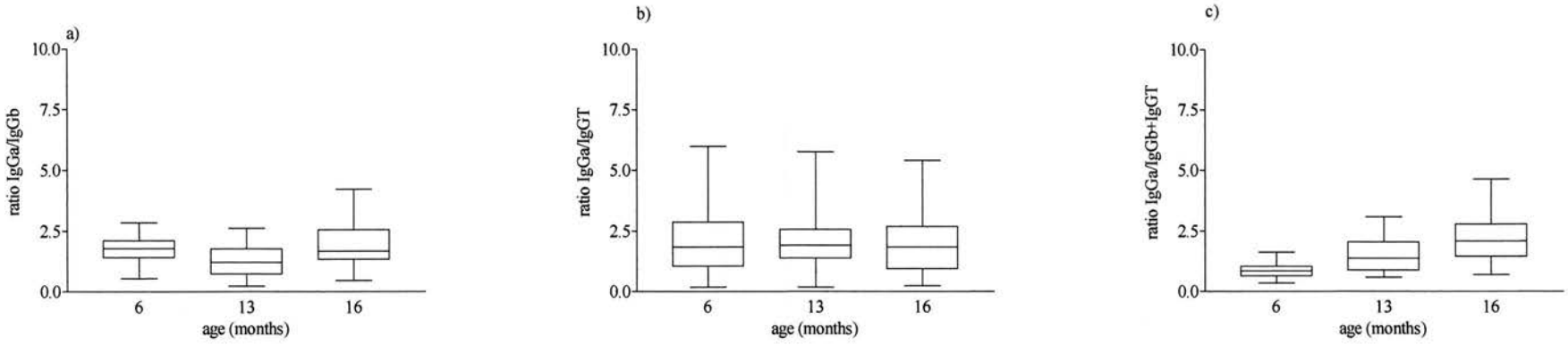
IgGa/IgGb

An overall increase was observed in specific IgGa/IgGb ratios across the sampling period with ANOVA P value at 0.03 and Tukey's post-tests at P = >0.005 between 6 and 13 months and 6 and 16 months. Between 13 and 16 months P = <0.05. Unpaired t-tests gave P values of P = 0.02, P = 0.07 and P = 0.03 between 6 and 13 months, 6 and 16 months and 13 and 16 months respectively.

IgGa/IgGT

An overall increase was observed in specific IgGa/IgGT ratios across the sampling period with ANOVA P value at <0.0001. Multiple comparisons using Tukey's post-tests gave P values of P = <0.001 between 6 and 13 months and 6 and 16 months. P = >0.05 between 13 and 16 months. Unpaired t-tests gave P values of P =< 0.0001 between 6 and 13 months 6 and 16 months. P = 0.76 between 13 and 16 months.

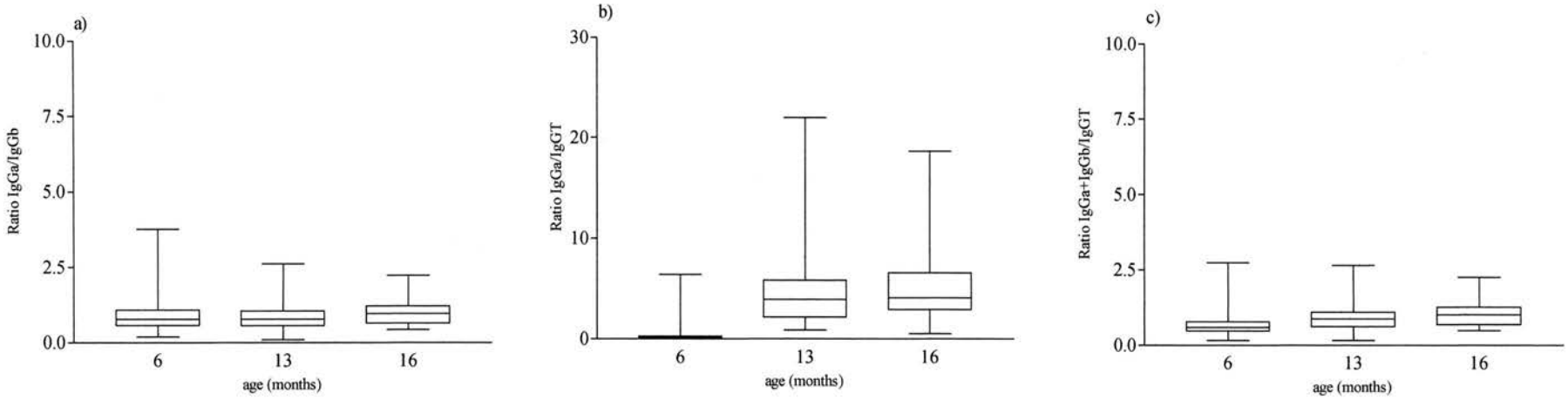
Fig. 3.4a



Boxplots demonstrating differences in specific IgG subclass ratios to BoNT/C: a) IgGa/IgGb b) IgGa/IgGT c) IgGa/IgGb+ IgGT

Significant increases were demonstrated between each data point of IgGa/IgGb + IgGT ratios.

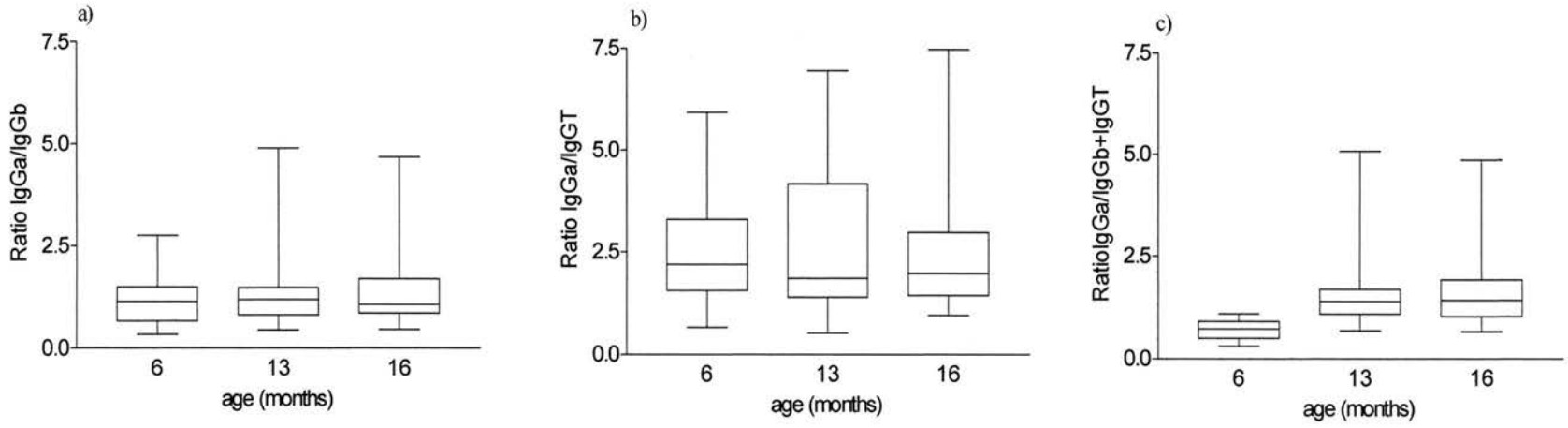
Fig.3.4b



Boxplots showing IgG subclass ratios against BoNT/D:a) IgGa/IgGb b) IgGa/IgGT c) IgGa/IgGb +IgGT

A significant difference was demonstrated between IgG subclass ratios IgGa/IgGT (P =<0.0001). An overall increase between IgGa/IgGb+IgGT ratios approached significance between 6m and 16m (P=0.07).

Fig.3.4c



Boxplots demonstrating differences in specific IgG subclass levels to SA: a) IgGa/IgGb b) IgGa/IgGT c) IgGa/IgGb+ IgGT

One – way ANOVA demonstrated an overall increase in ratios of specific IgGa/IgGb+IgGT with P = <0.0001 and Tukey's post-tests P = <0.001 between 6 and 13 months and 6 and 16 months and P = >0.05 between 13 and 16 months.

IgGa/IgGb+IgGT

One way ANOVA demonstrated a significant overall increase in the ratio of IgGa/IgGb+IgGT over time-with P=< 0.0001. Tukey's post-tests gave P=>0.01 between 6 and 13 months and 13 and 16 months, P = <0.001 between 6 and 16 months. Unpaired t-tests gave P values of P=0.004 and P=0.01 between 6 and 13 months and 13 and 16 months and P =<0.001 between 6 and 16 months.

BoNT/D

IgGa/IgGb

No significant change was demonstrated between specific IgGa/IgGb ratios over the sampling period with ANOVA P=0.97 and all Tukey's post tests at P =>0.05.

IgGa/IgGT

Overall ANOVA for specific IgGa/IgGT levels demonstrated a P=<0.001 with Tukey's post tests giving P= <0.001 between 6 and 13 months and 6 and 16 months and P = >0.05 between 13 and 16 months. Unpaired t-tests gave P=<0.0001 between 6 and 13 months and 6 and 16 months but no significant difference between 13 and 16 months (P = 0.7).

IgGa/IgGb+IgGT

One way ANOVA demonstrated no significant overall increase in the ratio of IgGa/IgGb+IgGT over time-with P= >0.15. Tukey's post-tests gave P=>0.05. Unpaired t-tests demonstrated an almost significant increase between 6months and (P = 0.07) and a trend toward an increase in level between 6 and 13 months (P = 0.11).

3.1.7 *IgG subclass ratios against surface antigens*

IgGa/IgGb

No significant difference was observed in specific IgGa/IgGb using ANOVA (P= 0.7).

IgGa/IgGT

ANOVA demonstrated an overall increase in ratios of specific IgGa/IgGT with P = <0.0001 and Tukey's post-tests P = <0.001 between 6 and 13 months and 6 and 16 months and P = >0.05 between 13 and 16 months.

Unpaired t-tests showed P=<0.0001 between 6 and 13 months and 6 and 16 months and P = 0.7 between 13 and 16 months.

IgGa/IgGb+IgGT

ANOVA demonstrated an overall increase in ratios of specific IgGa/IgGb+IgGT with P = <0.0001 and Tukey's post-tests P = <0.001 between 6 and 13 months and 6 and 16 months and P = >0.05 between 13 and 16 months. Unpaired t-tests showed P=<0.0001 between 6 and 13 months and 6 and 16 months and P = 0.8 between 13 and 16 months.

3.2.1 Longitudinal study of adult horses

Very little is known about IgG levels in healthy horses against clostridial antigens and neurotoxins. This study was designed to examine the IgG levels of a herd of clinically healthy horses over the course of 12m-to determine if there was any seasonal difference or if different management practices affected IgG level.

3.2.2 *Samples and study*

Forty horses (from a herd of 144) were used from a herd kept specifically for bleeding for blood products-samples received were excess from these samples and so no licence was required. Horses were sampled at two weekly intervals over the course of a year, from the end of September 2002 to the beginning of September 2003. Horses were not bled if they were on antibiotics, of poor body condition or in the same week as their tetanus and equine flu vaccinations, given every other year.

Animals at the premises were retired or recuperating from surgery or lameness. Horses were chosen for this study by the staff, on the basis that they would probably be there for the duration of the study and that they were 'good doers' and would therefore provide regular samples. The full set of samples was not obtained every fortnight, either for the reasons given above, due to staff changes and in the summer, when fewer horses were needed to be bled due to the increase in packed cell volume (PCV). Due to holidays, samples were not taken over the Christmas break (between 16th December and 16th January, or the first week in February).

Horses were out at grass part-time from the end of February and all horses turned out full-time from the middle of March. When housed indoors, horses were barn kept and fed big-bale horsehage *ad liberatum* and hard feed when necessary. Horses had access to the barns in the summer although no horsehage was fed during the months May through to September.

Age of horses

The ages of the horses were normally distributed with mean = 11.53, median = 11.50 and a range of 5 - 19.

3.2.3 *Analysis of median IgG levels of the herd over time.*

Median values of IgG levels against the neurotoxins and SA can be seen in Fig.3.5 and descriptive statistics are detailed in Table 3.1.

Differences between medians of sample sets were calculated using Mann-Whitney test as data was not normally distributed before or after transformation.

BoNT/C

Significant increases were demonstrated in median anti-BoNT/C IgG levels between samples 7-8, 8-9, 11-12, 14-15 and 20-21 ($P = <0.05$ for each, $P = <0.0001$ between 11-12).

Significant decreases were demonstrated between samples 3-4, 9-10, 10-11, 13-14, 16-17, 17-18 and 19-20 ($P = <0.05$ for each).

Fig.3.5a

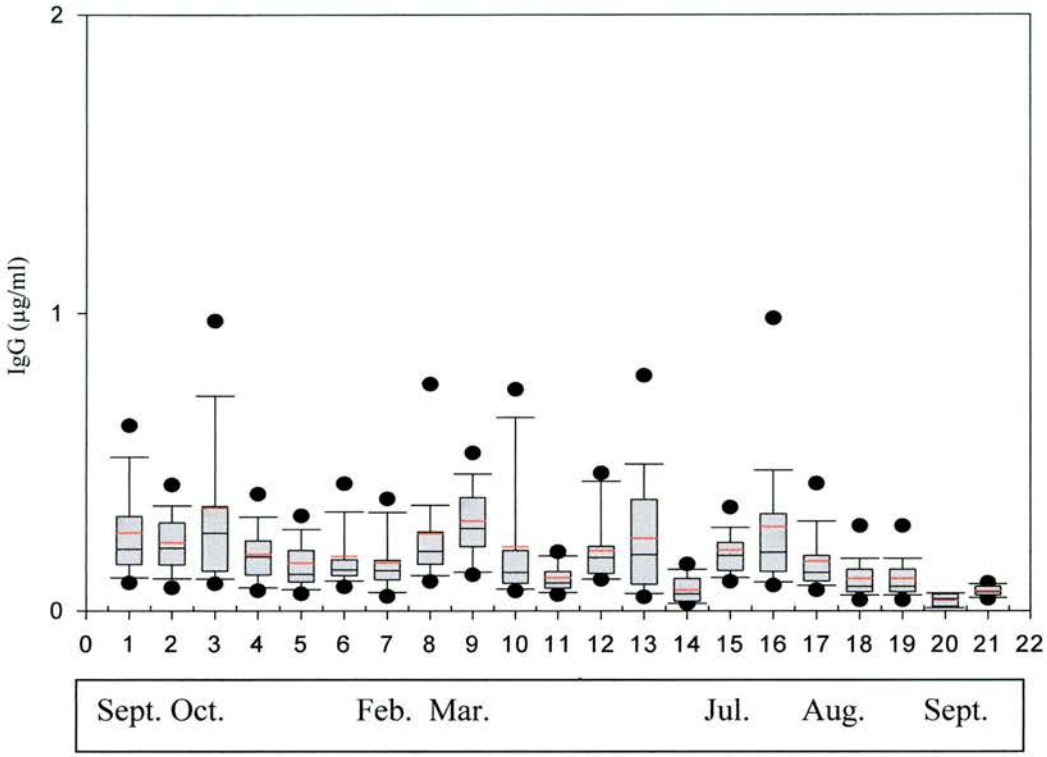


Fig.3.5b

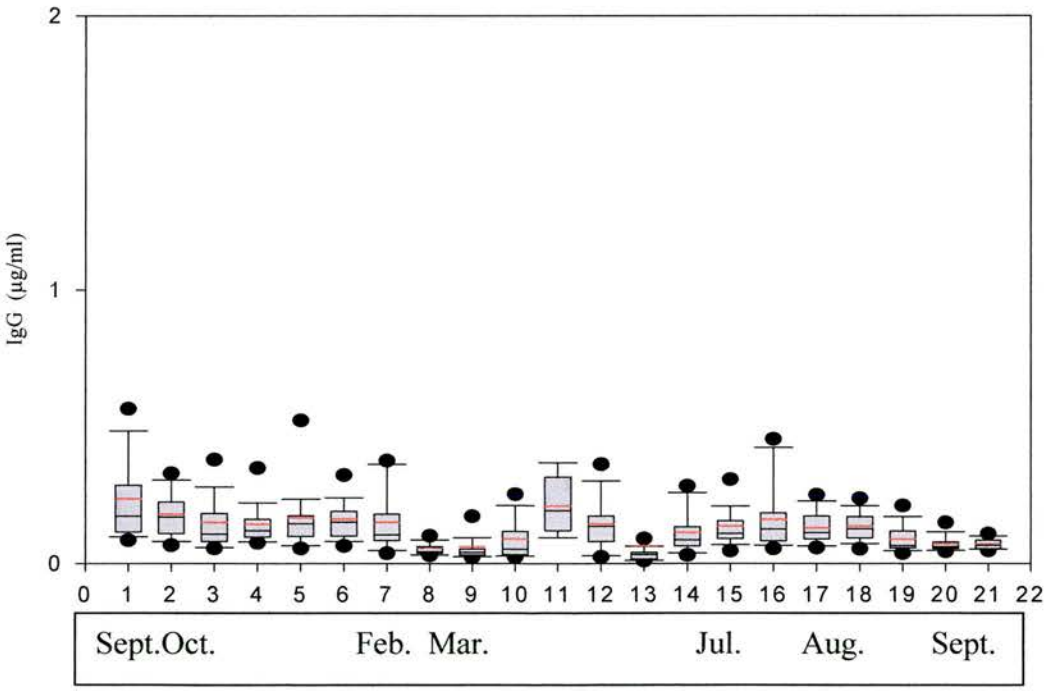
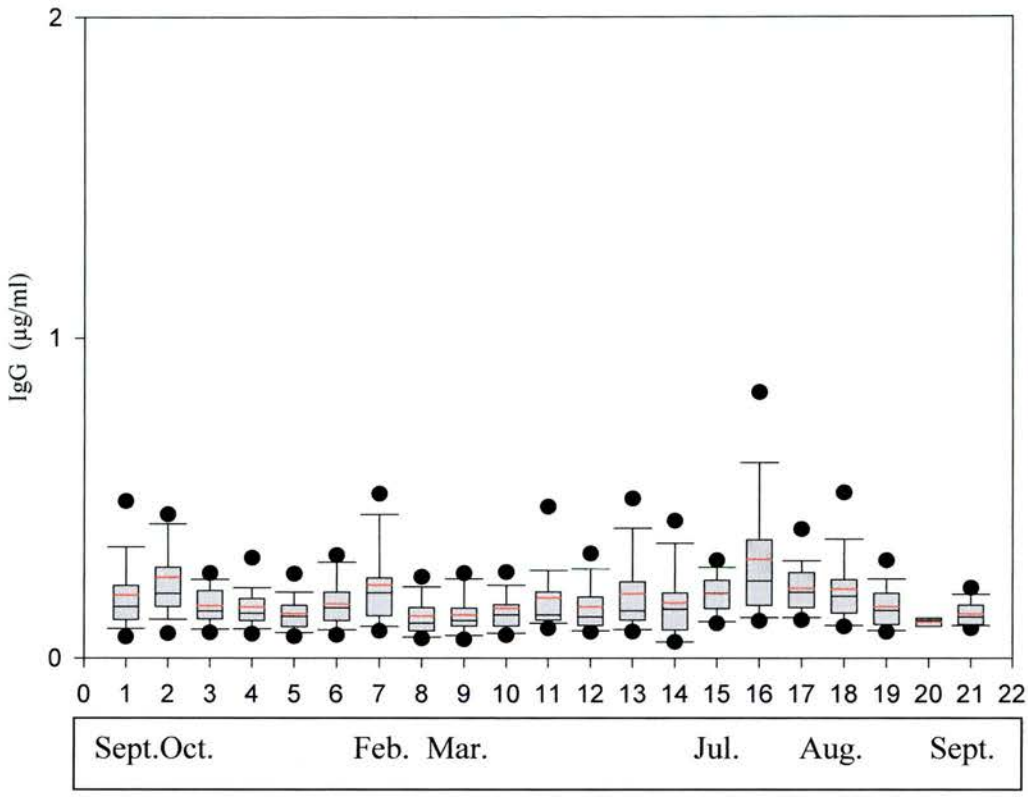


Fig.3.5c



Graphs depicting Boxplots of herd titres against a) BoNT/C b) BoNT/D and c) SA. See Table 3.1 for dates of samples.

Boxplots depict the 5th and 95th percentiles, mean (red lines) and median values for each data set.

Samples 1 and 2 were taken when the horses were still out at grass and they were bought in and barn-kept from October (sample 3) until the end of January (sample 6), part-time at grass from Sample 7and all horses were kept at grass fulltime from the middle of March (sample 9).

Table 3.1 Descriptive statistics from longitudinal analysis of herd IgG levels against a) BoNT/C b) BoNT/D c) SA (Sept. 02 – Jun 03)

Date	Sept.	Oct.		Nov.		Dec.	Jan.	Feb.	Mar.		Apr.		May		Jun	
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
a)																
N	37	40	27	32	37	33	31	37	25	32	28	29	29	10	30	25
Range	0.70-0.08	0.54-0.05	1.82-0.08	0.50-0.06	0.60-0 .05	0.86-0.07	0.55-0.01	1.30-0.10	0.70-0.11	0.90-0.06	0.29-0.05	0.47-0.10	0.79-0.04	0.16-0.02	0.83-0.09	1.29-0.08
Mean	0.26	0.23	0.35	0.19	0.16	0.18	0.16	0.26	0.30	0.22	0.11	0.20	0.24	0.07	0.21	0.20
Median	0.21	0.21	0.26	0.18	0.12	0.14	0.14	0.20	0.28	0.13	0.09	0.18	0.19	0.06	0.19	0.20
b)																
Range	0.87-0.08	0.53-0.06	0.49-0.05	0.41-0.07	0.62-0.05	0.49-0.05	0.48-0.03	0.30-0.02	0.41-0.02	0.33-0.02	0.40-0.08	0.38-0.02	1.04-0.00	0.29-0.03	0.52-0.04	0.48-0.04
Mean	0.24	0.18	0.15	0.14	0.17	0.16	0.15	0.06	0.06	0.90	0.21	0.14	0.06	0.11	0.14	0.16
Median	0.17	0.17	0.11	0.12	0.15	0.15	0.10	0.04	0.04	0.05	0.19	0.14	0.03	0.09	0.11	0.13
c)																
Range	0.66-0.06	1.17-0.02	0.35-0.06	0.41-0.07	0.29-0.06	0.38-0.06	0.62-0.05	0.40-0.05	0.27-0.04	0.81-0.07	0.84-0.07	0.44-0.08	0.63-0.07	0.44-0.05	0.31-0.11	0.89-0.90
Mean	0.20	0.25	0.16	0.16	0.14	0.17	0.23	0.13	0.13	0.16	0.19	0.16	0.20	0.17	0.20	0.31
Median	0.16	0.20	0.15	0.14	0.13	0.16	0.20	0.11	0.12	0.14	0.14	0.13	0.15	0.15	0.20	0.24

Table 3.1 Descriptive statistics from longitudinal analysis of herd IgG levels against a) BoNT/C b) BoNT/D c) SA (June 03-Aug 03)

Date	June	July		August	
Sample	17	18	19	20	21
N =	20	14	13	5	22
a)					
Range	0.55-0.07	0.32-0.03	0.18-0.03	0.06-0.01	0.10-0.04
Mean	0.17	0.11	0.08	0.04	0.07
Median	0.13	0.08	0.07	0.04	0.06
b)					
Range	0.27-0.05	0.24-0.05	0.21-0.04	0.15-0.04	0.11-0.04
Mean	0.13	0.14	0.09	0.07	0.07
Median	0.11	0.13	0.06	0.06	0.07
c)					
Range	0.48-0.09	0.57-0.12	0.32-0.08	0.13-0.09	0.23-0.08
Mean	0.22	0.22	0.16	0.11	0.14
Median	0.21	0.19	0.15	0.12	0.13

BoNT/D

Significant increases demonstrated between samples 10-11, 13-14 (P = 0.001 and <0.001 respectively). Significant decreases observed between samples 7-8, 12-13 (P = < 0.001 for each).

SA

Significant increase were observed between samples 1-2, 5-6 (P = 0.03 and 0.07 respectively) and an almost significant increases between samples 15-16 (P = 0.06). Significant decreases were observed between samples 2-3 and 7-8 (P = 0.03 and 0.0001).

The increase in anti-BoNT/C IgG coincided with the horses beginning to be turned out and corresponds with a drop in level of anti-SA IgG and the subsequent fall in anti-BoNT/C levels corresponds with an increase in anti-SA IgG level although the increases are not statistically significant. Other increases between samples 14-15 and 20-21 also correspond with increases of IgG to SA and BoNT/D although these are not significant rises in level against the latter two antigens. A decrease in IgG to BoNT/C between samples 9-10 corresponds to a slight increase of IgG to SA.

3.2.4 *Correlation between age and specific IgG level*

Results of this analysis is shown in Table 3.2 and Fig.3.6. Some positive and negative correlation was demonstrated between different samples and age against BoNT/C. No correlation was observed between age and anti-BoNT/D IgG level and

only two instances of positive correlation was demonstrated between age and anti-SA IgG level.

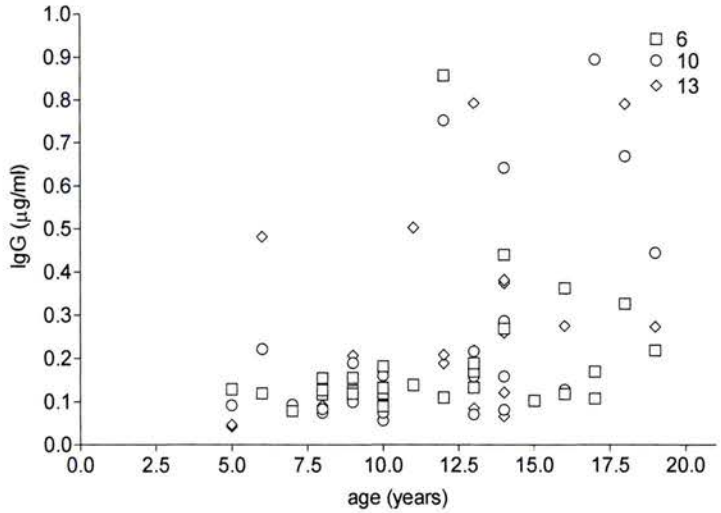
All correlations were calculated using the non-parametric Spearman's correlation coefficient.

Table 3.2. Results of age-specific IgG level in all animals sampled

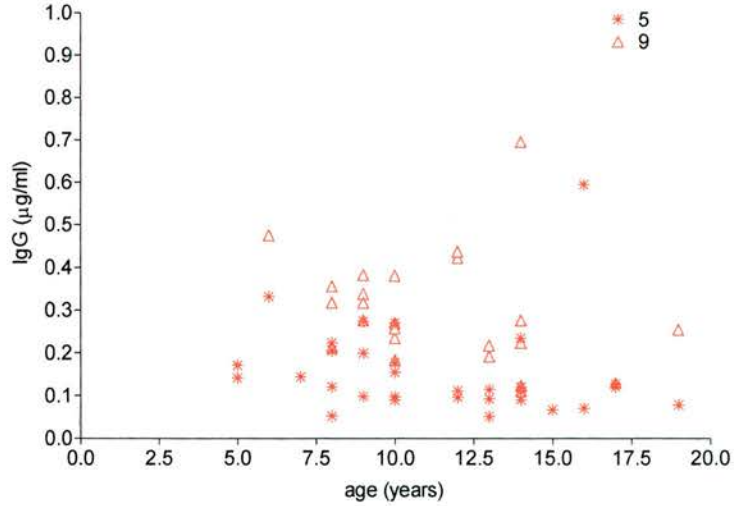
Sample no.	P value	Spearman's r	positive/negative
BoNT/C			
5	0.04	-0.36	negative
6	0.04	0.38	positive
9	0.04	-0.42	negative
10	0.01	0.47	positive
13	0.007	0.51	positive
SA			
1	0.03	0.36	positive
12	0.02	0.48	positive

It is interesting to note that negative correlations are demonstrated between age and anti-BoNT/C level when levels are comparatively low (sample 7) or high (sample 9). Positive correlation is again demonstrated when levels fall. This is also demonstrated in IgG to SA to a lesser extent and no negative correlation is observed with comparatively low or high levels. The lack of any sort of correlation between age and IgG to BoNT/D may be due to the much lower levels to this neurotoxin, which may be due to the rarity of BoNT/D producing organisms in the environment.

Fig.3.6 a)



b)



Graphs showing correlation between anti-BoNT/C IgG and age of all horses sampled a) positive correlation was demonstrated at samples 6, 10 and 13 b) negative correlation demonstrated at samples 5 and 9.

3.2.5 *Correlation between specific IgG levels*

In order to identify any correlation between fluctuations of specific IgG levels was carried out on each data set showing significant differences. Levels were not normally distributed before or after transformation so the non-parametric Spearman's correlation was used.

Sample 7

The results of this analysis can be seen in Fig. 3.7a. A positive correlation was demonstrated between anti-BoNT/C and anti-BoNT/D IgG levels ($P = 0.007$, Spearman's $r = 0.5$) and anti-BoNT/D and anti-SA IgG ($P = 0.0006$, Spearman's $r = 0.6$) but not between anti-BoNT/C IgG and anti-SA levels ($P = 0.13$, Spearman's $r = 0.30$).

Sample 8

A positive correlation was demonstrated between anti-BoNT/D and anti-SA IgG level ($P = <0.001$, Spearman's $r = 0.7$) but not between anti-BoNT/C and anti-SA IgG or anti-BoNT/C and anti-BoNT/D IgG levels ($P = 0.5$, Spearman's $r = 0.12$ and $P = 0.6$, Spearman's $r = -0.09$ respectively.

Sample 9

No significant correlation was demonstrated between any IgG levels for this sample point.

Sample 10

A positive correlation was demonstrated between anti-BoNT/D and anti-SA IgG level ($P = <0.007$, Spearman's $r = 0.44$) but not between anti-BoNT/C and anti-SA IgG or anti-BoNT/C and anti-BoNT/D IgG levels ($P = 0.33$, Spearman's $r = -0.2$ and $P = 0.13$, Spearman's $r = 0.2$ respectively.)

Sample 14

No correlation was demonstrated between any IgG level.

Sample 15

A positive correlation was demonstrated between anti-BoNT/D and anti-SA IgG level ($P = 0.03$, Spearman's $r = 0.41$) but not between anti-BoNT/C and anti-SA IgG or anti-BoNT/C and anti-BoNT/D IgG levels ($P = 0.9$, Spearman's $r = -0.2$ and $P = 0.14$, Spearman's $r = 0.14$ respectively.)

Sample 16

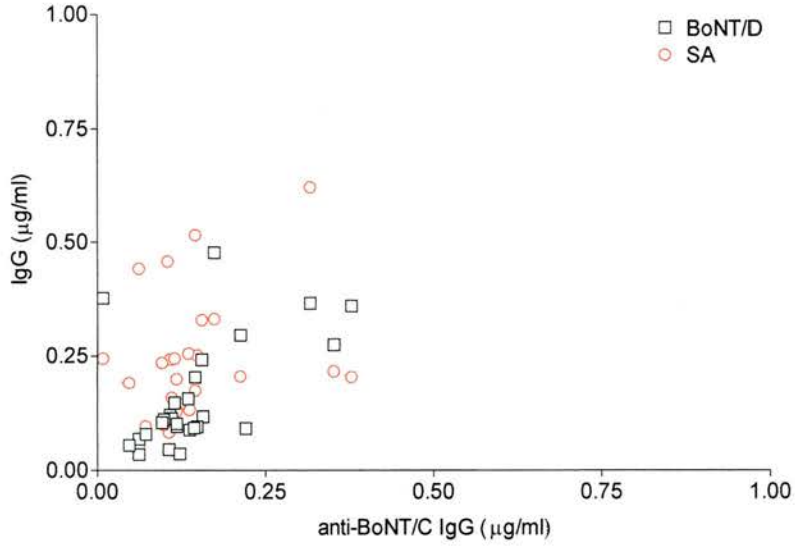
The results of this analysis can be seen in Fig.3.7b. A positive correlation was demonstrated between anti-BoNT/C and anti-BoNT/D IgG levels ($P = 0.03$, Spearman's $r = 0.53$) and anti-BoNT/D and anti-SA IgG ($P = 0.008$, Spearman's $r = 0.53$) and between anti-BoNT/C IgG and anti-SA levels ($P = 0.05$, Spearman's $r = 0.49$).

Sample 17

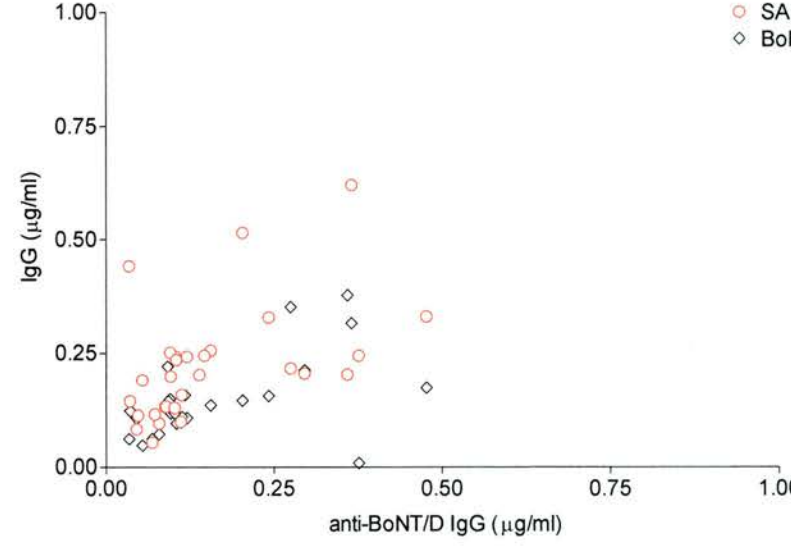
A positive correlation was demonstrated between anti-BoNT/D and anti-SA IgG level ($P = 0.01$, Spearman's $r = 0.6$) but not between anti-BoNT/C and anti-SA IgG or anti-BoNT/C and anti-BoNT/D IgG levels ($P = 0.7$, Spearman's $r = 0.15$ and $P = 0.13$, Spearman's $r = 0.14$ respectively).

Before IgG level to BoNT/C rises between samples 7-8, there is a positive correlation between IgG to both neurotoxins and between IgG to SA and BoNT/D although not between BoNT/C and SA. The correlation is lost between the neurotoxins as IgG to BoNT/C continues to rise in the next two samples (8-9). The

Fig.3.7a

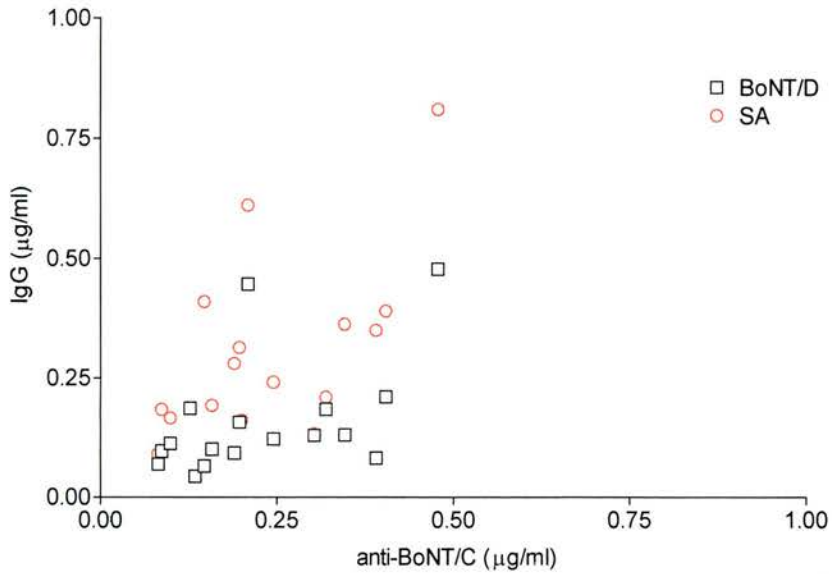


b)

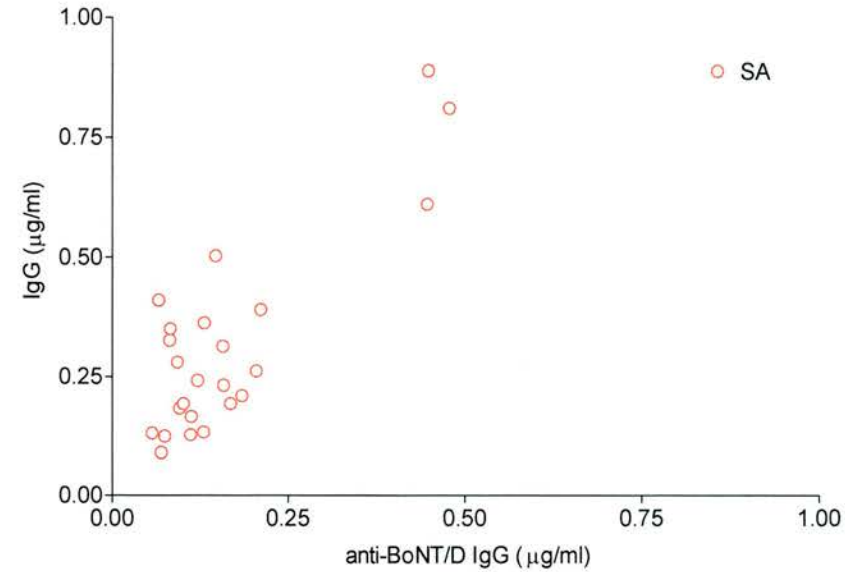


Correlation between IgG titres at sample 7. A positive correlation was demonstrated between anti-BoNT/C and anti-BoNT/D IgG titres (P = 0.007, Spearman's r = 0.5) and anti-BoNT/D and anti-SA IgG (P = 0.0006, Spearman's r = 0.6) but not between anti-BoNT/C IgG and anti-SA titres (P = 0.13, Spearman's r = 0.30).

Fig. 3.7c)



d)



Correlation at Sample16. Significant, positive correlations were demonstrated between all titres as calculated using Spearman's correlation coefficient. A positive correlation was demonstrated between anti-BoNT/C and anti-BoNT/D IgG titres (P = 0.03, Spearman's r = 0.53) and anti-BoNT/D and anti-SA IgG (P = 0.008, Spearman's r = 0.53) and between anti-BoNT/C IgG and anti-SA titres (P = 0.05, Spearman's r = 0.49).

only positive correlation demonstrated between all combinations is at sample 16 (Fig. 3.7b) when all levels are comparatively high.

3.2.6 Analysis of IgG levels in selected horses

With such a large data set, specific levels were not analysed for every horse over the 12m sampling period. An anti-tetanus ELISA was developed to depict IgG levels in horses against a known encountered antigen and to demonstrate that the ELISAs were representative. Nine horses were chosen on the basis of tetanus vaccinations occurring during the sampling period and encompassing the age range. Table 3.3 lists ages and breed of the selected animals.

3.2.6.1 IgG levels against TetNT

IgG levels against TetNT can be seen in Fig.3.8. An anti-tetanus ELISA was used to demonstrate that the ELISA methodology was representative of what was happening to IgG levels. These samples were assayed three times, in duplicate and error bars on the graphs depict the SEM. The two week period that the vaccination was given was known, but not the exact date. Large peaks of specific IgG were demonstrated in the two weeks after the vaccination and horses took varying times for the level to drop e.g. from 10wks (1468) to 26 wks (1159).

Fig. 3.8a)

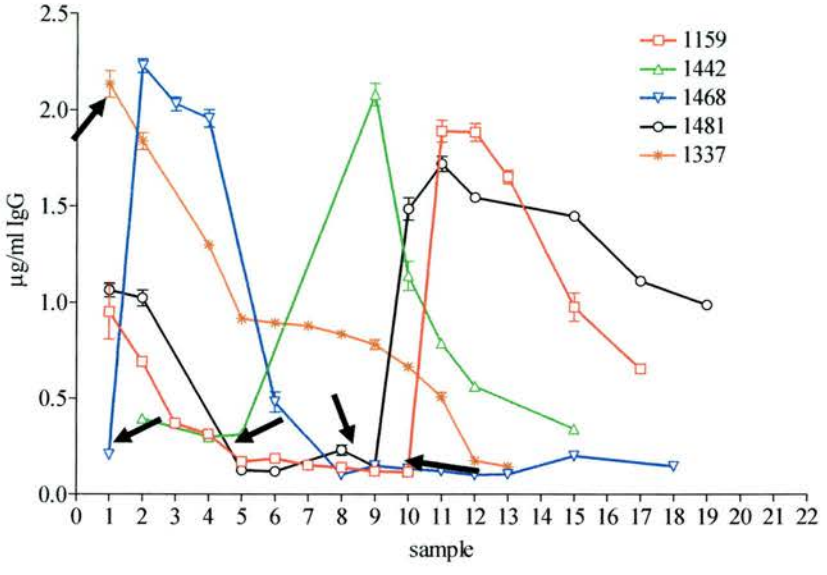
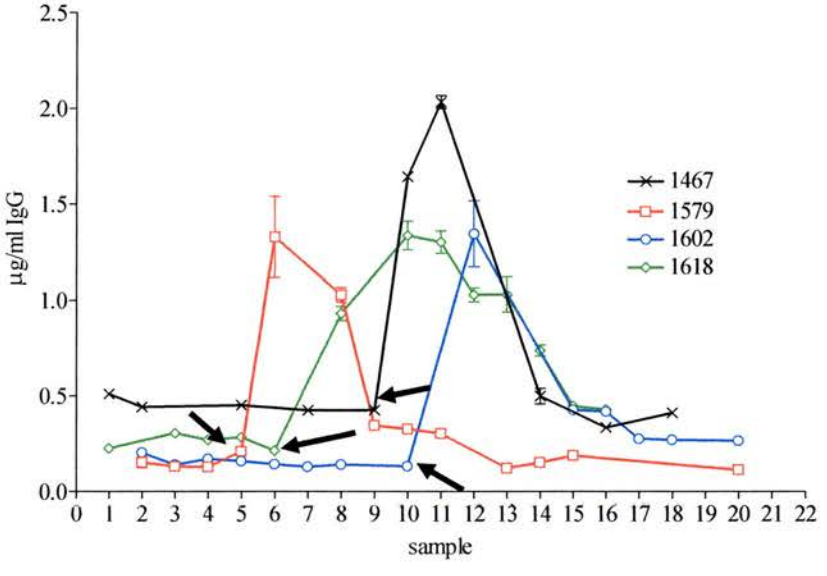


Fig.3.8b)



Graph depicting anti-tetanus IgG in animals following vaccination. Horses were split into two random groups a) and b).

Arrows depict the sample before vaccination-except for 1337 which had been vaccinated at the beginning of September. Animals were not sampled the week they received their vaccinations. Error bars demonstrate the SEM. These samples were assayed three times, in duplicate. Horses shown were chosen on the basis of vaccinations occurring during sampling.

Table 3.3. Ages and breeds of animals used in specific IgG analysis

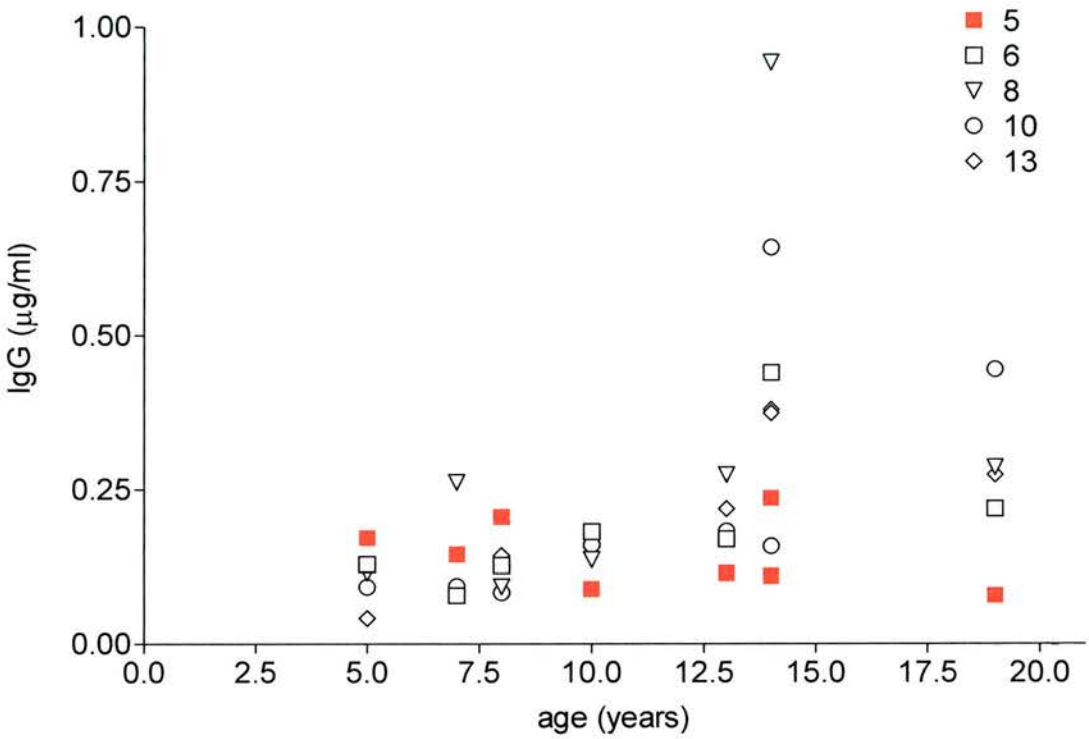
Case no.	Age(y)	breed/type
1159	19	cob
1337	14	¾ TB
1442	14	TB
1467	14	¾ TB
1468	13	TB
1481	10	½ TB
1579	8	½ TB
1602	7	TB
1618	5	TB

TB = Thoroughbred

3.2.6.2 Age-specific IgG level correlation

No correlation was demonstrated between age and anti-BoNT/D level in the nine selected animals. Results of correlation analysis with BoNT/C and SA can be seen in Table 3.4 and Fig.3.9.

Fig. 3.9



Age-IgG correlation between nine selected animals and anti-BoNT/C IgG level. Of the 21 samples, positive correlation was demonstrated between age and IgG titre in samples 6, 8, 10 and 13. No correlation was observed in sample 5, which is included for comparison.

3.9

Table 3.4. Results of age-specific IgG level in all animals sampled

Sample no.	P value	Spearman's r	positive/negative
BoNT/C			
6	0.03	0.82	positive
8	0.05	0.79	positive
10	0.05	0.77	positive
13	0.02	0.83	positive
SA			
12	0.02	0.93	positive

Positive correlation between age and IgG to BoNT/C was again demonstrated at samples 6, 10 and 13 and additionally at sample 8.

3.2.6.3 Longitudinal analysis of IgG levels

Longitudinal analysis of individual horses is illustrated in Fig.3.10a-f.

Horse 1159

Little correlation is demonstrated between IgG levels of this horse. An increase in IgG to SA at sample 12 does correspond with an increase in anti-BoNT/D IgG but no increase is observed in anti-BoNT/C t the same sample point. An increase in anti-BoNT/C at sample 16 does correspond with a slight rise in IgG to BoNT/D and a fall in anti-SA IgG.

Horse 1337

A peak of IgG to BoNT/C at sample 6 is followed by a peak of anti-BoNT/D and anti-SA at sample 7. There are further peaks of anti-BoNT/C IgG at samples 8 and 12 with a corresponding peak of anti-BoNT/D also at sample 12. Further analysis showed a significant correlation between BoNT/D and SA IgG levels but not BoNT/C and SA levels (Table 3.5).

Horse 1442

An increase in IgG to BoNT/C is demonstrated at sample 10 along with a peak in anti-SA. IgG to BoNT/D remains fairly constant and further analysis demonstrated a strong correlation between anti-BoNT/C and anti-SA IgG levels (Table 3.5).

Horse 1468

Comparatively high levels against all BoNT/C and SA were demonstrated initially but a large peak of anti-BoNT/C at sample 13 has no corresponding peak of anti-SA or anti-BoNT/D.

Horse 1481

Comparatively high initial levels of IgG against all three antigens are demonstrated and a large peak of IgG against all three is also seen at sample 16. This animal demonstrated a strong correlation between all combinations of tested antigens (Table 3.5, Fig.3.11).

Horse 1467

A peak of IgG to BoNT/C is demonstrated at sample 6, followed by peaks of IgG to BoNT/D and SA at samples 7 and 8 respectively. A further peak of anti-BoNT/C and BoNT/D is seen at sample 15 but no peak of SA is seen.

Horse 1579

A large peak in IgG to BoNT/C at sample 3 corresponds with a drop in IgG to BoNT/D and SA. Likewise, a peak of anti-BoNT/C at sample 9 does not correspond with peaks of anti-SA or anti-BoNT/D.

Horse 1602

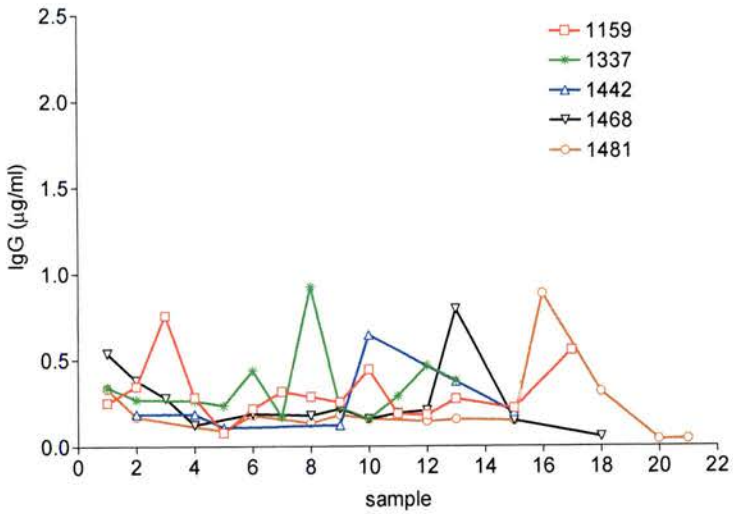
Small peaks are demonstrated at sample 5 against all three antigens. A small peak of anti-BoNT/D at sample 18 corresponds with a drop in IgG against the other two antigens.

Horse 1618

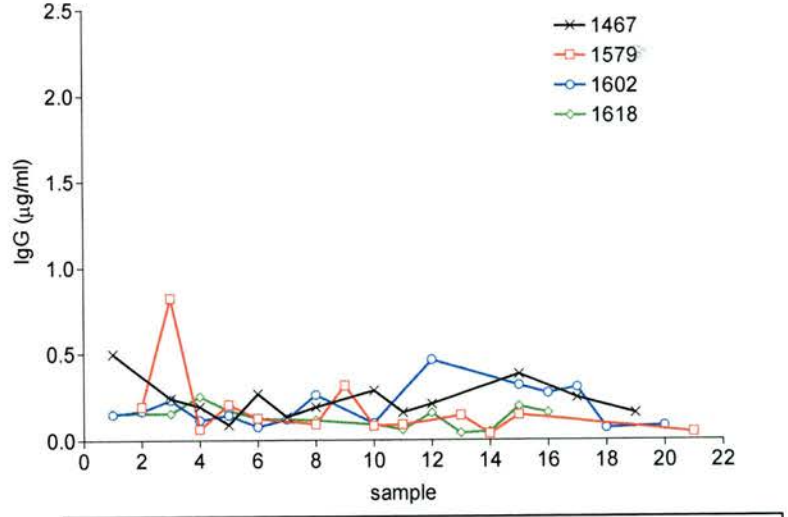
A peak of IgG to BoNT/D at sample 4 corresponds with a smaller peak of IgG to BoNT/C. This animal demonstrated a strong correlation between anti-BoNT/C and anti-BoNT/D (Table 3.5).

Fig.3.10a

i)



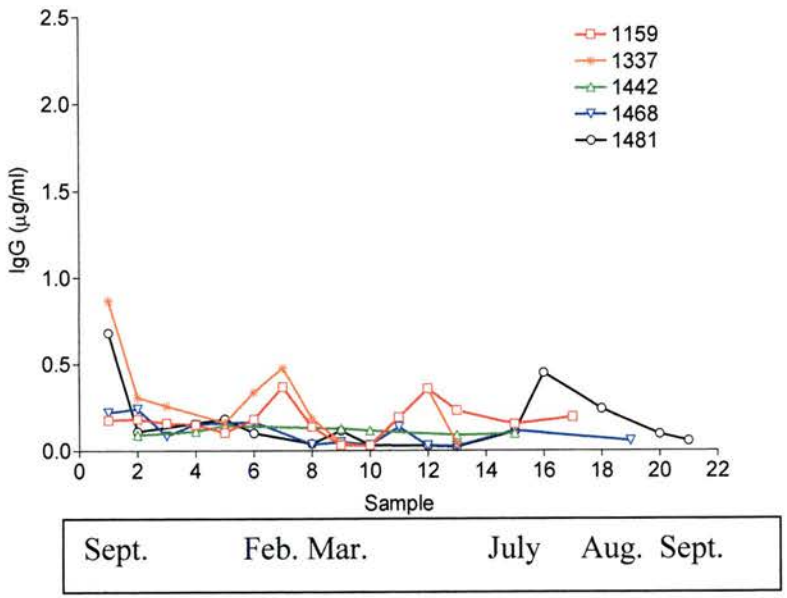
ii)



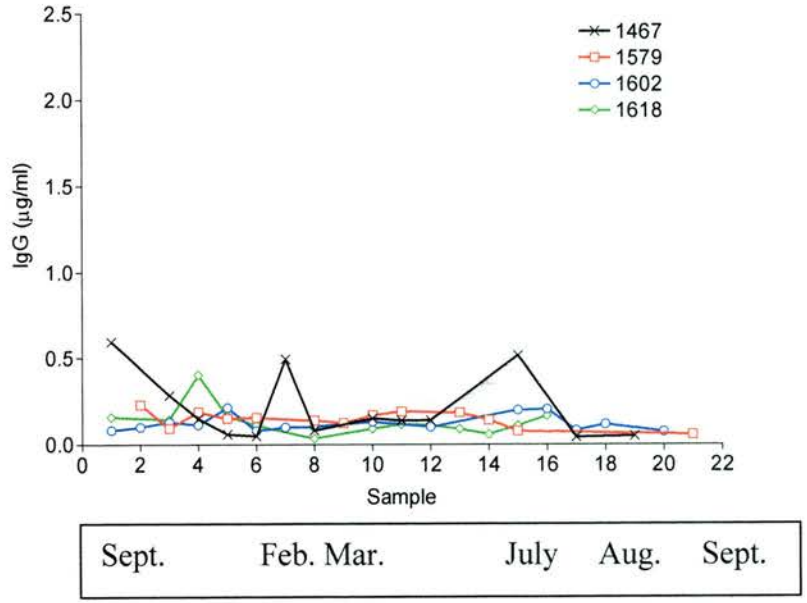
Longitudinal sampling of selected horses against BoNT/C. Horses were selected on the basis of having their anti-tetanus vaccination within the sampling period.

Fig.3.10b

i)

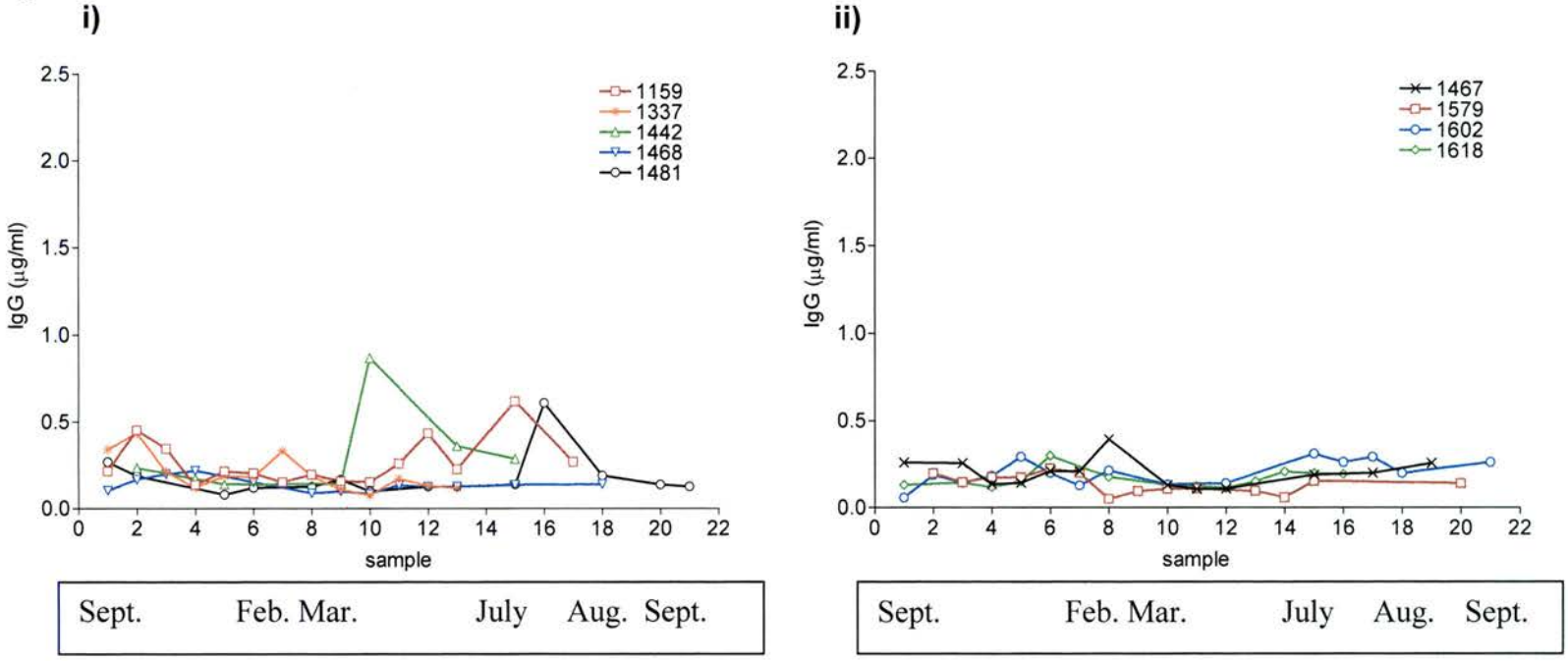


ii)



Longitudinal analysis of IgG levels against BoNT/D of selected horses. Horses were selected on the basis of having their anti-tetanus vaccination within the sampling period, thus allowing examination of ELISA methodology to see if it was representative of IgG fluctuations

Fig.3.10c



Longitudinal analysis of IgG titres to SA of selected animals. Horses were selected on the basis of having their anti-tetanus vaccination within the sampling period, thus allowing examination of ELISA methodology to see if it was representative of IgG fluctuations.

3.2.6.4 Specific IgG-IgG correlation of horses over time

Results of this analysis are detailed in Table 3.5 and case 1481 is shown in Fig.3.11.

Table 3.5 Significant correlations between IgG levels over time

Horse	antigens	P value	Spearman's r
1337	BoNT/D & SA	0.02	0.69
1442	BoNT/C & SA	0.005	0.87
1481	BoNT/C & BoNT/D	0.025	0.60
	BoNT/C & SA	0.02	0.62
	BoNT/D & SA	0.02	0.63
1618	BoNT/C & BoNT/D	0.01	0.66

3.2.6 *Analysis of median specific IgGT OD values*

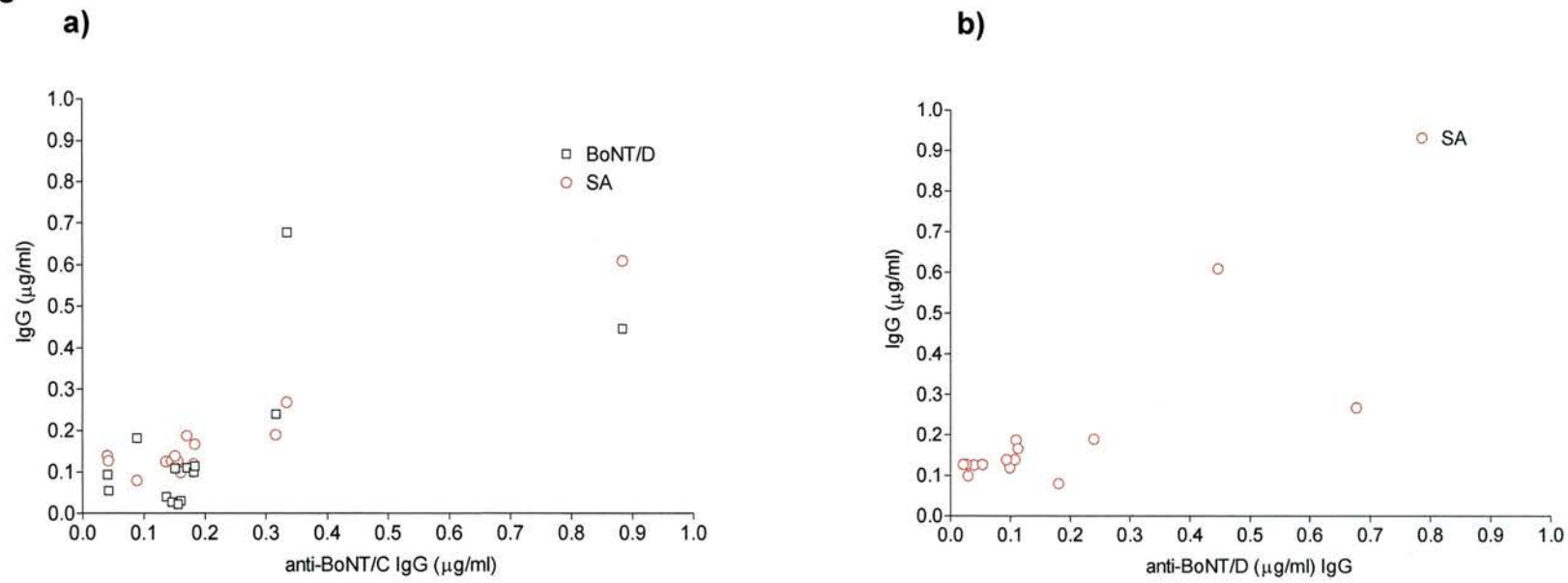
Median values of IgG levels against the neurotoxins can be seen in Fig.3.12 and descriptive statistics are detailed in Table.3.6.

Differences between medians of sample sets were calculated using Mann-Whitney test as data was not normally distributed before or after transformation.

BoNT/C

Significant differences were demonstrated between sample sets using Mann-Whitney U-tests but only one significant difference was calculated using one way ANOVA (Kruskall-Wallis with Dunn's post-test) between OD values of sample 2 and 3

Fig.3.11



Graphs showing correlation between levels of specific IgG of horse 1481:

a) correlation between anti-BoNT/C and anti-BoNT/D and anti-SA (P = 0.03 r = 0.60, r = 0.66, P = 0.02 respectively) and b) anti-BoNT/ D & SA r = 0.63, P = 0.02.

against BoNT/C. Significant differences were demonstrated between each sample set except samples 3-4, 13-14 and 17-18 onwards. All P values <0.005 except that between sample 5-6 (P = 0.01).

BoNT/D

Significant differences were demonstrated (Mann-Whitney U-test) between samples 1-2, 5-6, 7-8, 10-11, 12-13, 16-17. P values were all <0.05.

There is no corresponding increase in IgGT to BoNT/C to the increase in IgG seen in samples 7-9 although there is an increase in IgGT in samples 4-6.

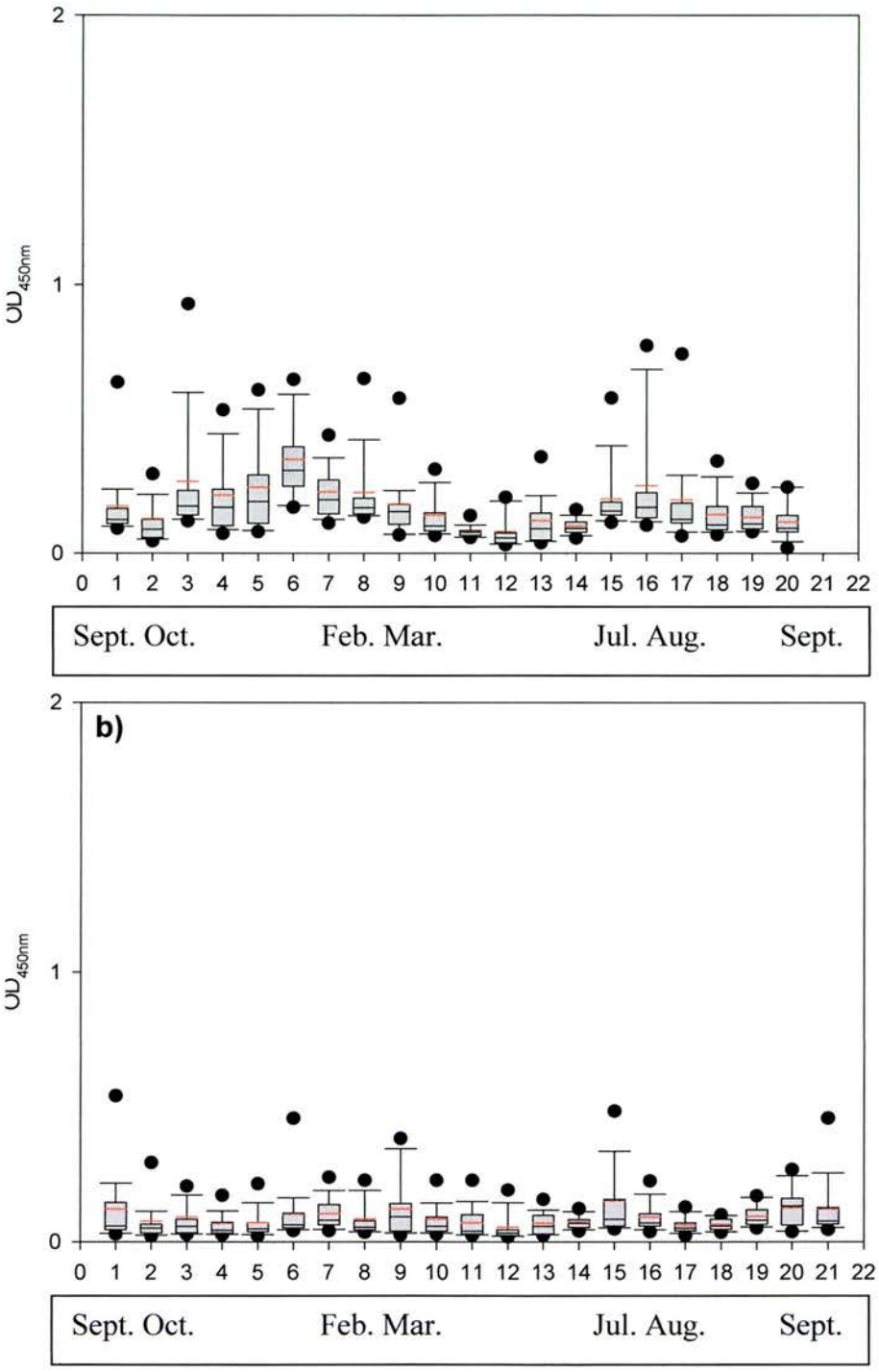
3.2.7 *Specific IgGT-IgGT level correlation*

Results of these analyses are shown in Table 3.6. Strong correlation was observed between most data sets.

3.2.8 *Longitudinal analysis of specific IgGT levels of specific horses*

Results of this analysis are shown in Fig.3.13. There is no correlation between IgGT OD value and specific IgG level. IgGT accounts for only 13% of total IgG (Sheoran et al, 2000) and was not quantified in this study. Large peaks of specific IgGT occurred within two week periods in different animals against both neurotoxins although the largest peaks occurred against BoNT/C. Peaks of anti-BoNT/C rarely coincided with peaks of anti-BoNT/D. Exceptions to this are Horse 1481 at sample 9, Horse 1159 at sample 15, Horse 1602 at sample 16 and Horse 1579 at samples 8 and 10. Horse 1442 showed little change in IgGT OD levels and also was one that demonstrated correlation between IgG to BoNT/C and SA. The other horse to demonstrate correlation between both neurotoxins and SA (1481) demonstrated a large peak of anti-neurotoxin IgGT at sample 9.

Fig.3.12a)



Boxplots showing IgG(T) OD values against a) BoNT/C b) BoNT/D.

Significant correlation was demonstrated between IgGT OD values in 16 out of 21 samples.

Table 3.6 Descriptive statistics from longitudinal analysis of herd IgGT OD values against a) BoNT/C b) BoNT/D c) correlation between specific IgGT OD values

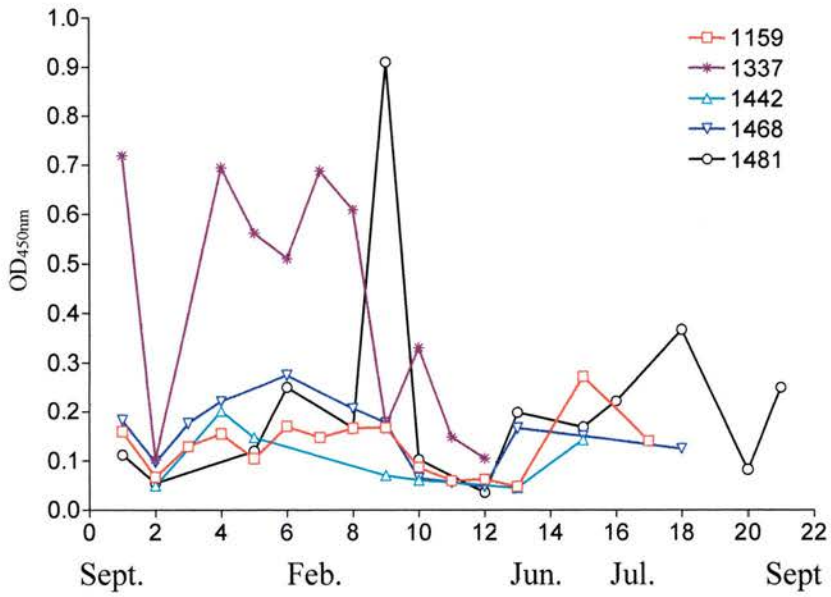
Date	Sept.	Oct.		Nov.		Dec.	Jan.	Feb.	Mar.		Apr.		May		Jun	
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
a)																
N	37	40	27	32	37	33	31	37	25	32	28	29	29	10	30	25
Range	0.72-0.07	1.21-0.04	1.24-0.12	0.69-0.07	1.11-0.08	0.78-0.14	0.70-0.09	0.71-0.13	0.91-0.06	0.73-0.06	0.15-0.06	0.28-0.03	0.47-0.04	0.16-0.06	0.62-0.11	0.92-0.10
Mean	0.17	0.13	0.27	0.22	0.25	0.35	0.23	0.23	0.18	0.14	0.08	0.06	0.09	0.09	0.16	0.17
Median		0.13	0.09	0.18	0.17	0.19	0.31	0.20	0.17	0.15	0.10	0.08	0.06	0.09	0.09	0.16
0.10																
b)																
Range	0.75-0.02	0.60-0.01	0.75-0.03	0.50-0.02	0.32-0.02	0.54-0.03	0.26-0.04	0.53-0.03	0.39-0.02	0.45-0.02	0.33-0.02	0.25-0.02	0.21-0.02	0.12-0.04	1.01-0.03	0.26-0.03
Mean	0.12	0.08	0.09	0.07	0.07	0.10	0.10	0.08	0.12	0.08	0.07	0.05	0.07	0.07	0.15	0.09
Median	0.06	0.05	0.06	0.04	0.05	0.06	0.08	0.05	0.09	0.06	0.04	0.03	0.06	0.07	0.08	0.07
c)																
r	0.48	0.72	0.74	0.70	0.42	0.47	0.43	0.54	0.66	0.98	0.97	0.98	0.69	0.25	0.60	0.64
P value	0.002	<0.001	<0.001	<0.001	0.01	0.007	0.01	0.006	0.002	<0.001	<0.001	<0.001	<0.001	0.25	0.005	0.007

Table 3.6 cont'd

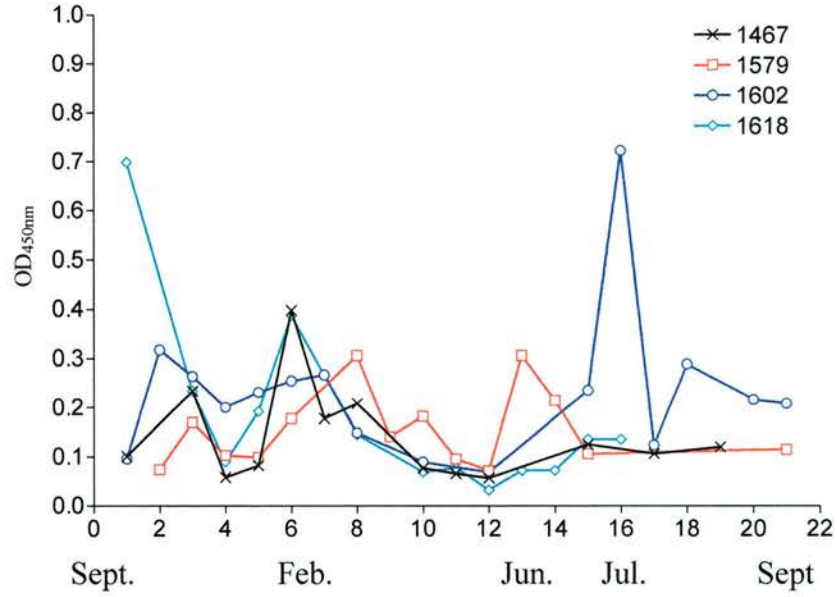
a) BoNT/C b) BoNT/D c) SA (June 03-Aug 03)

Date	June	July		August	
Sample	17	18	19	20	21
N =	20	14	13	5	22
a)					
Range	1.18-0.06	0.38-0.07	0.26-0.08	0.25-0.01	0.30-0.06
Mean	0.20	0.14	0.13	0.12	0.13
Median	0.13	0.11	0.11	0.09	0.11
b)					
Range	0.14-0.02	0.10-0.03	0.17-0.05	0.27-0.04	0.49-0.04
Mean	0.06	0.06	0.09	0.13	0.12
Median	0.05	0.06	0.08	0.13	0.08
c)					
r	0.20	0.64	0.75	-0.02	-0.02
P	0.3	0.007	0.01	0.95	0.41

Fig.3.13 a)

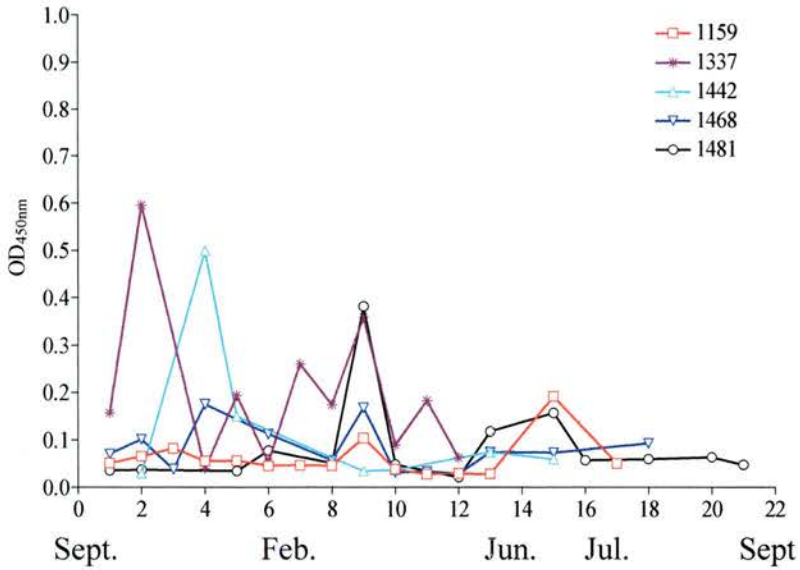


b)

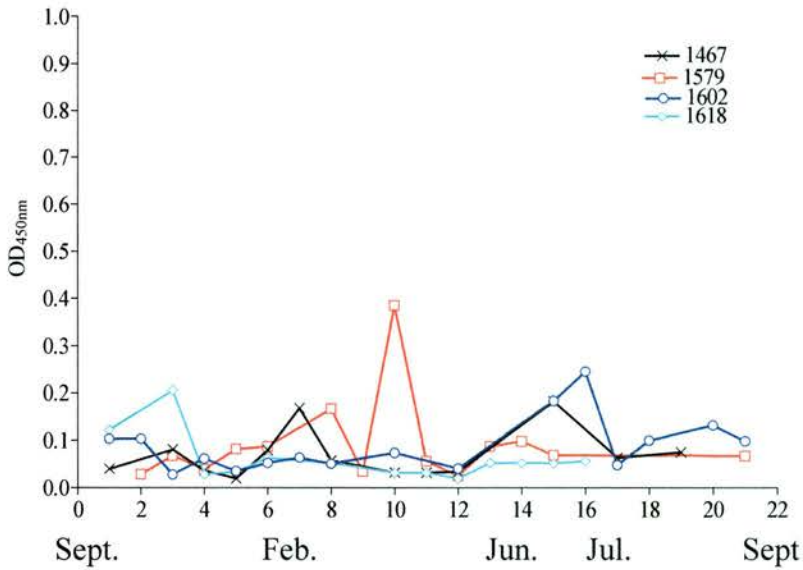


Graphs showing longitudinal IgG(T) OD levels to BoNT/C of selected horses. Large peaks in specific IgG(T)were observed in most of the horses during the sampling period.

Fig. 3.13c



d)



Graphs showing longitudinal IgG(T) OD levels to BoNT/D. Horse 1481 demonstrated peaks of specific IgG(T) against both neurotoxins at sample 9.

3.3 Discussion

3.3.1 Sampling and statistical analysis

Only three data points were obtained for the pony study. It is unfortunate that no other samples were obtained as four data points would have been sufficient to carry out correlation/linear regression of the levels with age (Motulsky, 1996). If the data points had been equally spaced a relationship between age and antibody level could have been calculated using linear correlation. These animals were still immature at the time of last sampling and it would have been interesting to follow the serology for a further year. However, given the difficulty in obtaining licensing for the sampling of healthy animals, finding a suitable group of animals and the cost of sampling such a group, such studies are difficult to organise and any samples are therefore of use. As with all the statistics in this thesis, arguments can be made for and against the use of parametric versus non-parametric tests. Unpaired t-tests were used to examine any differences between data points that may not have been highly significant using one - way ANOVA.

The study of adult horses would have been improved if more animals had been under the age of five but the nature of the premises meant that animals were generally recuperating from surgery or had retired from work. Access to such a large herd with the added benefit of no licensing required was a good opportunity. A control group on GS associated premises would have enabled further comparisons. However, comparisons needed to be made between chronically affected horses, acutely affected horses and co-grazing controls so some idea of normalcy was required i.e. IgG levels

in animals not associated with GS. Additionally, the known vaccination status of these horses enabled a control, in the form of a anti-TetNT ELISA, to be developed.

3.3.2 *Specific IgG of ponies*

The increase in specific IgG seen against all three antigens gives an indication that antibody acquisition increase with age as found by McCarthy et al (2004). The seemingly increased susceptibility of younger animals (those under 7 years of age) to grass sickness has been taken as a sign of the infectious aetiology of the disease. The horse is considered mature physically at ~6 years of age and therefore perhaps it is not surprising that an adult level of immunity is reached by this time. However, a level of immunity to different antigens would presumably be due to abundance of that antigen in the environment and subsequent exposure to it. There is little work published on the natural acquisition of antibody to environmental antigens, most of the literature is concerned with exposure to organisms such as *Rhodococcus equi* (Hooper – McGrevy, 2003), whereby exposure to the organism leads to clinical disease and no work has been published on the sampling of healthy horses longitudinally against enteric organisms. Although the means increase overall with increasing age with this population, there is also some fluctuation within the ranges of specific IgG levels between 13 and 16months - noticeably in anti-BoNT/D and anti-SA. This indicates that levels can rise and fall within individuals, possibly due to other factors such as exposure to other antigens/subclinical infection caused by other organisms or by the (undisclosed) nature of the other study being carried out at the time.

Correlation between anti-SA and anti-BoNT/C did demonstrate a positive relationship between exposure to *C. botulinum* type C and exposure and subsequent immune response to BoNT/C. An inverse relationship has been demonstrated between anti-SA and anti-BoNT/C level in adult co-grazing animals and a high anti-SA level has been statistically shown to be protective against EGS (McCarthy et al, 2004).

The lack of correlation between anti-BoNT/C and anti-BoNT/D IgG suggests that there is little/no detectable cross-reactivity between the two neurotoxins although correlation in antibody levels has been found between the neurotoxins in adult horses. Although some correlation was demonstrated at 13 months, this is not seen at 16 months when comparatively high levels of anti-BoNT/D are observed. Generally, anti-BoNT/D levels are lower than BoNT/C indicating that BoNT/D producing organisms are less prevalent in the environment. The relatively high anti-BoNT/D levels of these ponies indicate greater exposure to the type D organism. These premises were previously associated with cases of grass sickness (within five years of the beginning of the study) which is known to increase the risk of grass sickness (Doxey et al, 1991) and although type D has never been implicated in GS through toxin ELISA this is due to the lack of reagents and the possibility of involvement of the organism cannot be ruled out. These results may indicate that the Type D producing strain is more prevalent at these premises whereas Type C or a combination of the two may exist elsewhere.

IgG subclasses levels were measured by use of OD values and not quantified as specific IgG (mg/ml) so although some parallels between OD and IgG level can been seen it would be misleading to draw too many parallels between IgG and IgG subclass levels.

Specific IgGa increased with age with all three antigens although a decrease in the lower ranges against BoNT/C and SA demonstrates that specific subclass levels can fluctuate.

IgGb is the most abundant IgG subclass in equine serum (Sheoran et al, 2000) and therefore any rise in specific IgGb would go some way in explaining a rise in total specific IgG and this appears to be the case in both anti-BoNT/D and anti-SA IgGb levels. A decrease in the median anti-BoNT/C IgGb level at 16m seems to be cancelled out by a large increase in IgGa subsequently still leaving an overall increase in total specific anti-BoNT/C IgG. The ranges of specific anti-SA IgGb hardly changed across the sampling period and although the herd median decreased against BoNT/C between 13m and 16m, the range did not change substantially. The median anti-IgGb against BoNT/D increased between the same sampling points although the higher range of the sample fell.

IgGT is the subclass thought to be involved in neutralisation of bacterial toxins (Sheoran et al, 2000) and although IgGT increases with age against both SA and BoNT/C it does not against BoNT/D. The increase in specific anti-BoNT/D IgG must therefore be accounted for by increases in specific IgGa and IgGb. The strain used

for the SA ELISA is positive for the C2 toxin by both PCR and immunoblot (data not shown) although reagents are not currently available to assay directly for anti – C2 antibodies, so it is not know if the presence of the C2 toxin accounts for any rise in IgG or which subclass reacts with it most.

The lack of either a positive or negative correlation between levels of specific subclasses of IgG may possibly be due to the limits of the experimental design – quantification of total subclass levels and then expressing specific IgG as a percentage may help in this respect as it would with ratios of IgG subclasses. A Th1 response has been demonstrated to be effective in horses against *R. equi* (Hooper – McGrevy et al, 2003). Although *C. botulinum* is not an intracellular organism, and as such should produce a mixed Th1/2/0 response, it was decided to look at subclass ratios as very young animals show a bias toward a Th2 response (IgGb, IgGT) over a Th1 response (IgGa) (Hooper – McGrevy et al, 2003). As IgGb and IgGT are both Th2 type subclasses it is perhaps more relevant to look at the IgGa/IgGb + IgGT ratios. A rise in the ratios of IgGa/IgGb + IgGT does occur overall against BoNT/C and SA but not against BoNT/D, suggesting that the animals did develop more of a Th1 response with age. However, without knowing what the ponies were being used for at the time or without quantifying the subclass levels, it is not possible to come to any firm conclusion.

3.3.3 *Specific IgG of horses*

The sampling period was chosen so that any seasonal differences in IgG level could be observed, or the possible effect of management practices. Turnout of horses was staggered in both terms of time and which horses were turned out but the overall increase in IgG to BoNT/C over samples 7-9 and decrease in IgG to SA at samples 7-8 interesting. It has been demonstrated (McCarthy et al, 2004) that higher levels of IgG to SA associated with protection. A sudden increase in exposure to Group III organisms due to turnout may have led to the drop in IgG to SA, allowing increased exposure to BoNT/C. Hunter et al (2001) showed that toxin could be detected in samples from controls, albeit at very low concentrations. Although not always statistically significant, levels of IgG to SA did fall when levels of IgG against BoNT/C increased and vice versa, indicating a relationship between the two within this group of horses.

Correlation analysis is affected by outliers in a data set. Outliers were left in because large fluctuations in specific IgG levels have been observed in CGS cases and it needed to be determined if similar fluctuations would be observed in controls. Within the data set certain levels of specific IgG correlate with age of the data set with two of the three antigens (BoNT/C and SA) and these correlations become negative if levels fall or rise too far beyond that level. There is no age-IgG correlation between IgG to SA and BoNT/D. This may be because BoNT/D is a rarer antigen although some animals do show higher IgG levels to BoNT/D than BoNT/C at certain data points, indicating exposure. The age-IgG level correlation is repeated in the nine horses chosen and a similar pattern demonstrated.

Specific IgG-IgG correlation was examined to try and determine any relationship between anti-SA IgG and anti-BoNT IgG. No significant inverse relationship was demonstrated at any data point and correlation between IgG levels to SA and BoNT/C was less common than correlation between the neurotoxins and BoNT/D and SA. A certain level of correlation between IgG levels to the two neurotoxins might be expected due to some cross-reactivity although this was not observed in the pony study.

Longitudinal analysis shows that a peak in IgG against any of the antigens does not always correspond with a rise or fall in the level of another. This independence may be due to rarity of neurotoxin producing organisms in the environment, due to them sometimes coexisting together and the detection of cross-reacting antibodies to other non-neurotoxin producing clostridia by the ELISA.

When looking at the individual animal's response to their tetanus vaccinations it is clear that the ELISA methodology used is representative of the IgG response against all of the antigens used. Clear IgG responses are observed after each animal's vaccination against tetanus which then fall with time. Obviously immune responses to environmental antigens are far more complex than against a standardised parentally administered one but the assay served as a useful control in this study and in studies of cases of chronic grass sickness.

Fluctuations in herd medians of specific IgGT levels were also demonstrated which did not correlate with specific IgG level although this may be partly due to IgGT not being quantified. These fluctuations were also observed in the 9 individual horses.

Immune responses in the horse are poorly understood with investigations being involved with parasites and intracellular pathogens. No study involving the equine immune reponse either to enteric pathogens or to environmental bacteria has been published to the best of my knowledge. This study was a useful exercise in assessing normal fluctuations of IgG levels against the main antigens of interest and is later used as a comparison when studying the IgG responses of chronically affected animals. A control in the form of an anti – tetanus toxoid assay was used to demonstrate that the fluctuations in IgG levels to these environmental antigens were real. However, immune responses to environmental antigens are complex and both of the studies could have been improved upon by quantifying the IgG subclasses and the pony study being for longer and having more data points.

Chapter IV

Comparative serology of AGS cases and healthy co-grazing controls

Results

4.1 *Samples and study*

A focused study was required to maximise the chance of isolating *C. botulinum* type C/D and also to examine any possible differences between susceptible animals and premises-matched controls. Samples were collected from healthy non-GI controls- that is horses euthanased for conditions not related to the GI tract- and also from AGS cases and their co-grazers. As well as serological studies (both serum and secretory IgA), samples were taken from the GI tract of cases at post-mortem for bacteriology and histopathology. Environmental samples were also collected from the pastures involved.

Seven horses with acute grass sickness went to post-mortem between August 2002 and August 2003. Cases were selected on basis of time between onset of clinical signs and diagnosis of acute grass sickness; only those diagnosed and euthanased within 36h of onset of clinical signs were selected for this study.

Serum was taken pre and postmortem from cases and serum from go-grazers was taken within 24 h. Blood samples were collected in sterile vacutainers containing no additives and allowed to clot overnight at 4°C. Serum was collected after centrifugation at 1000g for 6 min. The number of co-grazers varied from case to case and their features are summarised in Table 4.1.

Table 4.1 Acute cases and co-grazing controls

Case	Co – grazing control	age (y)	breed/type	sex
02-352		5	cob	MN
	1	10	thoroughbred	MN
	2	15	cob	MN
	3	7	warmblood	MN
	4	13	cob	MN
	5	10	Cleveland Bay	MN
	6	21	thoroughbred cross	MN
	7	15	thoroughbred cross	
02-351		15	Highland	F
	1	15	Highland cross	F
	2	14	Welsh Section A	MN
	3	14	Welsh cross	F
	4	15	Fjord	F
	5	7	Highland cross	F
	6	14	Welsh cob	F
	7	16	Cleveland Bay cross	F
02-376		5	Icelandic	MN
	1	5	Icelandic	MN
	2	6	Icelandic	MN
	3	13	Icelandic	MN
	4	10	Icelandic	MN

Table 4.1 Acute cases and co-grazing controls cont'd

Case	Co – grazing control	age (y)	breed/type	sex
03-392		5	thoroughbred cross	MN
	1	14	-	MN
	2	4	-	MN
	3	8	-	MN
	4	15	-	MN
	5	11	-	MN
	6	11	-	MN
	7	5	-	MN
	8	25	-	MN
	9	8	-	MN
	10	10	-	MN
	11	21	-	MN
	12	21	-	MN
	13	5	-	MN
	14	12	-	MN
	15	15	-	MN
03-409		3	pony	MN
	1	6	-	MN
03-425		5	thoroughbred cross	MN

MN = male neutered - = unrecorded

Case 03-425 had no co-grazers and was omitted from any statistical comparisons. However the sample was used in specific IgG-IgG correlation studies. A sample from the seventh case was not received.

Specific IgG levels against BoNT/C, BoNT/D and *C. botulinum* type C (a neurotoxin negative strain) surface antigens were compared between cases and controls as was secretory IgA. Levels of IgG subclasses were also examined against these antigens. As means of control, IgG levels against tetanus toxoid and surface antigens of a *C. tetani* (TetNT negative strain) were also assayed. All of the cases and controls were vaccinated with tetanus toxoid and the TetSA assay was used as an indicator of soil exposure.

4.1.1 *Comparisons of age groups*

Ages of the two groups were compared as it is well established that animals of seven years of age and under are more susceptible to GS (Doxey et al, 1991). Antibody acquisition to SA and BoNT/C has also been shown to plateau at this age (H. McCarthy, personal communication) and therefore it is important that the ages of the control groups be taken into account. The analysis is summarized in Table 4.2.

Table 4.2 ***Comparison of age groups***.

Statistics	AGS	Co-grazing controls
N	6	34
Mean	6.33	12.09
Median	5.0	12.50
Range	15-3	25-4

Both age groups were normally distributed. The co-grazing control group were significantly older (P = 0.014, Student's unpaired t-test).

4.1.2 *Total specific IgG*

Total specific IgG of the acute cases and their co-grazers are summarised in Table 4.3a and 4.3b. Values were computed using GraphPad Instat which tests for Gaussian distribution using a variation of the Kolmolgorov and Smirnov test. Only levels against BoNT/C&D were normally distributed among the co-grazers, anti-SA levels were not normally distributed even after transformation. All levels were normally distributed in the AGS groups.

Table 4.3a. Total specific (μg/ml) IgG of AGS cases.

	Anti-SA	Anti-BoNT/D	Anti-BoNT/C	Anti-tetSA
Mean	0.248	0.128	0.242	0.289
Median	0.223	0.120	0.220	0.205
Range	0.467 – 0.056	0.184- 0.082	0.350- 0.035	0.576- 0.169

Table 4.3b. Total specific (μg/ml) IgG of co-grazing controls.

	Anti-SA	Anti-BoNT/D	Anti-BoNT/C	Anti-tetSA
Mean	0.448	0.160	0.205	0.467
Median	0.310	0.147	0.176	0.407
Range	2.170- 0.099	0.440- 0.027	0.522- 0.025	1.484- 0.031

4.1.3 *Comparison of median specific IgG levels*

No significant differences were observed between AGS and co-grazing controls for any antigen although differences approaching significance were seen between levels against SA and TetSA (P = 0.06 and P = 0.09 respectively). Outcomes of the median comparisons can be seen in Table 4.4 and Fig 4.1.

The co-grazers demonstrated a range of specific IgG that reached below that of the AGS cases against all antigens with the exception of SA.

Table 4.4 Group median comparisons

	Median			
Antigen	AGS	Co-grazers	test	P value
SA	0.223	0.310	t-test, Welsh's	0.06*
BoNT/D	0.120	0.147	unpaired t-test	0.54
BoNT/C	0.220	0.176	unpaired t-test	0.77
TetSA	0.205	0.407	unpaired t-test	0.09

*SD of the two groups significantly different, P = 0.02

4.1.4 *Comparison between different herds*

There were some differences of the total specific IgG between herds. The herd of co-grazers of Case 02-351 had significantly higher levels of anti-BoNT/C than the herds of co-grazers of case 02-376 and 02-392 and a higher level against BoNT/D than the herd of case 02-352. The other significant results are detailed in Table 4 .5.

Fig 4.1. Boxplots showing differences in specific IgG in AGS cases and co-grazing controls. a) BoNT/D b) BoNT/C c) SA d) TetSA

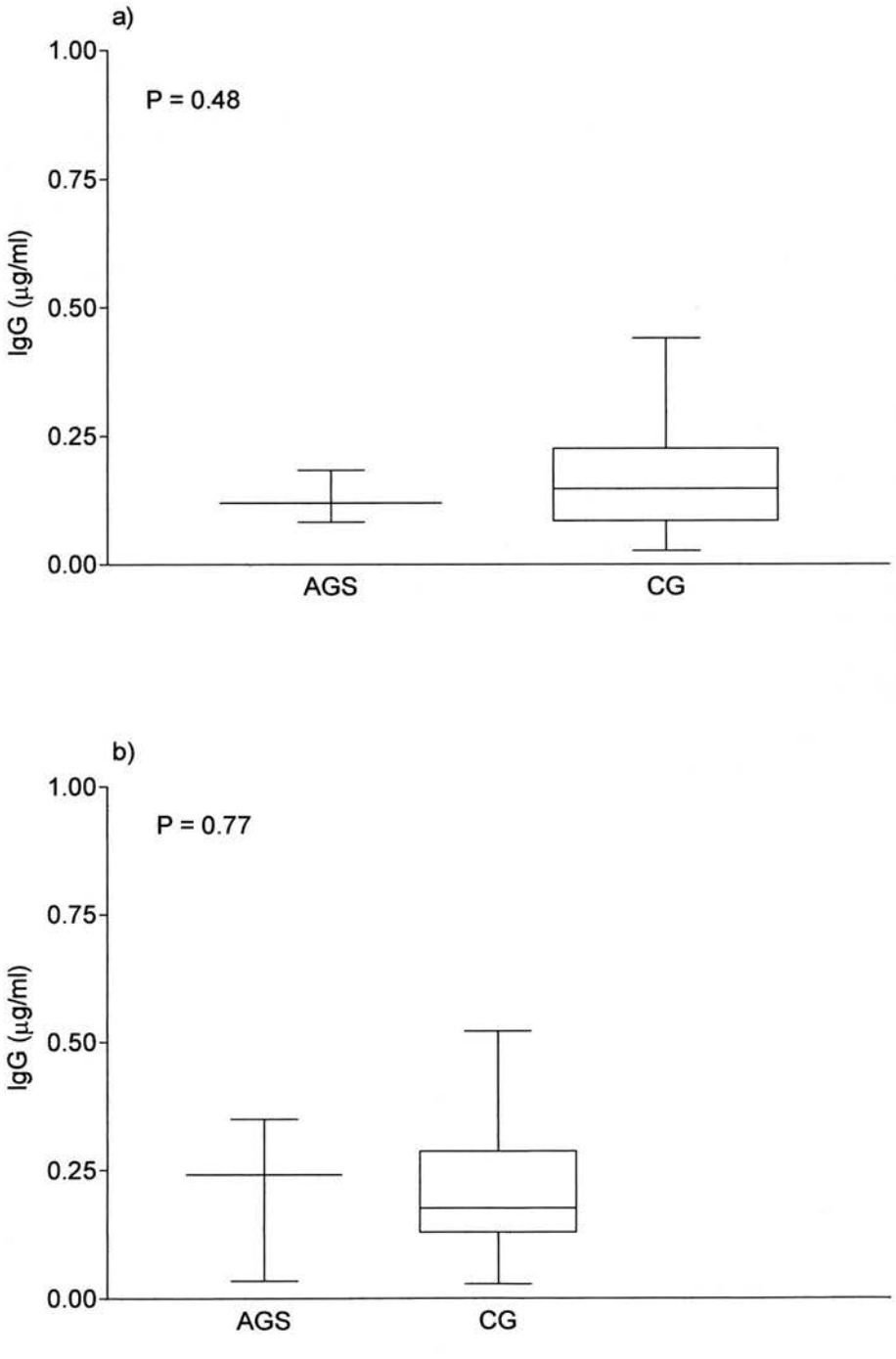
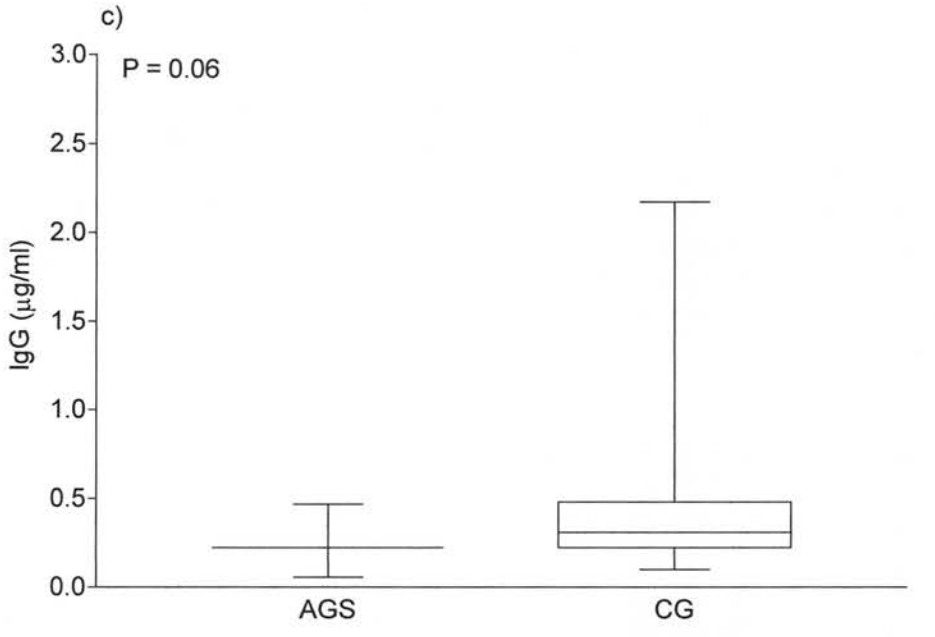


Fig 4.1


c)
P = 0.06
IgG (µg/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
AGS
CG

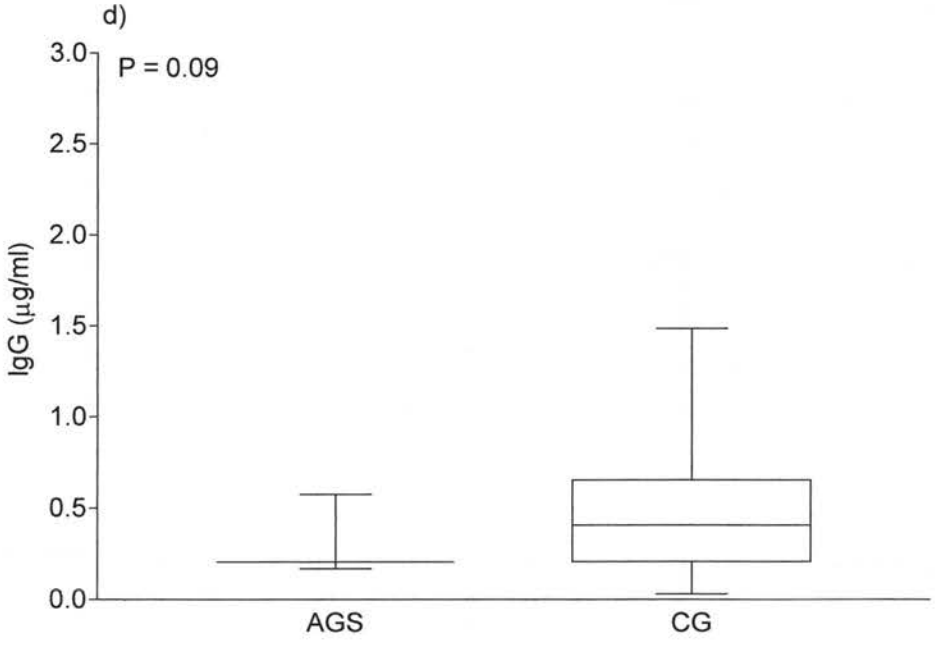

d)
P = 0.09
IgG (µg/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
AGS
CG

Table 4.5. Significant differences in total specific IgG levels between herds

Antigen	Herd	P value
BoNT/C	02-351 vs 02-376	P<0.05
	02-351 vs 03-392	P<0.05
BoNT/D	02-351 vs 02-352	P<0.01
TetSA	02-351 vs 02-376	P<0.01
	02-351 vs 03-392	P<0.05

4.1.5 *Comparison within herds*

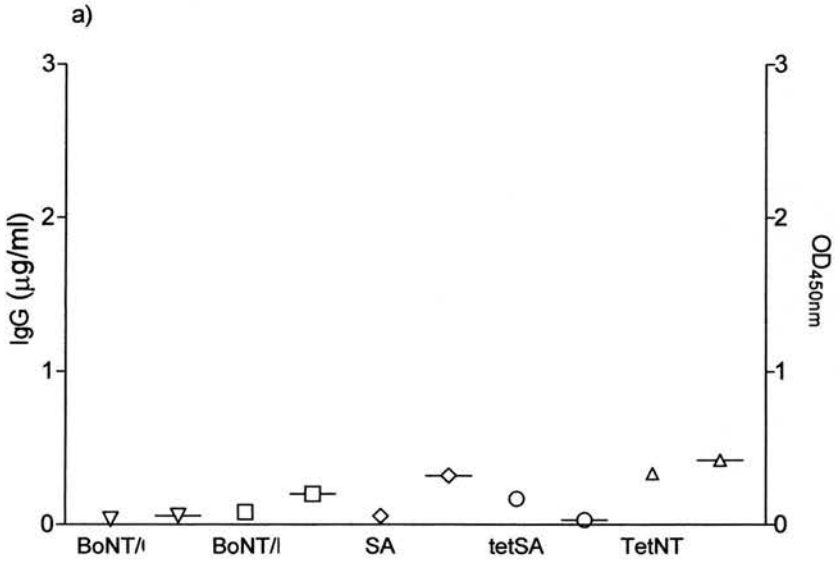
Due the range in specific IgG levels and the differences in level between herds, the individual cases were compared to their respective herds and these can be seen in Fig.4.2 a-e.Three of the five cases had lower level against BoNT/C, BoNT/D and SA than their respective herd median (cases 02-409,02-392 and 02-351) and of these cases the latter two had lower levels against TetSA. With the exception of the anti-TetSA level of case 03-409, in no instance does the level of the corresponding case go above the value of the upper range of the herd.

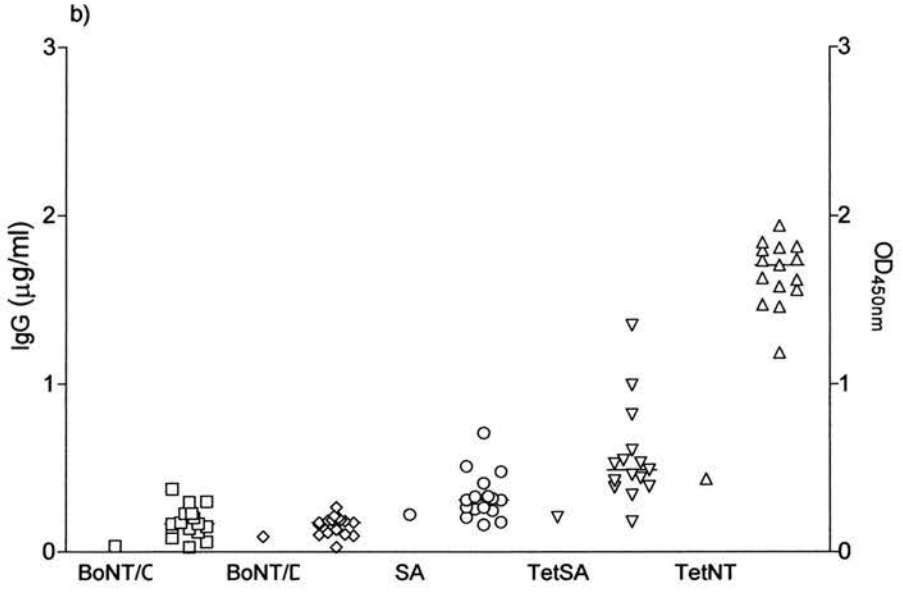
4.1.6 *Correlation between age and IgG level*

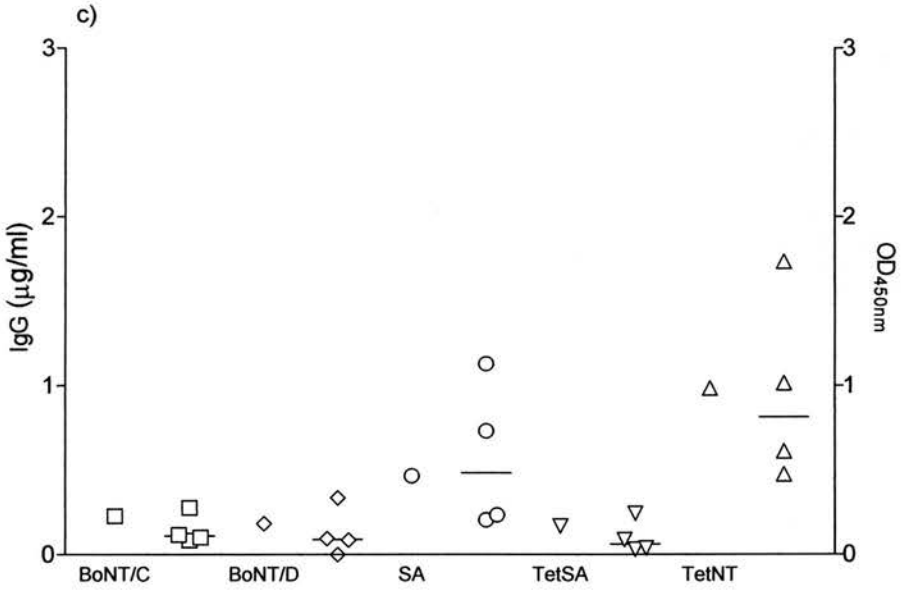
No correlation was demonstrated between age and specific antibody levels in this group of healthy controls. However, only six of the co-grazers were below seven years of age and none below 4y and this would have affected the analysis. Statistics of this analysis can be seen in Table 4.6.

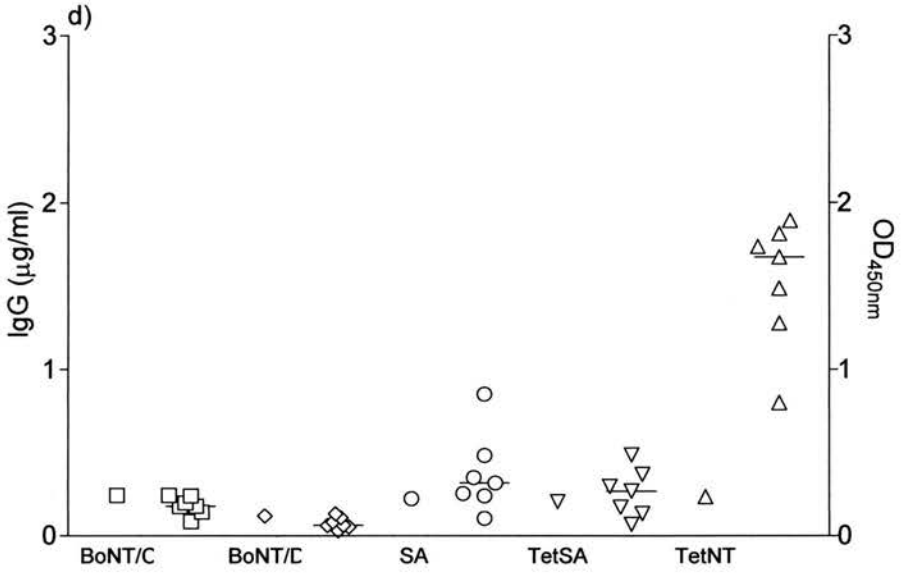
Fig 4.2a-e.Comparisons between specific IgG levels between AGS cases and their respective herds a) Case 03-409 b) Case 03-392 c) 03-376 d) 03-352 e) 03-351.

All levels expressed as µg/ml except anti-TetNT, which are expressed as an OD value.




b)
IgG (µg/ml)
OD450nm
0
1
2
3
BoNT/C
BoNT/D
SA
TetSA
TetNT


c)
IgG (µg/ml)
OD450nm
0
1
2
3
BoNT/C
BoNT/D
SA
TetSA
TetNT


d)
IgG (µg/ml)
OD450nm
0
1
2
3
BoNT/C
BoNT/D
SA
TetSA
TetNT

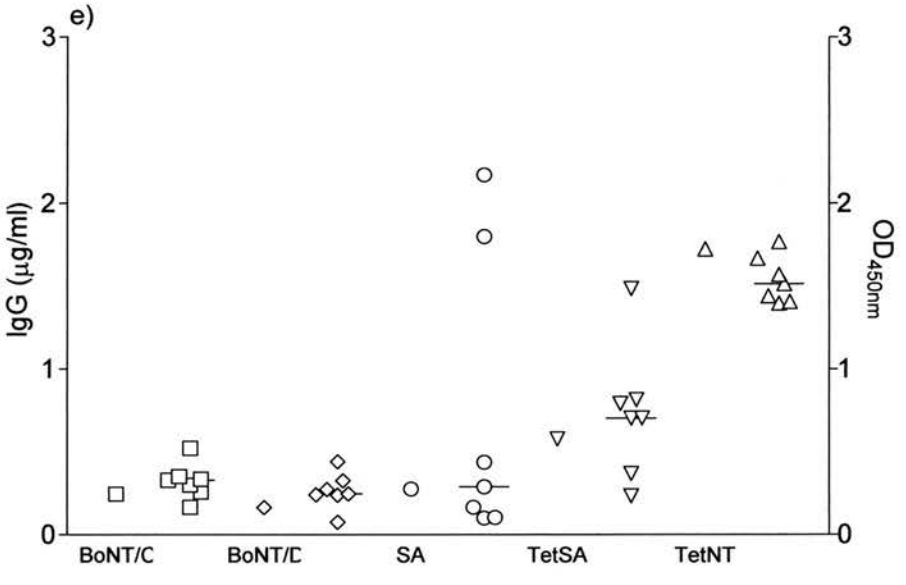

e)
IgG (µg/ml)
OD450nm
0
1
2
3
BoNT/C
BoNT/D
SA
TetSA
TetNT

Table 4.6 Correlation between age and IgG level

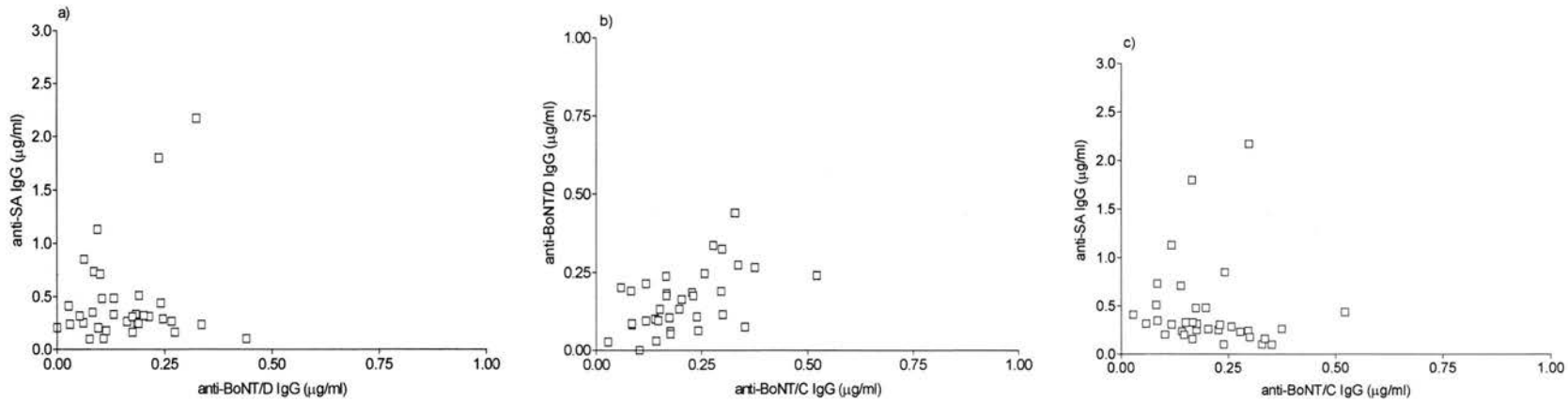
Antigen	test	r^2	P value
BoNT/C	Pearson's	0.065	0.145
BoNT/D	Pearson's	0.002	0.8
SA	Spearman's	0.116	0.54
tetSA	Pearson's	0.021	0.145

4.1.7 *Correlation between specific IgG levels.*

In co-grazing controls, significant correlation was demonstrated between anti-BoNT/C level and anti-BoNT/D level (Pearson's r = 0.5195; r^2 0.2698; P = 0.0016). (Fig.4.4) A significant inverse correlation was observed between anti-BoNT/C level and anti-SA level (Spearman's r = -0.3782; P = 0.0274). No correlation was observed between anti-BoNT/D and anti-SA level (Spearman's r = - 0.02995; P = 0.8665). These results are graphically represented in Fig. 4.3a-c.

In the AGS group the only significant correlation was between anti-BoNT/C and anti-BoNT/D IgG levels (Pearson's r = 0.05834; P = 0.02; r^2= 0.07805). No correlation was observed between anti-BoNT/D and anti-SA levels (Pearson's r = 0.494; P = 0.3192; r^2 = 0.2441) or between anti-BoNT/C level and anti-SA levels (Pearson's r = 0.461; P = 0.3575; r^2= 0.2125.

Fig.4.3 a – c.



Correlation between specific IgG titres in co-grazing controls a) anti-BoNT/D & anti-SA b) anti-BoNT/C & anti-BoNT/D c) anti-BoNT/C and anti-SA.

Significant correlation was demonstrated between anti-BoNT/C titre and anti-BoNT/D titre (P = 0.002) and a significant inverse relationship between anti-SA and anti-BoNT/C (P = 0.03) despite some animals demonstrating relatively high anti-BoNT/C IgG levels.

4.2 *IgG Subclasses*

4.2.1 *Comparison of subclasses between AGS and co-grazers*

Collective data was transformed to give a normal distribution and median comparisons were therefore calculated using Student's unpaired t-tests.

AGS cases demonstrated significantly less specific IgGa and IgG(T) against BoNT/C ($P = 0.006$ in both instances) and significantly less IgGa and IgGb against BoNT/D ($P = 0.06$ and $P = 0.09$ respectively). Against SA, AGS cases demonstrated a trend towards lower IgGa levels ($P = 0.2$) and a trend towards higher IgG(T) levels ($P = 0.09$).

4.2.2 *Comparison between individual cases and herds*

Results of this analysis can be seen in Fig. 4.4. Cases 03-409, 03-351 and 03-392 all demonstrated lower IgGabT against neurotoxins than the median level of their respective herd and these three cases also showed higher IgGb and IgGT against SA than the median levels of their herds.

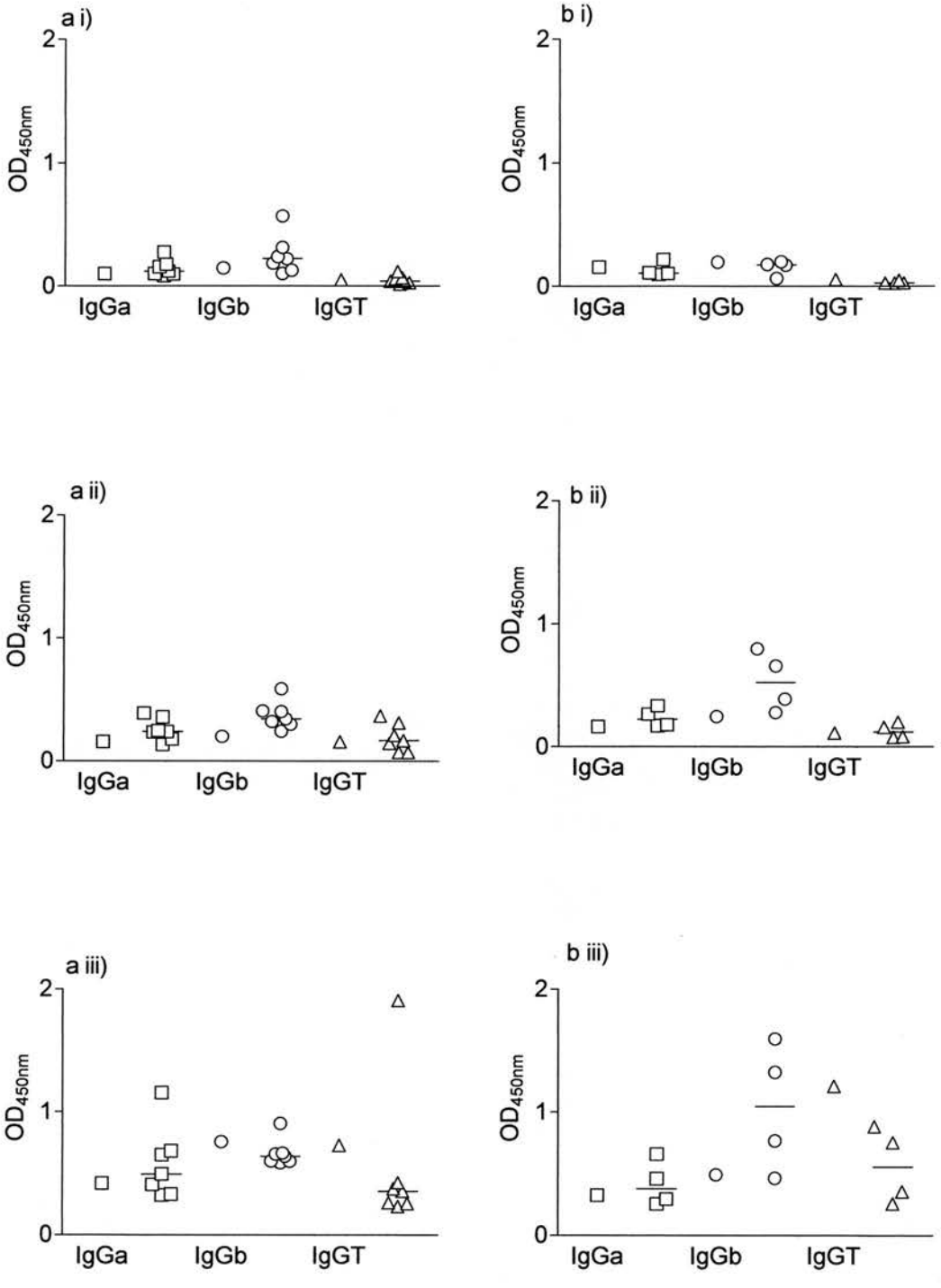
Case 02-352 had lower IgGabT against neurotoxins than the median level of its herd but higher IgGb and IgGT against SA than the median levels of its herd.

Case 02-376 had higher levels of IgGabT against BoNT/D than the median level of its herd but lower IgGabT against BoNT/C and lower IgGab against SA, although a higher IgGT level than all of its co-grazers.

4.2.3 *Specific IgG subclass correlation*

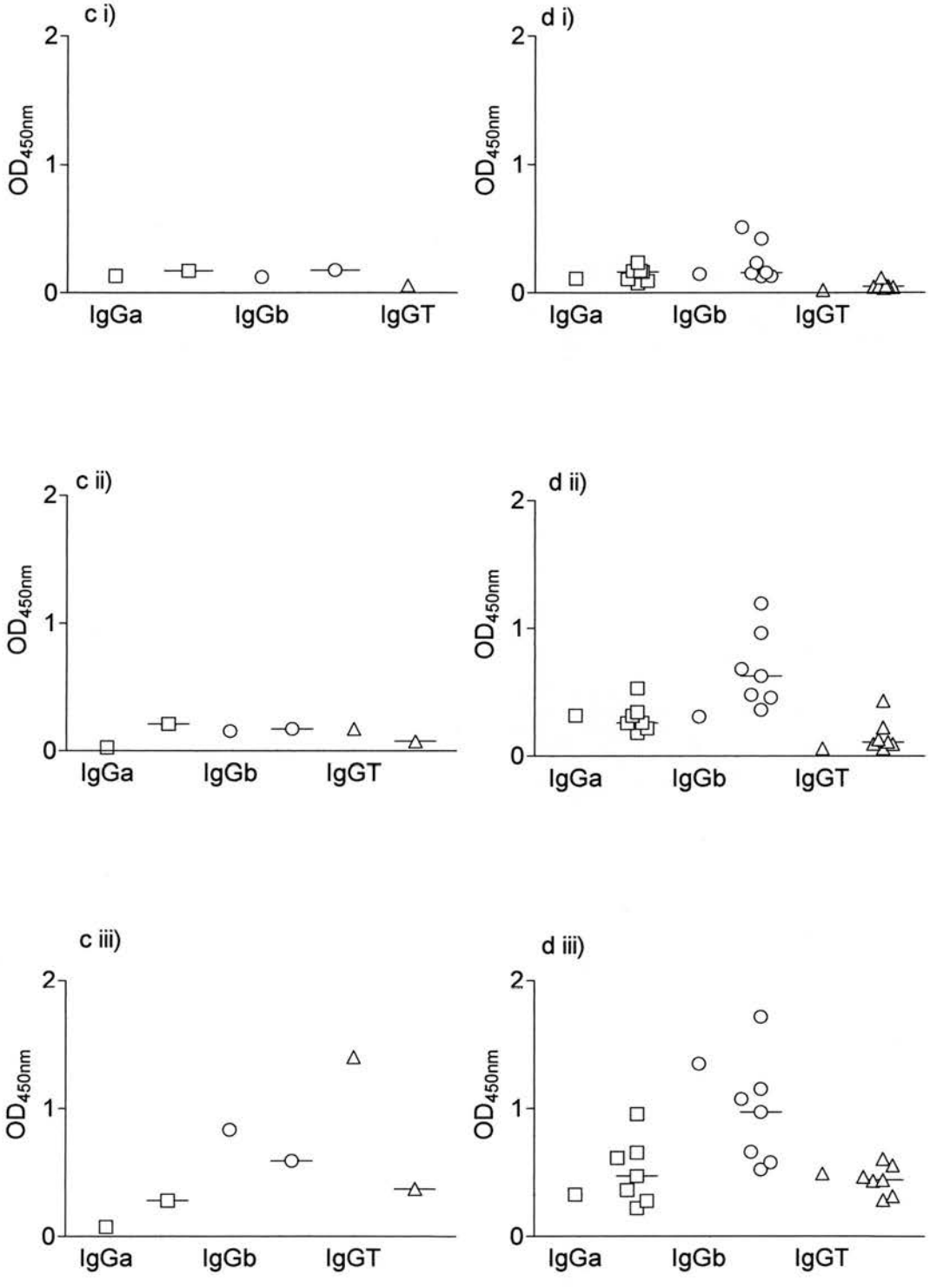
No correlation was demonstrated between any subclass for any antigen.

Fig. 4.4. a - b



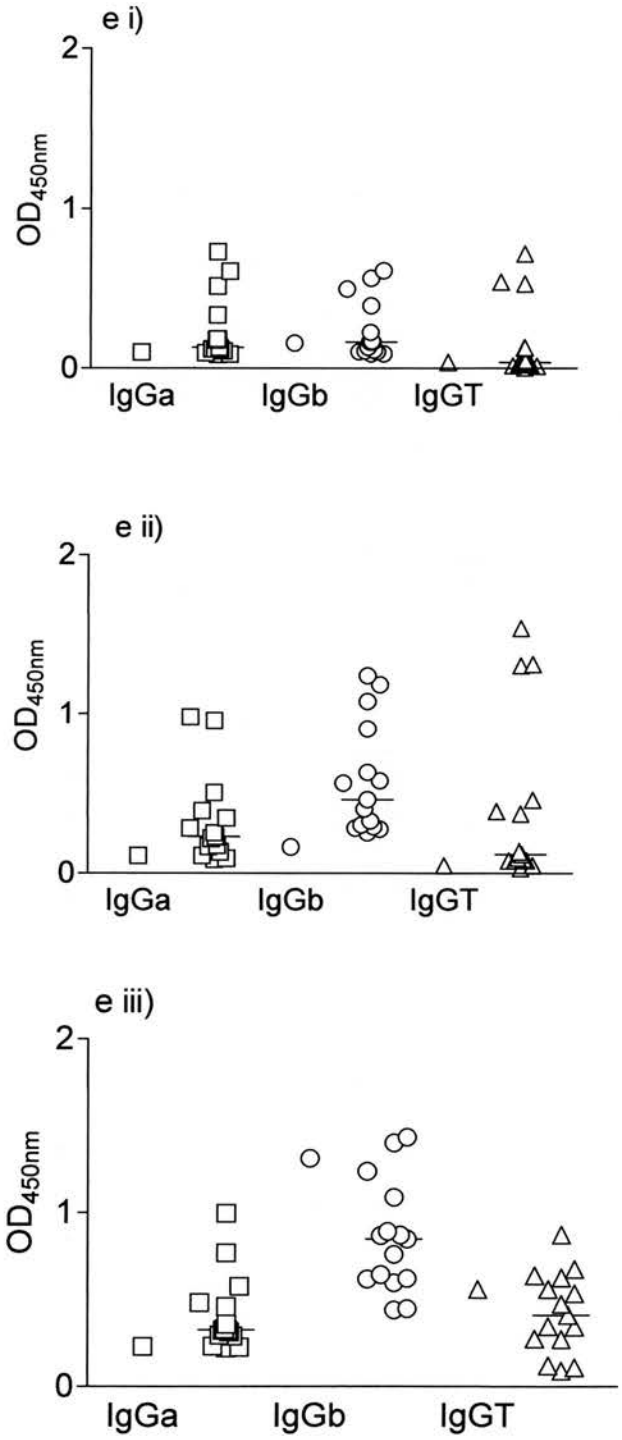
Case 02-352 (Fig. 4.4a) and Case 02-376 (4.4b) compared to herd medians against i) BoNT/D ii) BoNT/C and iii) SA

Fig. 4.4. c - d



Case 03-409 (Fig. 4.4b) and Case 02-351 (4.4c) compared to herd medians against i) BoNT/D ii) BoNT/C and iii) SA

Fig. 4.4. e



Case 03-392 compared to herd medians against i) BoNT/D ii) BoNT/C and iii) SA

4.3 *IgA detection: samples & study*

Samples received from cases are detailed in Table 4.7.

In addition to the seven acute cases described in **4.1.1**, a further 5 acute cases went to post-mortem in the season of 2003. Ileum samples were taken from these for IgA extraction and are included in analysis when mentioned. Four other horses that were euthanased for reasons other than GI problems were sampled post-mortem in the same way as the seven original acutes and are referred to as non-GI controls.

Eighteen faecal samples from the co-grazers of three of the seven acute cases were also collected for IgA extraction, these samples are summarised in Table 4.7.

The samples consisted of tissue and/or contents. Samples from the small intestine consisted of tied-off pieces of intestine. Samples of the intestinal wall and of the contents (where there were contents) underwent saponin extraction or faecal extraction respectively and these were also used to compare IgA yield extracted by the two techniques. Samples from the large intestine and stomach consisted of contents only.

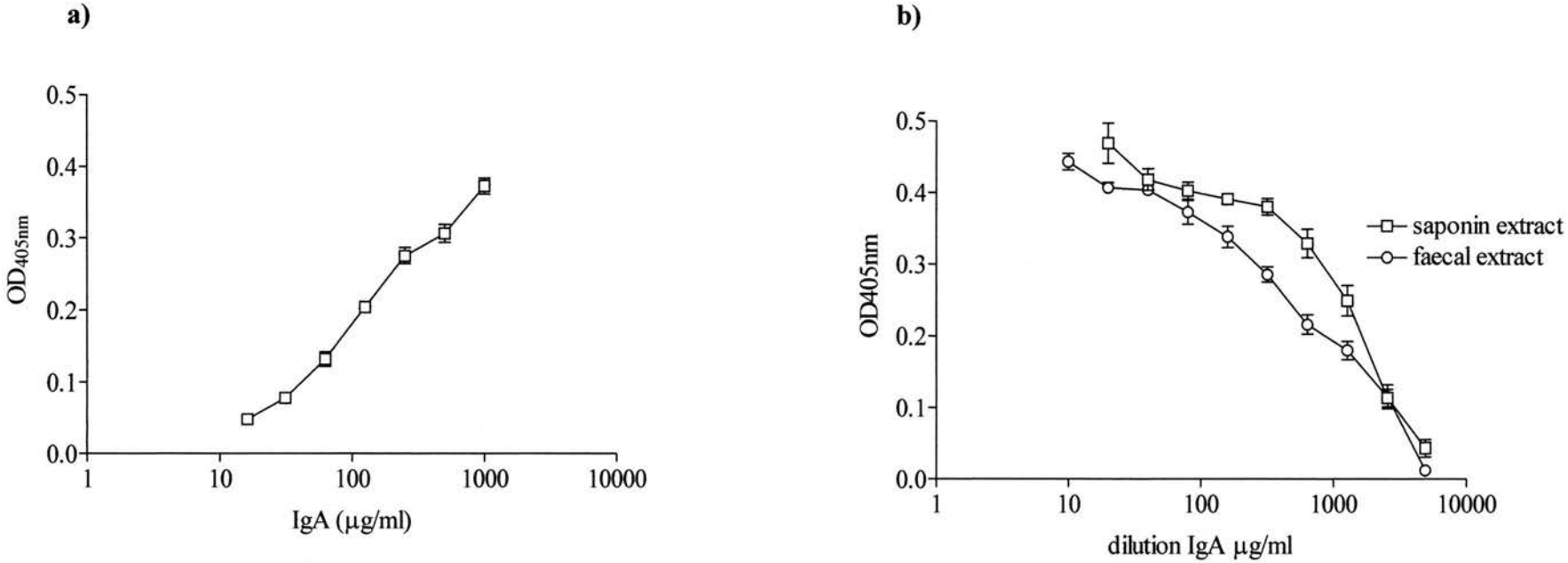
All statistical analysis was performed on Graphpad Prism V3.03. This software tests for Gaussian distribution using a variation of the Kolmolgorov and Smirnov test. Normality testing could not be carried out on the control horse samples due to their small number and so the non-parametric Mann-Whitney U test was employed to test for differences between cases and group medians.

Table 4.7. Samples for IgA extraction from the different cases.

Case	Pharynx	Stomach	Duodenum	Jejunum	Ileum	Caecum	Colon	Rectal	Faeces
02-352	-	-	+	+	+	+	+	+	+*
02-351	+	+	+	+	+	+	+	-	+
02-376	+	+	+	+	+	+	+	-	-**
03-342	-	+	+	+	+	+	-	-	-
03-392	+	+	+	+	+	+	+	+	+***
03-409	+	-	-	+	+	+	+	+	-
03-425	+	+	+	+	+	+	+	+	-
02-182	+	+	+	+	+	+	+	-	-
02-176	+	+	-	+	+	+	+	+	-
02-537	+	+	+	+	+	+	-	+	+
02-431	+	+	+	+	+	+	+	-	+
03-359	-	-	-	-	+	-	-	-	_
03-658	-	-	-	-	+	-	-	-	-
03-445	-	-	-	-	+	-	-	-	-
03-710	-	-	-	-	+	-	-	-	-
03-408	-	-	-	-	+	-	-	-	-
N† =	5/4	5/4	6/3	7/4	12/4	7/4	6/3	4/2	3/2

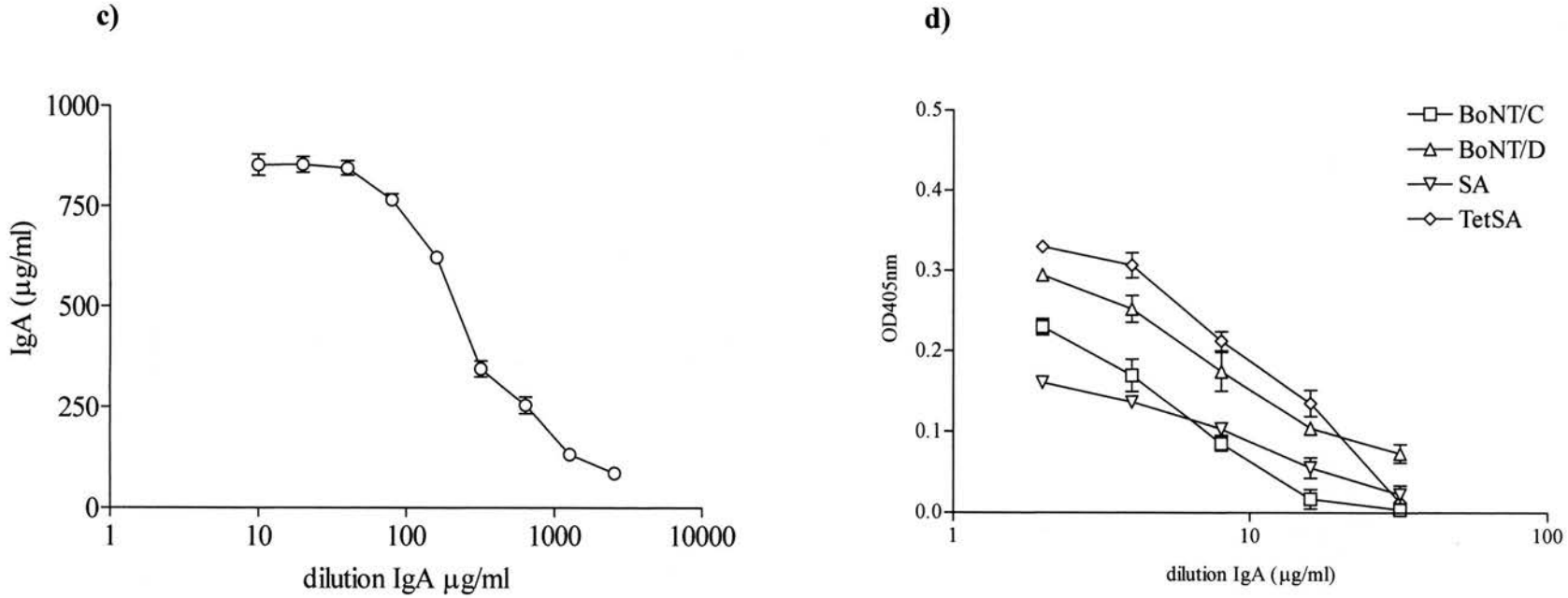
Also sampled: *5 co-grazers sampled **4 co-grazers sampled ***9 co-grazers sampled † N = cases/controls

Fig.4.5



Graphs showing the a) standard curve of the IgA of the Bethyl Laboratories reference serum b) dilution curve of a saponin extract of IgA and a faecal extract of IgA. The saponin sample was chosen to be used as an intraplate control for specific IgA ELISA due to its abundance. Error bars depict the SD.

Fig. 4.5



Graphs showing c) standard curve of the chosen control samples and d) dilution curves of that sample against the different antigens. A 1 in 8 dilution was used as the preliminary dilution for each sample assayed.

against all antigens for the extraction technique involved. A sample was identified and its dilution curve for total IgA can be seen in Fig.4.5c and for specific IgA in Fig.4.5d. In initial assays this sample was assayed on three separate occasions and error bars on the graphs depict SD. Samples were subsequently assayed in quadruplicate and averages of these OD values used for IgA quantitation. Optimal dilution was at 1 in 8 for the majority of samples although some were repeated at 1 in 4 and 1 in 16.

4.3.2 *Total IgA: comparison between extraction methods*

Cases

Significant differences were demonstrated within samples depending upon extraction method used ie significantly more total IgA was obtained from duodenal tissue than duodenal contents ($P = 0.01$) and between ileal tissue than ileal contents ($P = 0.01$). No significant difference was demonstrated between jejunal tissue and jejunum contents ($P = 0.12$).

Controls

The only samples that had sufficient numbers to allow this analysis were samples of ileal tissue and ileal contents ($P = 0.11$).

Cases and control samples combined

No significant differences were demonstrated between IgA yield between like samples of cases and controls. Descriptive statistics are outlined in Table 4.8.

Comparisons could not be made between faecal and rectal samples between groups due to insufficient numbers of samples. As some sample numbers were low, the above analyses were combined. Significantly higher total IgA was obtained from tissue samples rather than contents from the same part of the small intestine (SI). The results of this analysis are detailed in Table 4.9.

Table 4.9 Statistics of comparison of extraction methods: total IgA(μg/ml/g sample)

	duodenum	duodenal contents	jejunum	jejunal contents	ileum	ileal contents
N =	9	3	11	4	16	9
Range	2.852-0.566	0.478-0.009	4.044-0.067	0.302-0.025	4.127-0.087	0.376-0.012
Median	0.975	0.076	0.991	0.1208	1.022	0.077
Mean	1.314	0.188	1.270	0.1422	1.423	0.100
P =	0.009		0.02		0.0002	

4.3.3 *Specific IgA: comparison between AGS cases and Non-GI controls.*

Descriptive statistics for specific IgA can be found in Tables 4.10. P values between total specific IgA are shown in Table 4.11.

Table 4.11. Differences between total specific IgA from AGS cases and controls: cases having more IgA than controls

	BoNT/C	BoNT/D	SA	TeTSA
Pharynx	P = 0.11	P = 0.02	P = 0.2	P = 0.06
Stomach	P = 0.004	P = 0.39	P = 1.0	P = 0.8
Duodenum	P = 0.07	P = 0.10	P = 0.09	P = 0.9
Jejunum	P = 0.006	P = 0.006	P = 0.8	P = 0.5
Ileum	P = 0.006	P = 0.03	P = 0.006	P = 0.25
Caecum	P = 0.01	P = 0.07	P = 0.07	P = 0.65
Colon	P = 0.02	P = 0.10	P = 0.64	P = 0.86
Rectum	-	-	-	-
Faeces	-	-	-	-

4.3.4 *Specific IgA: comparison between AGS cases and Non-GI controls of specific IgA presented as percentage of total IgA yield.*

Descriptive statistics are shown in Tables 4.13a-i and P values in Table 4.12.

Table: 4.10a anti-BoNT/C IgA of AGS cases (µg/ml)

	Pharynx	stomach	duodenum	jejunum	ileum	caecum	colon	rectum	faeces
N =	5	5	6	7	12	7	6	4	5
Range	7.37-1.62	1.98-1.10	2.78-1.53	24.81-1.54	63.81-0.67	2.52-1.05	1.63-1.16	1.94-1.22	2.34-0.55
Median	2.17	1.33	2.78	2.20	8.23	1.39	1.34	1.39	1.36
Mean	3.39	1.44	6.94	5.85	16.41	1.57	1.34	1.52	1.35

Table: 4.10b anti-BoNT/C IgA of Control cases (µg/ml)

	Pharynx	stomach	duodenum	jejunum	ileum	caecum	colon	rectum	faeces
N =	4	4	3	4	4	4	3	2	2
Range	3.09-0.29	0.16-0.00	3.03-0.22	1.02 0.14	0.93-0.10	1.12-0.10	1.06 - 0.10	1.07-0.27	0.11-0.03
Median	0.88	0.14	0.12	0.41	0.14	0.74	0.15	0.67	0.07
Mean	1.29	0.10	1.22	0.49	0.33	0.68	0.44	0.67	0.07

Table: 4.13a anti-BoNT/C IgA of AGS cases (% total)

	Pharynx	stomach	duodenum	jejunum	ileum	caecum	colon	rectum	faeces
N =	5	5	6	7	12	7	6	4	5
Range	4.09-0.10	2.46-0.10	0.58-0.07	2.98-0.03	7.10-0.02	2.52-1.05	2.86-0.04	2.53 - 0.09	9.83-0.06
Median	1.58	0.99	0.31	0.43	0.93	1.39	0.70	2.53	0.40
Mean	2.00	1.06	0.33	0.75	1.62	1.58	1.13	1.00	2.30

Table: 4.13b anti-BoNT/C IgA of Controls (% total)

	Pharynx	stomach	duodenum	jejunum	ileum	caecum	colon	rectum	faeces
N =	4	4	3	4	4	4	3	2	2
Range	3.03-1.51	0.23-0.00	0.44-0.00	0.15-0.01	0.13-0.01	0.38-0.00	1.85 - 0.000	0.10 - 0.03	0.81-0.01
Median	0.53	0.15	0.04	0.04	0.07	0.24	0.01	0.07	0.41
Mean	1.06	0.13	0.13	0.06	0.04	0.22	0.47	0.07	0.41

Table: 4.13c anti-BoNT/D IgA of AGS cases (% total)

	Pharynx	stomach	duodenum	jejunum	ileum	caecum	colon	rectum	faeces
N =	5	5	6	7	12	7	6	4	5
Range	14.97-1.22	3.75-0.00	3.75-0.20	2.82-0.12	7.10-0.01	5.88-0.47	3.45-0.03	2.33-0.00	45.29-0.18
Median	4.12	0.61	0.45	0.71	1.86	1.29	0.46	0.20	0.60
Mean	2.85	1.05	0.78	2.20	2.31	2.14	0.93	0.69	9.68

Table: 4.13d anti-BoNT/D IgA of Controls (% total)

	Pharynx	stomach	duodenum	jejunum	ileum	caecum	colon	rectum	faeces
N =	4	4	3	4	4	4	3	2	2
Range	0.32-0.16	1.09-0.00	0.17-0.00	0.21-0.01	0.37-0.04	7.22-0.02	0.73-0.00	0.14-0.04	3.49-0.00
Median	0.26	0.59	0.00	0.08	0.12	0.20	0.46	0.09	1.74
Mean	0.25	0.57	0.04	0.10	0.16	1.91	0.19	0.09	1.74

Table: 4.13e anti-SA IgA of AGS cases (% total)

	Pharynx	stomach	duodenum	jejunum	ileum	caecum	colon	rectum	faeces
N =	5	5	6	7	12	7	6	4	5
Range	2.35-0.00	0.38-0.02	5.00-0.17	3.75-0.01	1.27-0.07	2.47-0.09	0.48-0.01	1.58-0.05	15.62-0.00
Median	0.58	0.05	0.34	0.12	0.31	0.43	0.08	0.29	0.32
Mean	0.74	0.16	1.14	1.05	0.42	0.79	0.37	0.55	4.06

Table: 4.13f anti-SA IgA of Control (% total))

	Pharynx	stomach	duodenum	jejunum	ileum	caecum	colon	rectum	faeces
N =	4	4	3	4	4	4	3	2	2
Range	0.69-0.01	0.73-0.00	0.06-0.00	0.28-0.02	1.55-0.19	1.06-0.02	0.09-0.00	0.14-0.04	0.00-0.00
Median	0.50	0.27	0.02	0.11	1.04	0.43	0.08	0.09	0.00
Mean	0.42	0.32	0.03	0.13	0.95	0.37	0.02	0.09	0.00

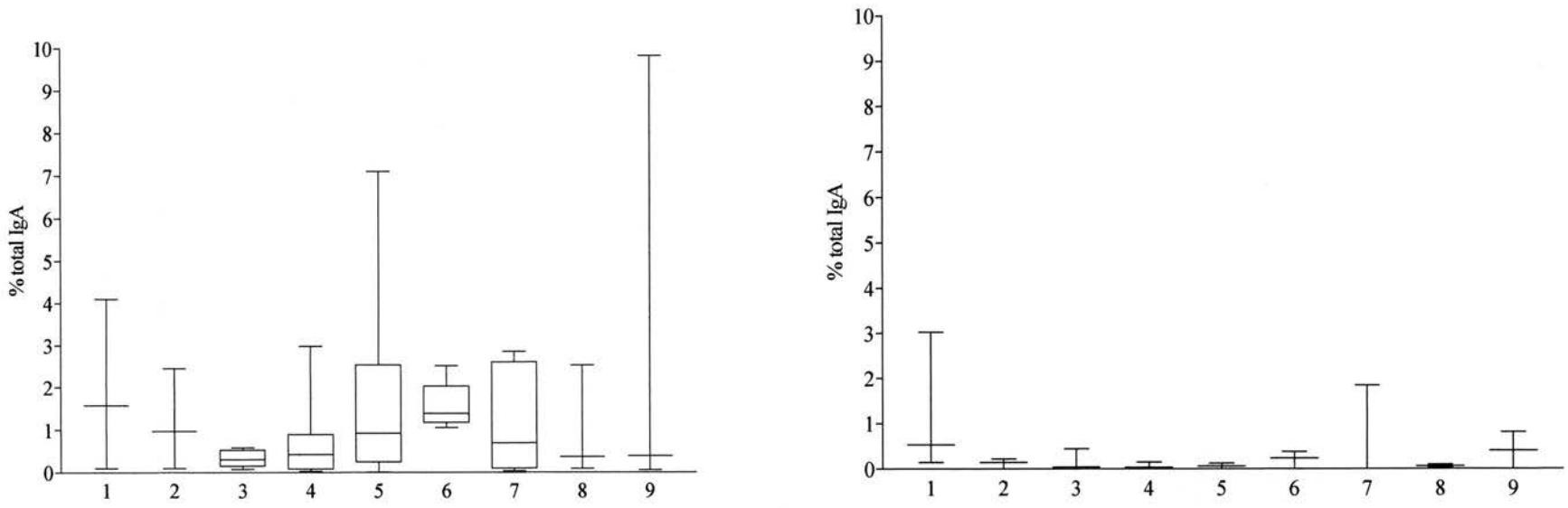
Table: 4.13g anti-TetSA IgA of AGS cases (% total)

	Pharynx	stomach	duodenum	jejunum	ileum	caecum	colon	rectum	faeces
N =	5	5	6	7	12	7	6	4	5
Range	5.27-0.13	0.82-0.00	4.22-0.12	3.78-0.03	9.62-0.03	1.78-0.03	0.93-0.00	63.72-0.00	37.03-0.01
Median	1.84	0.03	0.42	0.45	0.35	0.24	0.08	6.07	0.19
Mean	2.33	0.19	1.13	0.45	0.20	0.55	0.24	18.97	12.4

Table: 4.13f anti-TeTSA IgA of Controls (% total)

	Pharynx	stomach	duodenum	jejunum	ileum	caecum	colon	rectum	faeces
N =	4	4	3	4	4	4	3	2	2
Range	2.68-0.02	1.76-0.00	0.61-0.00	1.96-0.04	1.30-0.01	1.88-0.02	0.08-0.00	63.72-0.00	2.48-1.81
Median	0.50	0.27	0.02	0.11	1.04	0.43	0.08	0.09	0.00
Mean	1.03	0.60	0.29	0.58	0.43	0.64	0.02	18.97	6.28

Fig.4.6

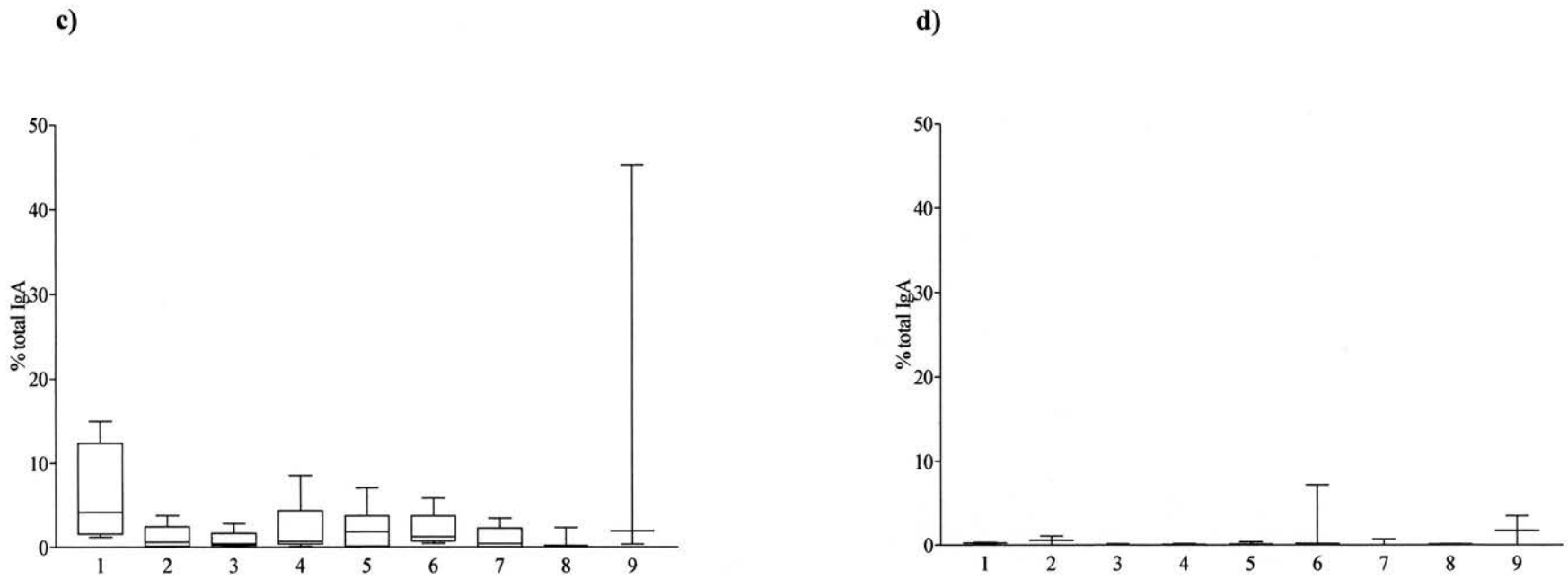


Boxplots showing specific IgA levels (% total IgA) found along the GI tract against BoNT/C in

a) AGS cases (n = 7) (b) Non-GI controls (n = 4). Sample sites 1-5 were mucosal IgA and sample sites 6-9 were luminal IgA.

Samples are as follows: 1= pharynx 2 = stomach 3 = duodenum 4 = jejunum 5 = ileum 6 = caecum 7 = colon 8 = rectum 9 = faeces

Fig. 4.6 cont'd

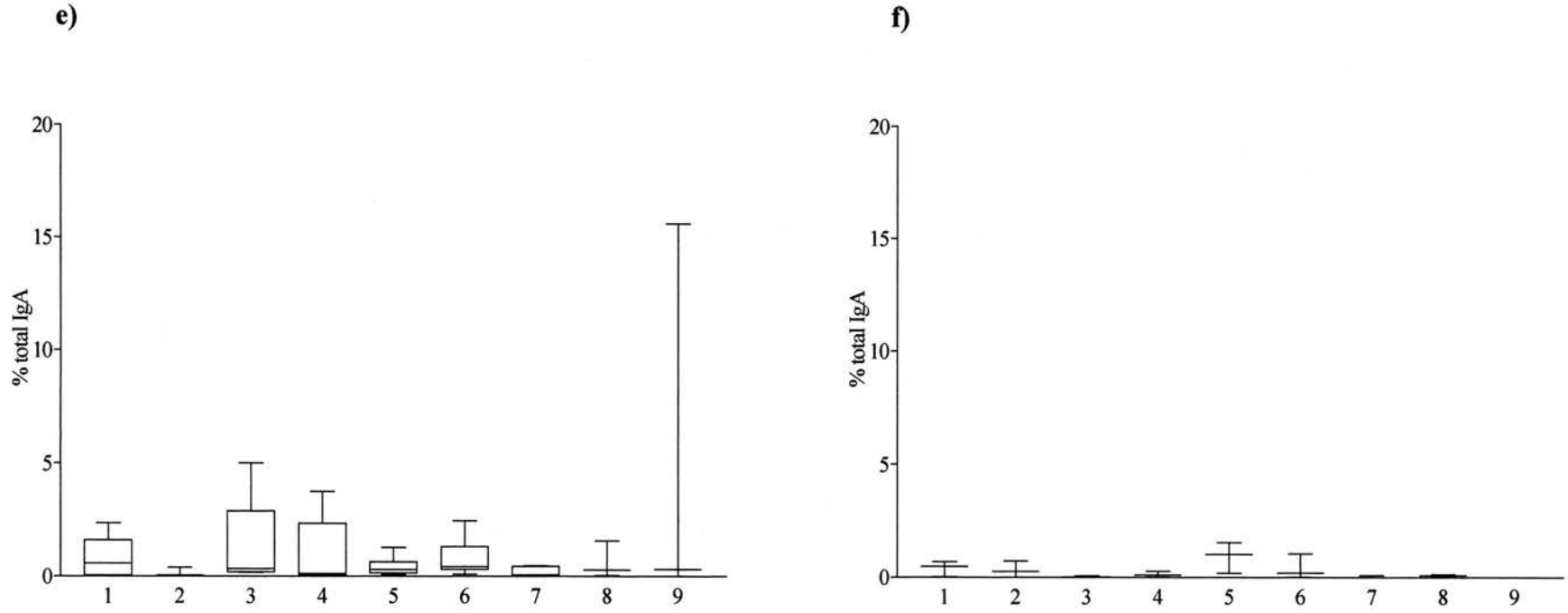


Boxplots showing specific IgA levels (% total IgA) found along the GI tract against BoNT/C in

c) AGS cases (n = 7) (d) Non-GI controls (n = 4).

Samples are as follows: 1= pharynx 2 = stomach 3 = duodenum 4 = jejunum 5 = ileum 6 = caecum 7 = colon 8 = rectum 9 = faeces

Fig. 4.6 cont'd

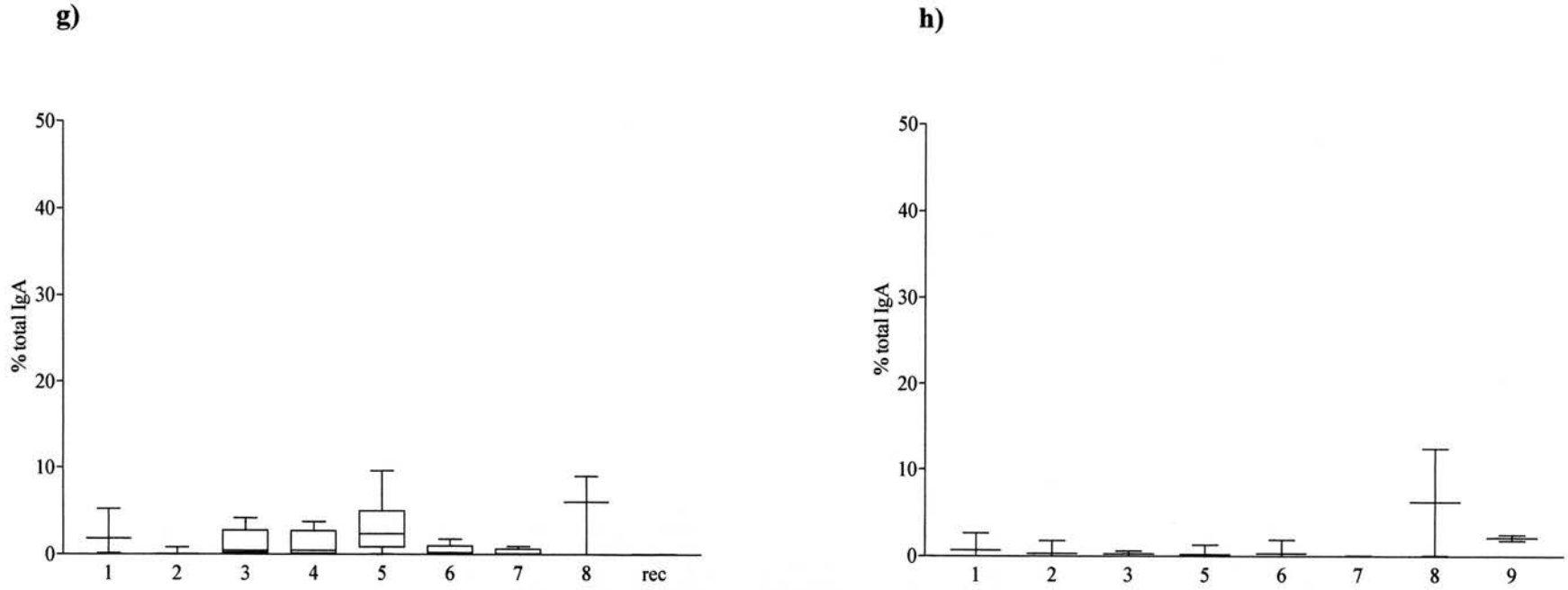


Boxplots showing specific IgA levels (% total IgA) found along the GI tract against BoNT/C in

e) AGS cases (n = 7) (f) Non-GI controls (n = 4).

Samples are as follows: 1= pharynx 2 = stomach 3 = duodenum 4 = jejunum 5 = ileum 6 = caecum 7 = colon 8 = rectum 9 = faeces

Fig.4.6 cont'd



Boxplots showing specific IgA levels (% total IgA) found along the GI tract against BoNT/C in

g) AGS cases (n = 7) (h) Non-GI controls (n = 4).

Samples are as follows: 1= pharynx 2 = stomach 3 = duodenum 4 = jejunum 5 = ileum 6 = caecum 7 = colon 8 = rectum 9 = faeces

4.3.5 *Specific IgA: detection of IgA along the GI tract of AGS cases*

Specific IgA concentration in the GI tracts of the seven acute cases is detailed in Fig 4.7a-d.

Case 02-352

The pharynx and stomach samples for this case were not analysed (due to insufficient sample and sample missing). Similar levels of IgA against the two neurotoxins were found throughout the GI tract with the highest levels of each detected in the ileum. The highest amounts of anti-SA were detected in the ileum and caecum samples and the highest amount of anti-TetSA detected in the jejunum and ileum.

Case 02-351

This case demonstrated higher levels of anti-BoNT/C than of anti-BoNT/D IgA. Specific IgA against neurotoxins were found in all samples present (rectal sample not assayed) with the highest levels found in the caecum and colon samples and very low levels were found in the ileum. However, the highest yield of total IgA was detected in this animal's ileum, and amongst the lowest total IgA from the caecum and colon. Likewise, the highest anti-SA and anti-TetSA IgA levels were also detected in the caecum.

Case 02-376

This case showed higher levels of anti-BoNT/C IgA than anti-BoNT/D IgA and anti-BoNT/C IgA was detected in every sample assayed (rectal and faecal samples

missing from data). This case had the second highest anti-BoNT/C IgA levels in the pharynx and the highest level of the whole group in the ileum with the corresponding total IgA detected in the samples being near the median of the group. The case's highest level of anti-BoNT/D IgA was also detected in the pharynx and ileum samples. This case also demonstrated the highest levels of anti-TetSA of the case group in ileal and pharynx samples and the highest anti-SA levels in the ileal sample.

Case 03-342

A similar pattern of anti-neurotoxin IgA can be seen in the samples of this case, with the highest levels detected in the ileal and caecal samples- higher levels of anti-BoNT/D than BoNT/C IgA is found in the caecum and colon. This case also demonstrated higher levels of antiSA IgA than TetSA IgA, with the highest levels detected in the jejunum.

Case 03-392

The highest percentage yield of anti-neurotoxin IgA was obtained from this case from its faecal sample although this was also one of the lowest total IgA yields from a sample to give a positive signal on a specific assay. Approximately 10 % and 45 % of the IgA detected was anti-BoNT/C and anti-BoNT/D specific respectively. This also accounts for the comparatively large percentage of specific IgA detected in the stomach and possibly the amount of anti-BoNT/C IgA detected in the caecum. Similar patterns are observed in levels of anti-SA and anti-TetSA IgA. Percentage yield of IgA are not significant against any antigen in this case from pharynx

samples, probably due to a comparatively large amount of total IgA extracted from this sample (upper range).

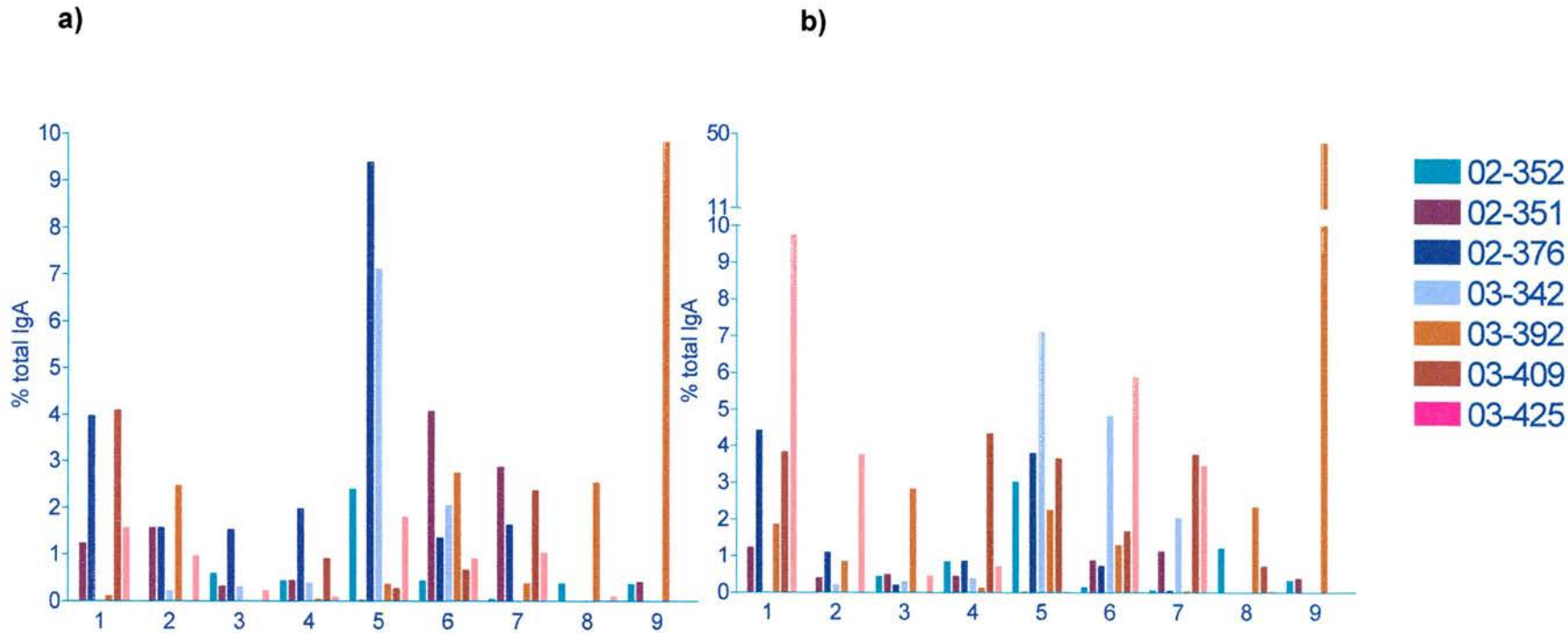
Case 03-409

This case demonstrated higher levels of IgA against BoNT/D than anti-BoNT/C with the highest levels detected in the pharynx, jejunum, ileum and colon. However, with the exception of the pharynx, these samples also demonstrate the lower end of the total IgA levels of the group. Similarly, the highest levels of anti-SA and anti-TetSA are also found in the pharynx and jejunum.

Case 03-425

This animal demonstrated higher levels of anti-BoNT/D overall, with the highest anti-BoNT/D IgA levels of the whole case group in the pharynx, stomach and caecum with total IgA from these samples being approximately around the median of the case group. This animal also demonstrated the second highest anti-SA IgA level in the pharynx, the highest level of the group from the stomach against SA and TetSA.

Fig. 4.7

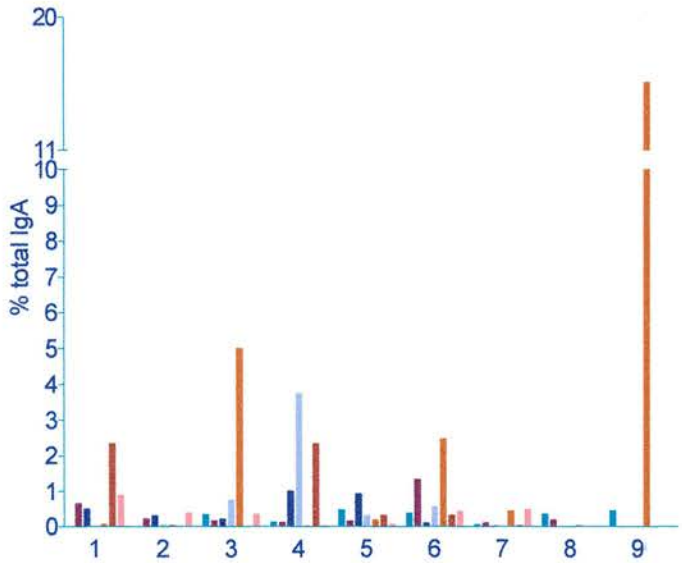


Graphs showing specific IgA along the GI tracts of AGS cases against a) BoNT/C b) BoNT/D

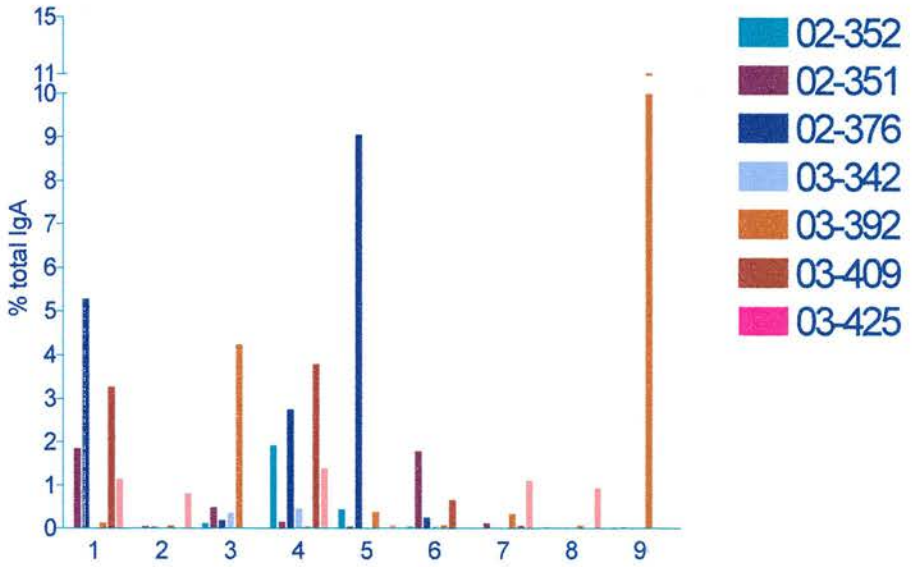
1= pharynx 2 = stomach 3 = duodenum 4 = jejunum 5 = ileum 6 = caecum 7 = colon
8 = rectum 9 = faeces

Fig. 4.7 cont'd

c)



d)



Graphs showing specific IgA along the GI tracts of AGS cases against c) SA d) TetSA

1= pharynx 2 = stomach 3 = duodenum 4 = jejunum 5 = ileum 6 = caecum 7 = colon
8 = rectum 9 = faeces

4.3.6 *Specific IgA: detection of IgA along the GI tract of Non-GI controls*

Data for specific IgA in the GI tracts of the four non-GI controls cases are detailed in Fig. 4.8a-d.

Case 02-182

The highest anti-neurotoxin IgA levels are found in the pharynx of this animal with other comparatively (to this case) high levels detected in the caecum. Increased levels of anti-SA and anti-TetSA are also found in the jejunum and caecum. Total IgA levels are unremarkable in the case of the pharynx and jejunum, but are at the lower end of the range from the caecum. No specific IgA was detected in the stomach, where a very low level of total IgA was detected.

Case 02-176

A high level of specific IgA against all four antigens was demonstrated in the pharynx of this animal, which also gave a yield of total IgA on the lower range. High levels of anti-BoNT/C was also detected in the caecum and ileum of the animal which also gave comparatively lower levels of total IgA. The highest anti-SA IgA level of the group is also found in this case and this corresponds to levels of anti-TetSA.

Case 02-431

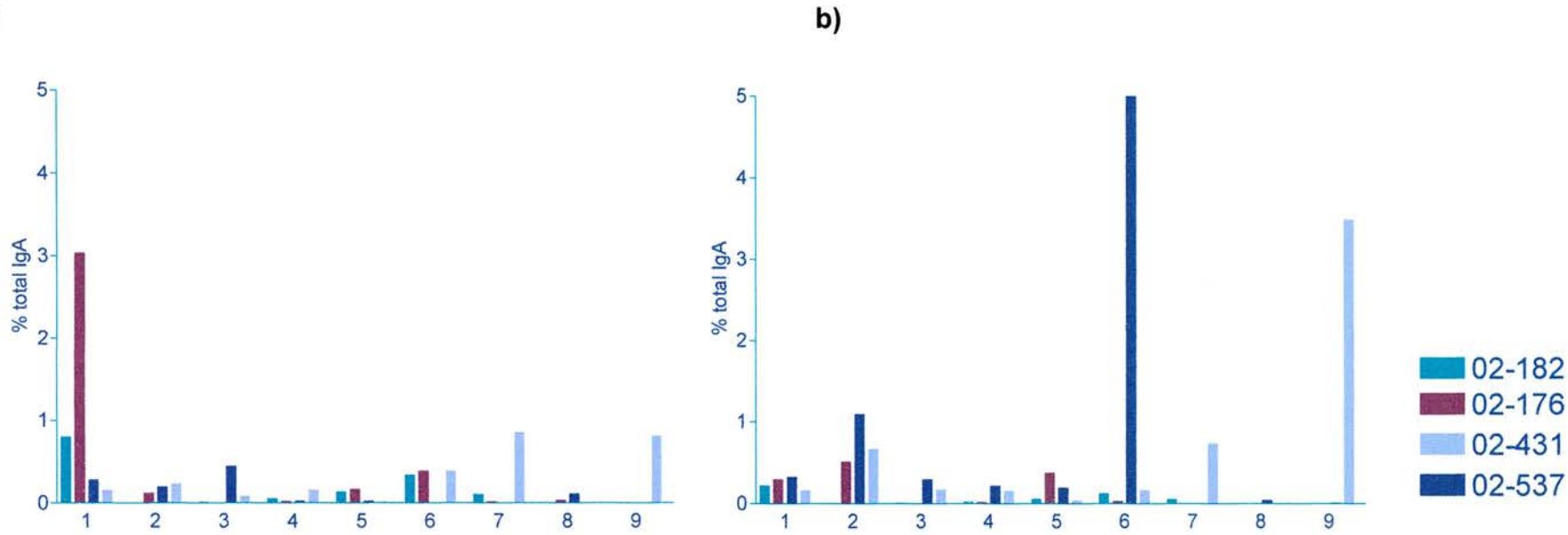
This case demonstrated a low level of anti-neurotoxin IgA in the pharynx and higher levels in the colon and faeces where very low amounts of total IgA were detected. However, the highest amounts of anti-TetSA was detected in the stomach and faeces of the whole group.

Case 02-537

Demonstrated the highest levels of anti-neurotoxin IgA from the jejunum of the whole group and high levels of anti-BoNT/D were demonstrated in the caecum and stomach. Total IgA levels were comparatively low in these samples. This case's level of anti-SA IgA was the second highest of the case group and total IgA from this sample was slightly above the median.

Overall, cases exhibited higher levels of specific IgA than non-GI controls although data must be carefully examined to check for bias caused by extremes of total IgA yield. Samples from the pharynx appear to be more representative due to most samples falling around the median of the group. Total IgA extraction may be more efficient and reproducible here due to the lack of gut contents that degrade IgA and the uniformness of the sample, which helps eliminate the error produced by varying thickness of the GI wall from different areas of the tract.

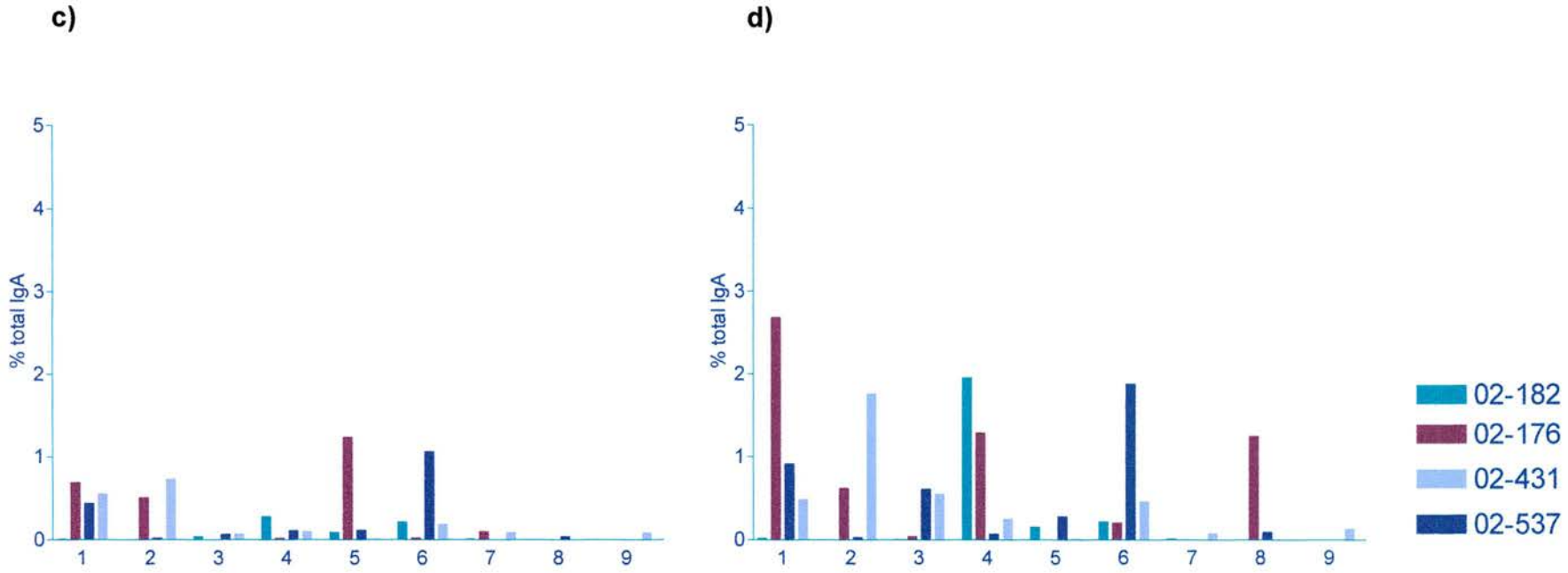
Fig. 4.8



Graphs showing specific IgA along the GI tract of Non-GI controls against a) BoNT/C b) BoNT/D

1= pharynx 2 = stomach 3 = duodenum 4 = jejunum 5 = ileum 6 = caecum 7 = colon
8 = rectum 9 = faeces

Fig. 4.8 cont'd



Graphs showing specific IgA along the GI tract of Non-GI controls against a) SA b) TetSA
Less specific IgA as a proportion of total IgA was demonstrated in the control horses.

4.3.7 *Specific IgA: detection of total and specific IgA from faecal samples of co-grazing controls.*

IgA from co-grazing controls was analysed collectively and on a herd-case basis. Descriptive statistics can be found in Table 4.14 and 4.15a-c respectively.

Table 4.14 Descriptive statistics of collective results from co-grazing controls(n=18)

	Total IgA (µg/ml)	BoNT/C	% total BoNT/D	SA	TetSA
Range	3697-23.68	9.71-0.02	8.03-0.02	14.86-0.00	4.40-0.00
Median	506.10	0.26	0.34	0.02	0.15
Mean	724.30	1.27	2.35	1.37	0.99

Table 4.15 Descriptive statistics of IgA detection from co-grazing controls

a) Case 02-376* (n=4)

	Total IgA (µg/ml)	BoNT/C	% total BoNT/D	SA	TetSA
Range	1322-107.30	4.95-0.10	4.30-0.09	0.11-0.01	0.24-0.01
Median	470.10	0.26	0.78	0.08	0.13
Mean	592.40	1.64	1.73	0.07	0.13

*faecal sample not available-caecal sample used

b) Case 02-392 (n=9)

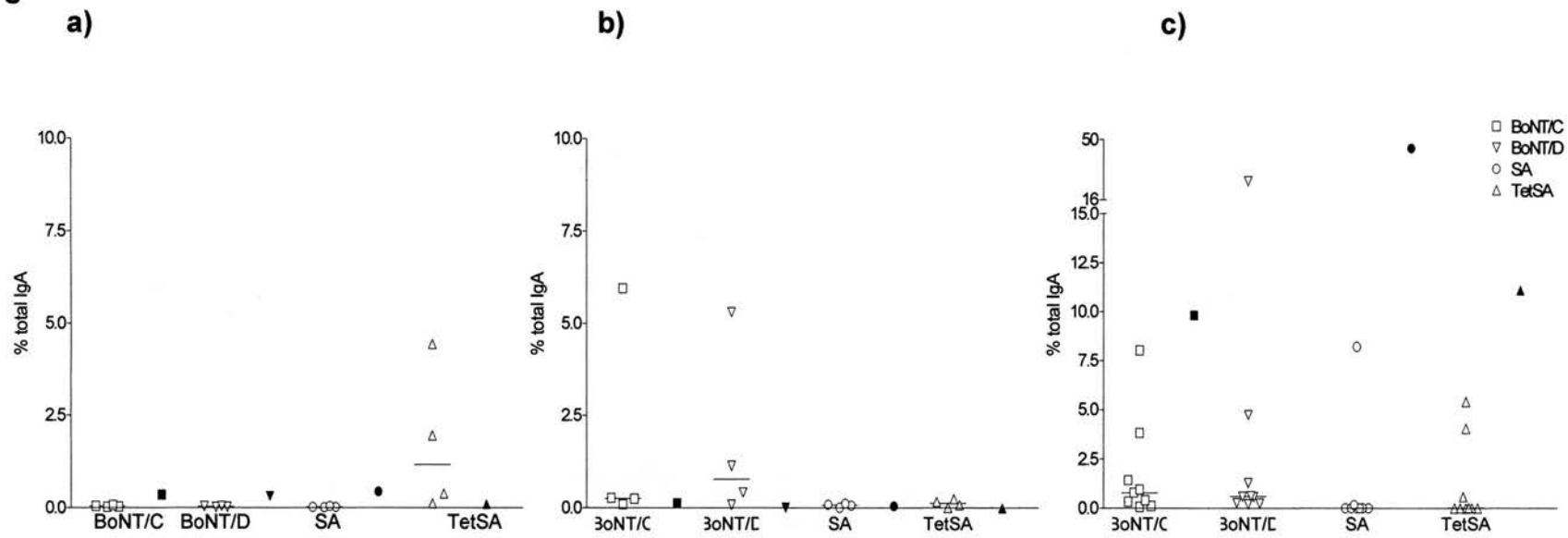
	Total IgA (µg/ml)	BoNT/C	% total BoNT/D	SA	TetSA
Range	1286.00-23.68	8.03-0.08	3.78-0.01	4.19-0.01	4.40-0.00
Median	239.70	1.02	0.60	0.00	0.01
Mean	436.8	1.772	3.903	2.695	1.121

c) Case 02-352 (n=5)

	Total IgA (µg/ml)	BoNT/C	% total BoNT/D	SA	TetSA
Range	3697.00-381.10	0.08-0.00	0.09-0.02	0.11-0.01	4.43-0.12
Median	1003.00	0.05	0.05	0.02	0.39
Mean	1351.00	0.05	0.05	0.04	1.43

Significant differences were demonstrated between the different herds. The non-parametric Kruskall-Wallis ANOVA test was used to allow multiple comparisons to be made. The herd of case 03-392 had significantly more total IgA extracted from samples than the other herds with Dunn's post test P = <0.05 against case 03-352, but P =>0.05 against 02-376. In spite of this, the herd of case 02-392 also had significantly more specific anti-BoNT/C IgA recovered than 02-352 (Kruskall-Wallis ANOVA P = 0.007, post-tests P = <0.05 against case 03-352, but P =>0.05 against 02-376). The same result was observed between anti-BoNT/D levels in the same

Fig. 4.9



Graphs showing comparison between specific IgA of cases and their respective co-grazers of cases a) 02-352 b) 02-376 c) 03-392.The solid symbols depict IgA of cases.

herds. No significant differences were demonstrated between the herds against anti-SA IgA or anti-TetSA IgA. However, the caecal sample of case 02-376 was used for comparison with its herd's faecal sample and this sample possessed a much higher total IgA yield (824.5 μg/ml compared to a herd median of 470.1).

Comparisons between individual cases and their respective herds can be seen in Fig. 4.9. Case 03-352 has higher levels of specific IgA against the neurotoxins and SA than the median value of its respective herd and case 03-392 has higher specific IgA levels than all the horses in its herd to all antigens. Case 02-376 had less specific IgA than the median of its herd.

4.4 Discussion

4.4.1 *Study design and analysis*

This study was a collaborative effort involving research centres around the UK and the study design was agreed upon by all involved. Although the number of AGS cases was adequate, more non-GI controls would have been more useful, particularly if they had been grazing animals. However, controls such as these are hard to come by.

Likewise, it is probably preferable to obtain premises-matched controls rather than purely age-matched ones. However studies have proved age is important in the disease, horses under the age of seven years being more susceptible (Doxey et al, 1991). A previous study (McCarthy et al, 2004) demonstrated that affected horses had significantly lower specific IgG levels than premises-matched controls,

reinforcing data previously found by Hunter et al (2001) that was not age or premises matched. While the lack of age-matched controls is not imperative it should be taken into consideration when looking at the data. The data was analysed using a multivariable model but nothing was found that could not be explained by the horses' ages i.e. lack of disease in this data set was due to the control group's higher age, not by a higher IgG titre (Tom Cave, personal communication).

4.4.2 *Comparisons of systemic IgG between cases and co-grazing controls.*

No significant differences in anti-neurotoxin IgG was demonstrated when taken collectively although three of the five cases had an IgG titre against BoNT/C and BoNT/D below the median of their respective herds and 5/5 animals had a titre below that of the upper range of their herd. The lack of significant differences may be explained by the small number of cases involved and the differences demonstrated between IgG levels of herds support the case for looking at the data on a case by case basis as this could also skew the data in this small data set.

Age and specific IgG correlation analysis was not useful in this study - a lack of data for horses under the age of 4y probably accounted for the lack of correlation with any antigen. Specific IgG-IgG correlation was more interesting however, with positive correlation demonstrated between anti-BoNT/C and anti-BoNT/D IgG titre and inverse correlation observed between anti-SA and anti-BoNT/C IgG levels. The extent of cross-reactivity between the two neurotoxins in not documented, although some might be expected, this has not been demonstrated using this ELISA in other studies (Chapter III). A strong correlation was found indicating that the neurotoxins

may be found together i.e. the Group III organisms and their bacteriophages may be encountered together in the environment and in the horses GI tract in health and disease (a positive correlation was seen in AGS cases also, although not as significant). However, the lack of correlation between anti-SA and anti-BoNT/D may be due to BoNT/D being more rarely encountered in the environment (Ian Poxton, personal communication). A significant inverse relationship between anti-SA and anti-BoNT/C IgG in co-grazing controls suggests that these animals have been exposed to the organism for longer and built up more of an immune response. This may be enough to protect the animal from developing GS in itself or possibly the lack of immunity coupled with the nutritional trigger causes the disease.

Significant differences in OD levels of IgG subclasses were demonstrated but no correlation was observed between any combination. A reasonable body of work on IgG subclasses in relation to respiratory pathogens has been published but nothing has been published on bacterial pathogens or bacterial flora of the GI tract to the best of my knowledge.

4.4.3 *IgA extraction and detection.*

IgA extraction from GI contents and faeces yielded less total IgA than GI tissue, making comparison between parts of the GI tract difficult where some consisted solely of contents. It is unlikely that those samples showing a high percentage of specific IgA and a low total IgA are true representations of the amount of specific IgA present *in vivo.*

Variation of thickness of the GI wall along the intestine also introduces experimental error into the saponin method and removal of just mucosa would be a more accurate method of quantifying IgA. Pharynx samples, with one exception, gave a much more

uniform amount of total IgA and therefore a potentially more representative account of specific IgA. The presence of higher levels of anti-BoNT/D in cases and a trend toward higher levels of anti-BoNT/C IgA in the pharynx is interesting. Some very acute cases lack the distinctive pathology of the disease, leading pathologists to hypothesize that toxin production may take place higher up the alimentary tract (KE Whitwell, personal communication).

Unfortunately there is no working toxin assay presently to identify the presence of BoNT/C and none exists for the detection of BoNT/D. Possibly, toxin production occurs in the proximal alimentary tract in acute cases but not in chronic cases leading to more severe disease and less typical pathology in the enteric nervous system.

All of the original seven AGS cases were confirmed as grass sickness by histopathology (KE. Whitwell, personal communiction). Two animals in particular (03- 409, 03 – 392) demonstrated interesting inflammatory changes and bacterial colonisation in sections taken from the ileum and jejunum. Case 03 – 409 did demonstrate relatively high levels of specific IgA against BoNT/D in the jejunum and ileum and against SA in the jejunum. Specific IgA demonstrated by case 02 – 392 was unremarkable when compared with the rest of the group. The discovery of bacterial colonisation warrants further investigation and fits in with the hypothesis of Hunter (2001) that toxin production could be highly localised. A highly focused study, allowing investigation by histopathology, gene detection from mucosa of clostridial toxins, IgA and cytokine production could be highly useful: both in identifying the species involved in the colonisation and in understanding the disease process and immunology.

Although significantly more specific IgA against BoNT/C was seen in cases from jejunum and ileal samples this was not the case in duodenal samples. Duodenum samples gave the lowest percentage yield against BoNT/C of all the samples in cases and had the third lowest yield in controls: although one of the highest medians for total IgA. Against BoNT/D, significantly more IgA was found in jejunal and duodenal samples but not in ileal samples despite a large difference in medians when looking at the data. However, if the medians are compared using an unpaired t-test (with Welsh's correction, see Chapter III) a significant P value is demonstrated ($P = 0.009$) Significance is not demonstrated between duodenal samples against BoNT/C between samples and controls using either test. Discrepancies between large intestinal samples may be due to the extraction technique not being representative. Although no significant difference was seen between pharynx samples against BoNT/C, the relatively high (when comparing to other samples) upper range indicates that there may be an association between high levels of specific IgA at this site in comparison with controls and this is supported by a significant difference between specific IgA against BoNT/D between cases and controls. A much higher upper range in specific IgA against SA in cases also indicates an association- supported by a less clear association in anti-TetSA in the pharynx of cases versus controls.

A significant difference was observed between specific IgA levels against SA in duodenal samples and approaching significance in ileal samples but this was not demonstrated in jejunum samples despite the higher upper range found in cases. A significantly higher level of anti-TetSA was found in the stomach samples of cases of cases and approaching significance in ileal samples.

Hunter (2001) suggested that the presence of higher levels of IgA in cases also indicates a degree of exposure to the organism prior to the onset of clinical signs and therefore toxin production may be a cause of gut stasis rather than an effect. This study was designed to allow comparisons between case and controls on the same pasture. Only three of the cases had co-grazers that were faecal sampled and out of these, cases 02-352 and 03-392 had higher levels of specific IgA against BoNT/C than their respective herds. Some co-grazers (Fig. 4.9) demonstrated comparatively high specific IgA levels and would have thus been exposed to the organism as well. Subclinical cases of GS are thought to occur (Doxey et al, 1998) or it maybe that certain animals exposed to the organism without the hypothesised nutritional/stress trigger, simply produce a normal immune response, clearing the infection.

In summary, no highly statistical significant differences were demonstrated between systemic specific IgG levels between AGS cases and their controls but case numbers were not very high. Interesting differences were observed between herds – demonstrating that IgG levels may be relative within a herd and that these differences may affect statistical analysis. Cases often demonstrated lower specific IgG or IgG subclass levels than the median of their respective herds, indicating less exposure to the organism of interest. Detection of IgA from GI contents is variable due to proteases that degrade it and results were more satisfactory using a method of IgA extraction from tissue rather than contents. However, significantly more specific IgA was detected in cases rather than in controls whereas total IgA extracted was comparable.

Chapter V

Serology of chronic grass sickness cases.

Results

5.1 *Samples and study*

Previous longitudinal studies of CGS cases demonstrated fluctuations in OD levels of specific IgG (Hunter 2001). However, it was not possible to determine whether these fluctuations were significant. ELISA kits for the quantification of equine IgG are now commercially available and these were used to quantify levels of specific IgG in chronic cases by comparison against a standard curve of total IgG. Levels of IgG would be measured against neurotoxins and SA thought to be involved in the disease and compared to levels of IgG against TetNT and TetSA. As the vast majority of horses in the UK are vaccinated with tetanus toxoid every other year an anti-tetanus ELISA would provide an opportunity to determine if any fluctuations in IgG were due to an immune response to involved antigens or non-specific immune responses. Samples were assayed for total specific IgG against BoNT/C, BoNT/D and tetanus toxoids (TetNT) and against EDTA extracted surface antigens of neurotoxin negative strains of *C. botulinum* type C (SA) and *C. tetani* (TetSA). OD values of specific IgG sublasses IgGa, IgGb and IgGT against BoNT/C and BoNT/D and SA were also assayed.

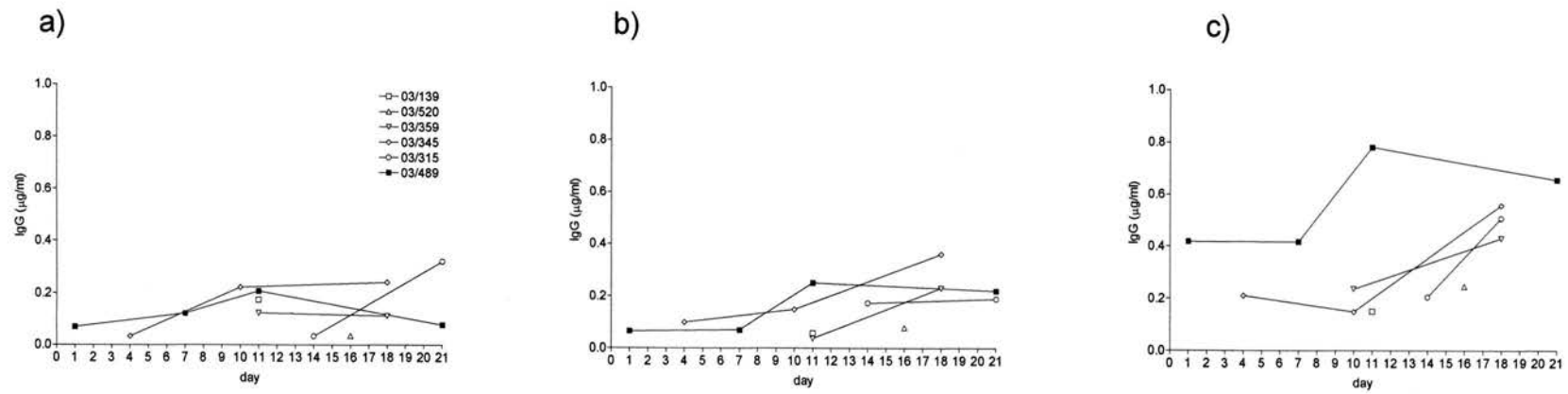
Table 5.1 shows the chronic cases used in the study. Twelve chronic cases of grass sickness, admitted to R(D)SVS in the season of 2003 were sampled longitudinally and samples assayed for specific IgG against BoNT/C, BoNT/D, SA and TetSA.

Table 5.1. Chronic cases sampled in 2003

Case number	Age	Breed/type	onset of clinical signs	admittance date	survived/euthanased
03/139	4y	Highland	18.02.03	26.02.03	euthanased 10.03.03
03/520	13y	Cob	25.06.03	27.06.03	euthanased
03/359	7y	Cob	03.05.03	11.05.03	euthanased 28.05.03
03/345	9y	Welsh Cob	04.05.03	06.05.03	euthanased 21.05.03
03/489	6y	unknown	14.06.03	14.06.03	euthanased*
03/376	5y	Irish sports horse	14.05.03	15.05.03	survived
03/315	15y	Coloured	26.04.03†	28.04.03	euthansed**
03/356	9y	Highland	09.05.03	10.05.03	survived
03/448	5y		03.06.03	-	survived
03/497	10y		23.06.03	23.06.03	survived
03/513	3y		06.06.04	-	survived
03/634	8y		~26.06.03	07.08.03	survived

†colic signs: colic surgery on admission *euthanased 4 months after discharge due to recurrent choke.
** euthanased at later date

Fig. 5.1



Graphs showing initial IgG titres in CGS cases that were euthanased with a) IgG against BoNT/C b) IgG against BoNT/D and c) IgG against SA. Time lines begin at date of onset of clinical signs (day 0 = onset of clinical signs). Cases 03-315 and 03-498 were discharged from hospital but euthanased at a later date.

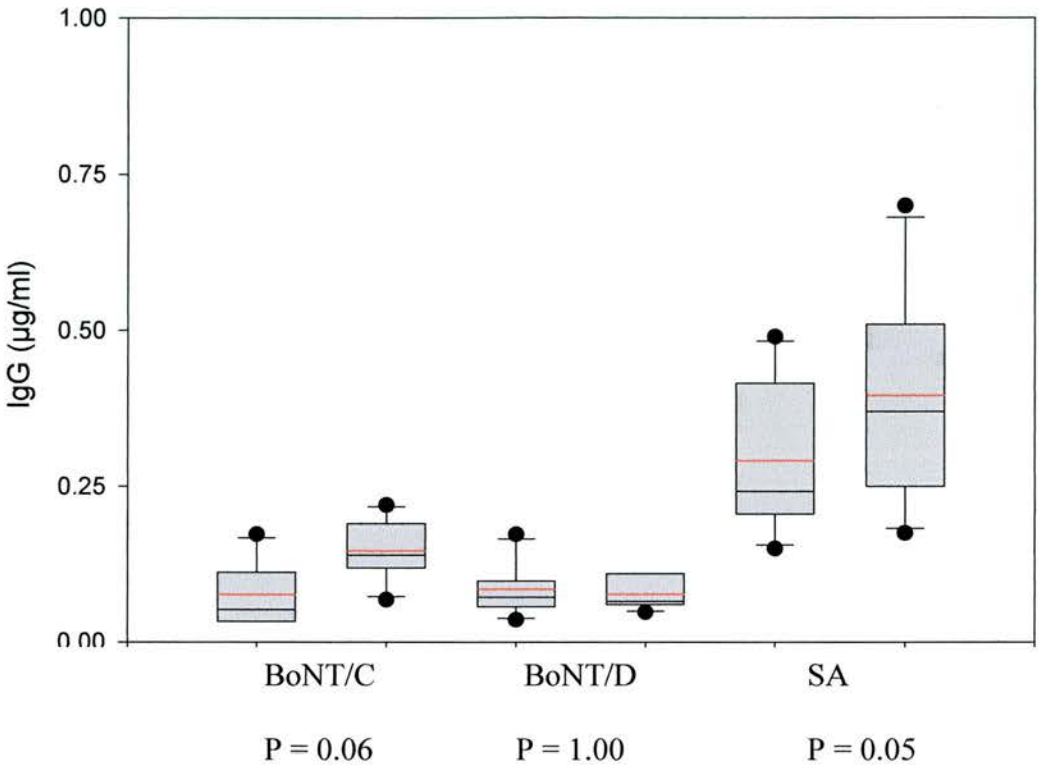
Four of the cases were euthanased at the hospital, two horses were euthanased at a later date, after returning home, and the remaining six animals survived.

A form was devised with the aid of clinicians, to provide relevant clinical and epidemiological information such as age, tetanus vaccination status and onset of clinical signs. A copy of the form can be found in Appendix 1.

5.1.1. *Analysis of the initial IgG levels of horses that were euthanased and comparison with surviving cases.*

Fig. 5.1 shows the IgG levels of animals that were euthanased, from Day 0 (onset of clinical signs) until death. The cases are plotted on the same timeline to enable comparison. The exception is Case 03-489 which was included in the analysis as the horse was eventually euthanased due to recurrent choke. The premise was to examine initial antibody levels of animals to look for a positive protective effect in the early stages of the disease. The remaining course of Case 03-489 immune responses can be seen in Fig.5.3c. Of the four horses sampled more than once, all demonstrated a rise in IgG titre to BoNT/C except 03-359. All cases also demonstrated a rise in titre to BoNT/D also, except case 03-315, which showed the most dramatic increase in anti-BoNT/C IgG titre. Likewise, all animals demonstrated some increase in anti-SA IgG over the course of sampling. Case 03-489 was sampled more than other cases and rises in specific IgG titre to all three antigens was followed by a drop in titre.

Fig.5.2



Boxplots illustrating the difference between initial levels of those horses euthanased (first plots of each set, n = 6) and those that survived (n = 6). Red lines demonstrate the mean and black lines the median of each data set.

The results of the comparison of initial levels of the two groups are shown in Fig.5.2. The non-surviving cases had significantly less initial specific IgG to SA than surviving cases (Mann-Whitney U test, P = 0.05) and almost significantly less IgG to BoNT/C than surviving cases (P = 0.06). There was no difference in levels to BoNT/D (P = 1.0). Cases 03-139, 03-359 and 03-520 were initially sampled much later during the course of the disease than the others (11 and 16 days respectively). The first two show comparatively high IgG levels to BoNT/C than other euthanased chronics although 03/520 does not.

5.1.2 *Longitudinal analysis of specific IgG levels in CGS cases that survived.*

The results of these analyses are graphically represented in Fig.5.3. IgG was quantified and plotted on the left hand axis as mg (specific IgG) /ml. IgG subclasses were not quantified and are plotted on the right hand axis and expressed as OD units.

Case 03-513

This case was the youngest animal sampled in this study (3y) and was first sampled at 7 days after the onset of clinical signs.

BoNT/C & TeNT

Anti-TeNT IgG increased very slightly after the first 18 days. The largest fluctuation is seen in OD of IgGb which corresponds in the relatively smaller fluctuation in IgG to BoNT/C.

BoNT/D

An overall initial increase is demonstrated in IgG at the end of sampling although corresponding fluctuations in IgG subclasses are not.

SA & TetSA

IgG decreases over the first 17 days and thereafter remains fairly level. However, large fluctuations are seen in the OD values of specific IgGa, IgGb and IgGT which are not reflected in the total specific IgG level.

Case 03-497

BoNT/C & TeNT

A slight increase in anti-TeNT was observed over the first 14 days. A drop was seen in anti-BoNT/C IgG and a corresponding fluctuation seen in IgGb.

BoNT/D

A peak in anti-BoNT/D IgG was demonstrated at 20 days. A drop in specific IgGb was seen at day 10.

SA & TetSA

Fluctuations in anti-SA IgG with peaks occurring at ~ 5-7 day intervals. These were largely mirrored by fluctuations in OD of IgGb and IgGa. A similar drop in anti-TetSA was seen at day 10 although not subsequently.

Fig.5.3a i) **ii)** **iii)**

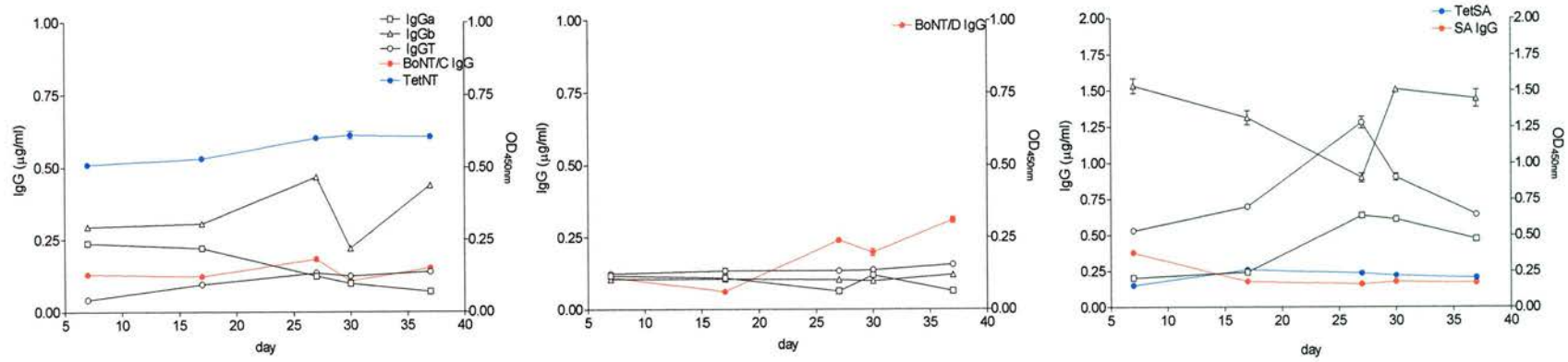
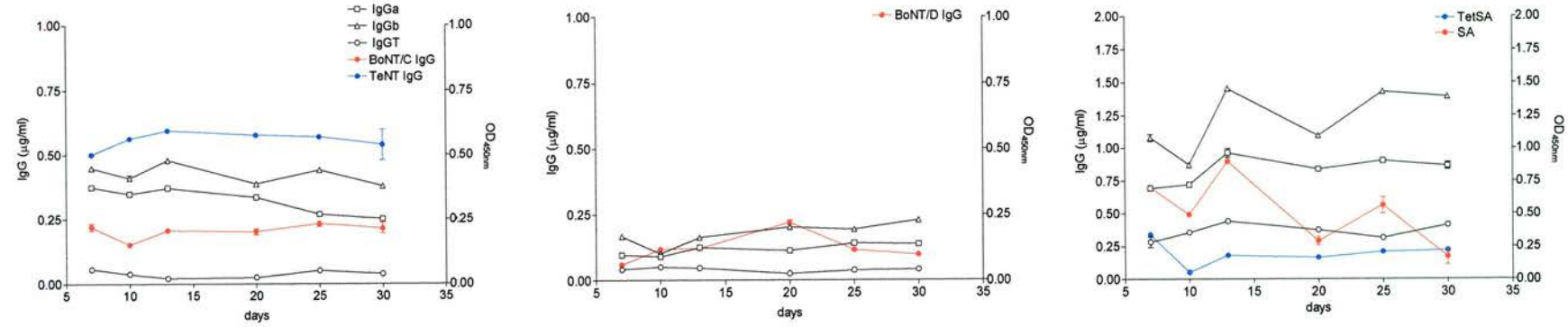


Fig.5.3b i) **ii)** **iii)**



Graphs of cases 03-513 (5.3a) and 03-497 (5.3b) showing longitudinal IgG levels and OD of IgG subclasses against i) BoNT/C and TetNT ii) BoNT/D iii) SA and TetSA.

Fig.5.3c i) **ii)** **iii)**

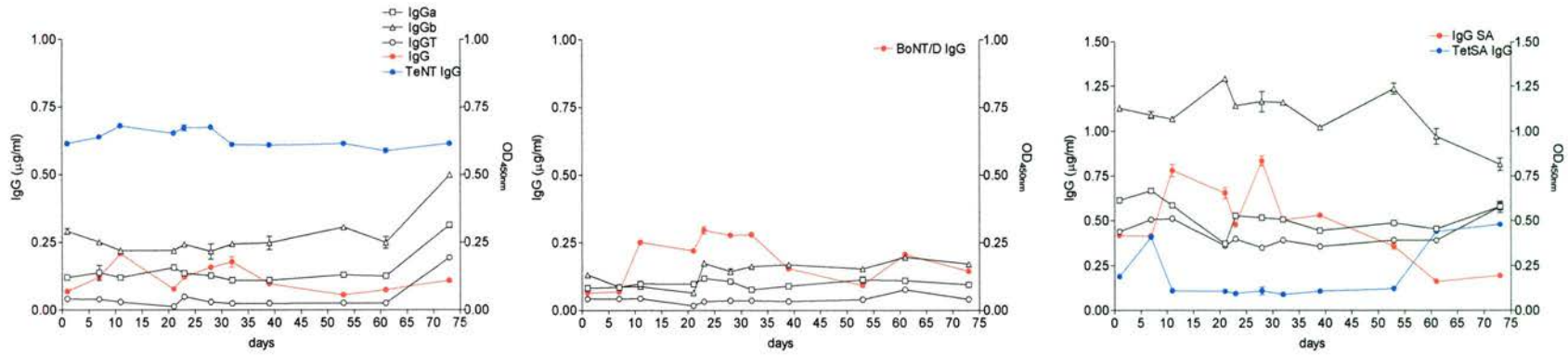
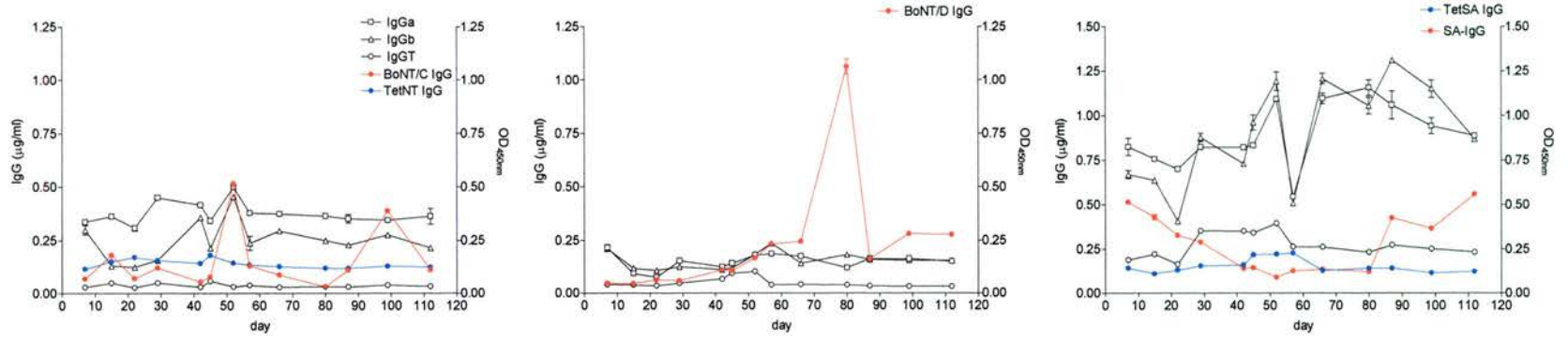
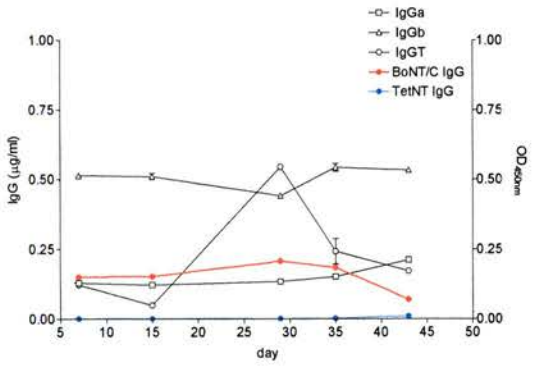


Fig.5.3d i) **ii)** **iii)**

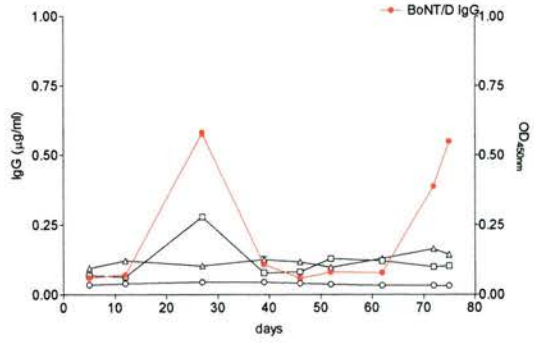


Graphs of cases 03-489 (5.3c) and 03-448 (5.3d) showing longitudinal IgG levels and OD of IgG subclasses against i) BoNT/C and TetNT ii) BoNT/D iii) SA and TetSA

Fig. 5.3e i)



ii)



iii)

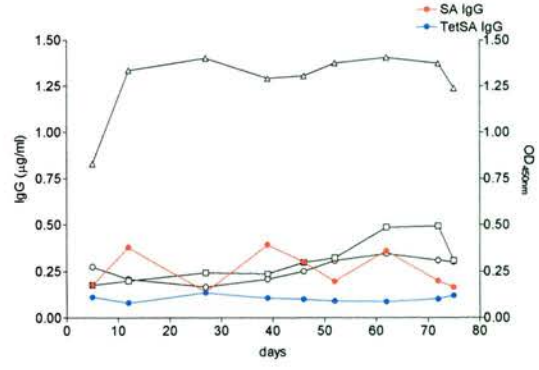
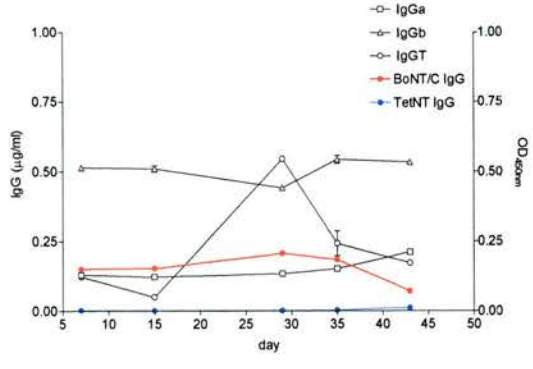
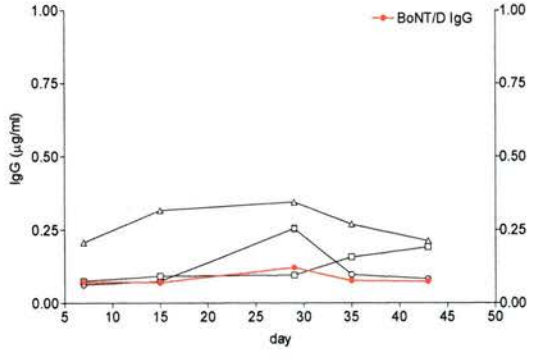


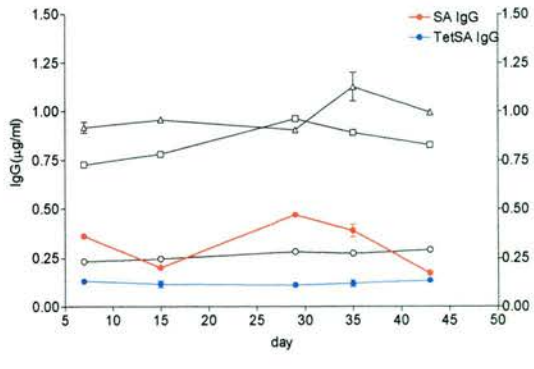
Fig. 5.3f i)



ii)

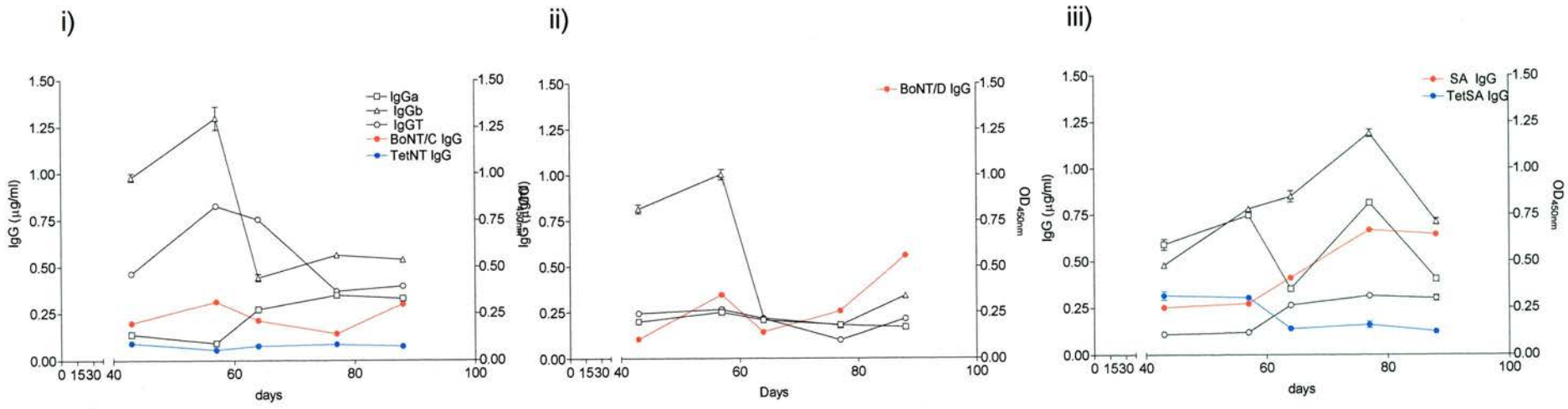


iii)



Graphs of cases 03-356 (5.3e) and 03-376 (5.3f) showing longitudinal IgG levels and OD of IgG subclasses against i) BoNT/C and TetNT ii) BoNT/D iii) SA and TetSA

Fig.5.3g



Case 03-624. Graphs showing longitudinal IgG titres and OD if IgG subclasses to i) BoNT/C and TeNT ii) BoNT/D iii) SA and TetSA.

Case 03-489

BoNT/C & TeNT

Peaks of anti-BoNT/C IgG were demonstrated at 10 and 32 days. All three subclasses showed an increase in OD at the last sampling. Anti-TeNT IgG remained relatively level over the time of sampling.

BoNT/D

An overall increase in anti-BoNT/D IgG was observed between 7 and 32 days with a further peak at 60 days. The IgG titre corresponds to the OD levels of the subclasses.

SA & TetSA

Fluctuations were seen in anti-TetSA IgG at the beginning and end of the sampling period. Between day 7 and day 40 a higher level of anti-SA was demonstrated. This case had high OD levels of IgGb and relatively high levels of IgG.

Case 03-448

BoNT/C & TeNT

Significant peaks of anti-BoNT/C IgG were demonstrated at day 52 and day 100 with smaller peaks at day 15 and day 30. The major peak at day 52 corresponds with peaks of OD levels of specific IgGa and IgGb. Anti-TeNT IgG remains largely unchanged. This horse had a higher level of specific IgGa than IgGb to BoNT/C.

BoNT/D

An increasing titre of anti-BoNT/D IgG is seen, resulting in a large peak at day 80.This does not correspond with any other anti-BoNT/D IgG subclass.

SA & TetSA

Anti-SA IgG gradually decreases from day 7 until day 50 after which it begins to increase. IgGa and IgGb mainly correspond to each other although not with anti-SA

IgG. A high level of anti-SA IgGa is maintained in this horse. Anti-TeTSA increases slightly between day 42 and day 57 but remains relatively constant after this.

Case 03-376

BoNT/C & TeNT

This animal was not vaccinated against tetanus. A significant peak of IgGT to BoNT/C is demonstrated at day 29 to BoNT/C. A very slight increase in anti-BoNT/C was seen although this had dropped at the time of last sampling.

BoNT/D

A peak of IgGa can be seen at day 29 which corresponds with a peak of IgG. A subsequent peak of IgG at the end of sampling is not followed by an increase in any of the subclasses.

SA & TetSA

Anti-TetSA IgG shows no fluctuations. A drop at day 15 of anti-SA IgG is followed by a peak at 29 days, corresponding with a peak of IgGa and a peak of IgGb can be seen at day 35.

Case 03-356

BoNT/C & TeNT

A small peak of anti-BoNT/C IgG is seen at day 62 and day 75. Anti-TetNT IgG drops after day 40.

BoNT/D

Significant peaks of anti-BoNT/D IgG can be seen at day 28 and day 75. A significant peak of IgGa can also be seen at day 27.

SA & TetSA

Anti-TetSA IgG levels remain low and unchanged throughout the sampling period. Anti-SA IgG demonstrates peaks and troughs in cycles of ~20 days. An increase in IgGa was observed up until day 70. This animal had relatively high levels of IgGb throughout the sampling period with a large increase between day 7 and day 12 and this appeared to be dropping at the end of sampling.

Case 03-634

This case had had suspected grass sickness since its arrival at the new owners and had been treated at home for ~42 days before being admitted to the LAH.

BoNT/C & TeNT

Anti-TeNT IgG does not fluctuate significantly throughout the sampling period. A peak of anti-BoNT/C IgG, IgGb and IgGT can be seen at day 57 although IgGa rises after this date.

BoNT/D

A large peak of total specific IgG and IgGb can be observed at day 57. After a drop IgG titre continues to rise up until end of sampling. Specific IgGb and IgGT had also risen at the last sampling.

SA & TetSA

A significant drop in anti-TetSA IgG is demonstrated at day 64. An overall increase is seen in anti-SA IgG which corresponds with a rise in specific IgGb, IgGT and IgGa OD levels.

5.2 *Discussion*

As discussed previously (Chapter IV) epidemiological evidence supports the hypothesis that EGS may have an infectious aetiology and that a protective immune response is demonstrated in older horses, those having prior contact with the disease and those animals that have remained on a particular pasture (Doxey et al, 1991, Wood et al, 1998). It is also accepted that the degree of pathology demonstrated in the various disease presentations is representative of the degree of toxic insult received (Scholes et al, 1993; Doxey et al, 1995). AGS is also associated with low levels of systemic IgG to neurotoxin and SA (Hunter et al 2001; McCarthy et al, 2004) and subclinical cases of GS are thought to occur (Doxey et al 1995). The importance of the state of the immune system at the time of exposure to the causative agent and subsequent severity of the disease is unknown. Hunter (2001) showed that CGS cases that did not survive had a lower specific IgG titre against SA than those that recovered, although this was not statistically significantly lower. The results presented here, that subsequently euthanased animals had significantly lower IgG levels to SA and almost significantly lower ($P = 0.06$) IgG levels to BoNT/C, suggest that the status of the immune system before infection may be important in the animal's prognosis. All of the euthanased cases that had been sampled more than once demonstrated rising levels against the antigens except one, suggesting that the

subsequent immune response has no effect on disease outcome. The surviving animals also demonstrate fluctuations in IgG levels to either BoNT/C or BoNT/D, often having higher levels at the end of sampling than at the start. Disease intervention treatments, such as passive immunisation may at best contribute some sort of damage limitation if given early enough, but may have no effect at all. The incubation period is unknown, although AGS cases do show higher levels of specific IgA against the antigens than controls (Hunter 2001, Chapter IV, this thesis) indicating that exposure happens some time before the onset of clinical signs. Unfortunately, some of the horses in this analysis were not sampled quickly after the onset of clinical signs (up until 42 days after) and when looking at the data from the surviving animals, IgG levels often drop in the first 14 days of sampling. This may be due to existing IgG being bound to free antigen (Hunter, 2001).There was no difference between IgG levels against BoNT/D although subsequent fluctuations in IgG indicate that low initial levels to this neurotoxin may be due to rarity of the BoNT/D producing organism/bacteriophage in the environment and lack of exposure to it beforehand. The extent of cross-reactivity of BoNT/C and BoNT/D is unknown and some horses show much higher levels of IgA and IgG to BoNT/D than BoNT/C, suggesting the presence of BoNT/D in some cases.

The serology of CGS has been examined before (Hunter 2001) although IgG was not quantified due to lack of reagents. Fluctuations in OD level alone give little evidence as to the significance of the rise or drop in levels. When looking at specific IgG (μg/ml) in different data sets (ie healthy horses, co-grazing controls, AGS and CGS cases) it does imply that the fluctuations demonstrated are significant – some

fluctuations are at least double the initial level and therefore the IgG level to that antigen is being affected in some way whether or not the organism has a causal role in the disease. Why the fluctuations are transient is unclear - increasing levels of IgG against the neurotoxins are only rarely observed although some CGS cases do demonstrate high levels in subsequent seasons (I. Poxton, unpublished data). However, this is the exception rather than the rule. That AGS cases produce IgA and CGS cases demonstrate such fluctuations suggest that animals begin to mount an immune response, but are subsequently immunosuppressed. It might be interesting to correlate IgG fluctuations with clinical observations, in particular to see if the peaks in specific IgG correspond with periods of GI stasis and therefore re-exposure to SA and or neurotoxin. An investigation into cytokine expression may also prove useful. An assay against TetSA was established to look for IgG against another ubiquitous soil organism and it is noteworthy that IgG to this antigen did not fluctuate as much as IgG levels against the BoNTs or SA in any animal at any time. The fluctuations would therefore not appear to be due to non-specific immune stimulation.

Little is known about the IgG subclass function in the horse. IgGa and IgGb are involved in opsonisation and IgGT in parasite and bacterial exotoxins. All the published literature is involved in viral diseases (Nelson et al, 1998; Mizukoshi et al, 2002) and intracellular bacteria such as *Rhodococcus equi* (Hooper – McGrevy et al, 2003). An infection involving an extracellular organism such as *C. botulinum* would involve both a Th1 and Th2 immune response and no differences / patterns were observed in IgG subclass ratios (data not shown). Quantification of subclasses may

be interesting if correlated with RNA expression of cytokines to try and establish the mechanism behind the apparent immunosuppression.

Chapter VI

Serology of challenge horses

6.1 *Samples and study*

This work is related to that in Chapter III and involves soil samples gathered from those premises that had the cases of AGS. This was part of a multi-centre research project and the oral challenges consisted of soil samples positive for different BoNTs. In Edinburgh, free toxin was detected before/after enrichment by toxin ELISA and mouse bioassays were used in Goettingen, to detect A/B/E positive or C/D positive enrichments. The challenge work was carried out in the Central Veterinary Laboratory in Dubai.

An initial study demonstrated that horses in Dubai had specific IgG OD levels to BoNT/C and SA comparable to British animals (data not shown). Several other horses were selected (not on the basis of pre-bleeds) for challenge work and the challenge protocols discussed and decided upon by the research groups involved. In addition to soil, some animals were given one/combination of other oral substances that were known/thought to be potential risk factors. These included ivermectin; oral antibiotics to alter GI flora and make it more amenable to clostridial growth and dextrin as a potential substrate for clostridial growth; iv corticosteroids to achieve some level of immuno-compromise (in order to mimic stress); oral inulin to attempt to mimic a seasonal increase in grass fructan levels; codeine phosphate to reduce GI motility and induce ileus; apricot kernels to act as a cyanide source (to mimic clover): omeprazole which is a proton pump to decrease gastric pH and thus

possibly increasing toxin survival through the gut. The challenge protocols for each horse are listed below in Table 6.1. Different soils/soil combinations were used and these are detailed in Table 6.2 and the graphs of antibody levels in individual horses in Figs 6.1-6.6.

Table 6.1 Challenge protocols for individual horses

Horse A

Week	1	2	3	4	5	6	7	8	9	10
Soil*	1	1	1	1	1	-	6	6	7	7
Antibiotics	-	-	+	+	+	+	+	+	-	-
Ivermectin	-	-	-	-	-	-	-	-	+	-

Horse B

Week	1	2	3	4	5	6	7	8	9	10
Soil*	1	1	1	3	-	4	4	4	6	6
Inulin	+	-	-	-	-	-	-	-	-	-
Steroids	-	-	+	-	-	-	-	-	-	-
Dextrin	-	-	-	-	-	+	+	+	+	+
Ivermectin	-	-	-	-	-	-	-	-	+	-
Codeine	-	-	-	-	-	-	-	-	-	-

Horse C

Week	1	2	3	4	5	6	7	8	9	10
Soil*	-	-	-	3	-	5	-	-	6	6
Inulin	+	-	+	-	-	-	-	-	-	-
Dextrin	-	-	-	-	-	+	+	+	+	+
Ivermectin	-	-	-	-	-	-	-	-	+	-
Codeine	-	-	-	-	-	-	-	-	+	-

Horse E

Week	1	2	3	4	5	6	7	8	9	10
Soil*	1	1	7	7	-	4	4	4	6	6
Inulin	+	-	-	-	-	-	-	-	-	-
Steroid	-	-	+	-	-	-	-	-	-	-
Dextrin	-	-	-	-	-	+	-	-	-	-
Kernels	-	-	-	-	-	-	+	+	+	-
Ivermectin	-	-	-	-	-	-	-	-	+	-

Horse F

Week	1	2	3	4	5	6	7	8	9	10
Soil*	-	-	-	7	-	5	-	-	6	6
Inulin	+	-	+	-	-	-	-	-	-	-
Dextrin	-	-	-	-	-	+	-	-	-	-
Kernels	-	-	-	-	-	-	+	+	+	-
Ivermectin	-	-	-	-	-	-	-	-	+	-

Horse G euthanasia

Week	1	2	3	4	5	6	7	8	9	10
Soil*	1	1	1							
Antibiotics	-	-	-							

Horse H

Week	1	2	3	4	5	6	7	8	9	10
Soil*	-	-	1	6						
Antibiotics	+	+	+	+						

* soil samples described in Table 6.2

Table 6.2 Details of soil samples

Sample no.	contents
1	soil mixture from 15 GS associated pastures
2	soil from a GS premises in Aberdeenshire
3	soil from a GS premises in Northumberland-BoNT/C directly detected by ELISA
4	soil in which BoNTs A,B and E detected by bioassay
5	soil in which BoNTs C/D detected by bioassay
6	soil from GS premises in Perthshire
7	worm casts from premises in Perthshire*

*same premises as in 6

6.2 Results

Graphs depicting longitudinal serological analysis are shown in Fig. 6.1-6.6. Specific IgG was quantified and plotted on the left hand axes as specific IgG (μg/ml). Specific IgG subclasses IgGa, IgGb and IgGT were not quantified and are therefore expressed as OD values and plotted on the right-hand axes. The only animal to demonstrate abnormal clinical signs was Horse G which was euthanased on Day 19 of the challenge. The results of this challenge are treated separately in section **6.3.**

6.2.1 Analysis of challenge animal serology

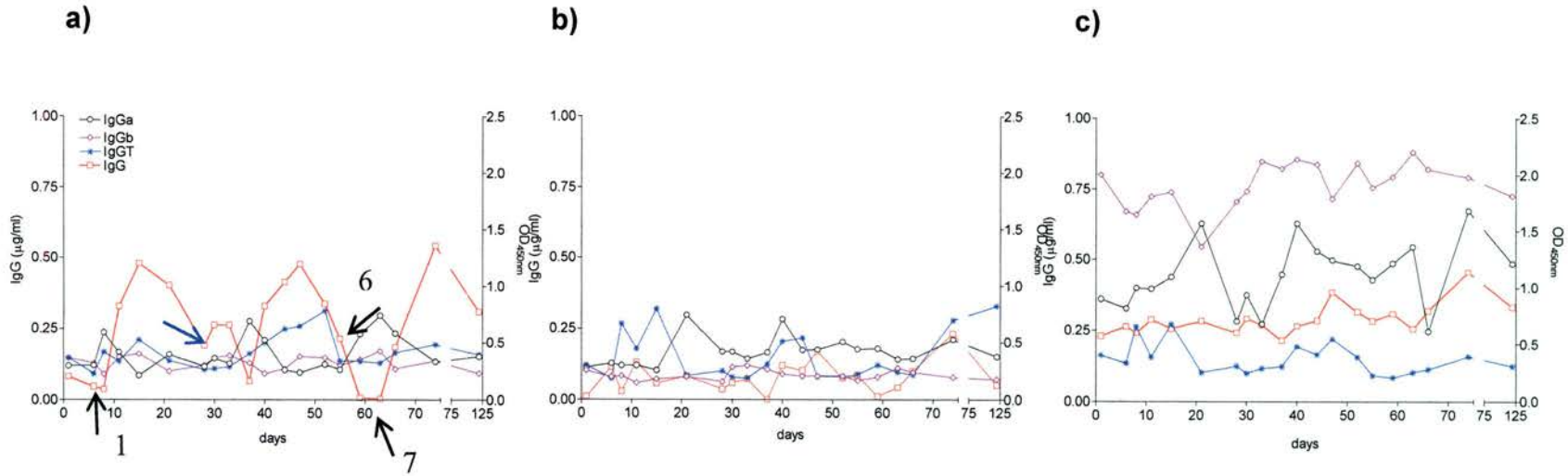
Horse A

Horse A (Fig. 6.1) was given the soil mixture from a mixture of GS premises (1) and demonstrated an increase in IgG to BoNT/C during the sampling period following the start of the challenge although changes in IgG to SA or BoNT/D were not as apparent. IgG to BoNT/C dropped during administration of sample 6 until the administration of a soil sample 7. Peaks of IgG to BoNT/D and SA are demonstrated at the end of the initial sampling period.

Horse B

Horse B (Fig.6.2) was also given the soil mixture 1 and also inulin in the first week. On Day 28 soil and steroids were given. Soil mixture number 4 and dextrin was given from Day 40 and this is also followed by a drop in IgG to all three antigens. Specific IgG increased against BoNT/C following administration of soil mixture 6, which was given in conjunction with codeine phosphate and then dextrin. IgG to BoNT/C and BoNT/D was higher at the end of the challenge period than at the beginning but IgG to SA at the end of sampling was slightly lower.

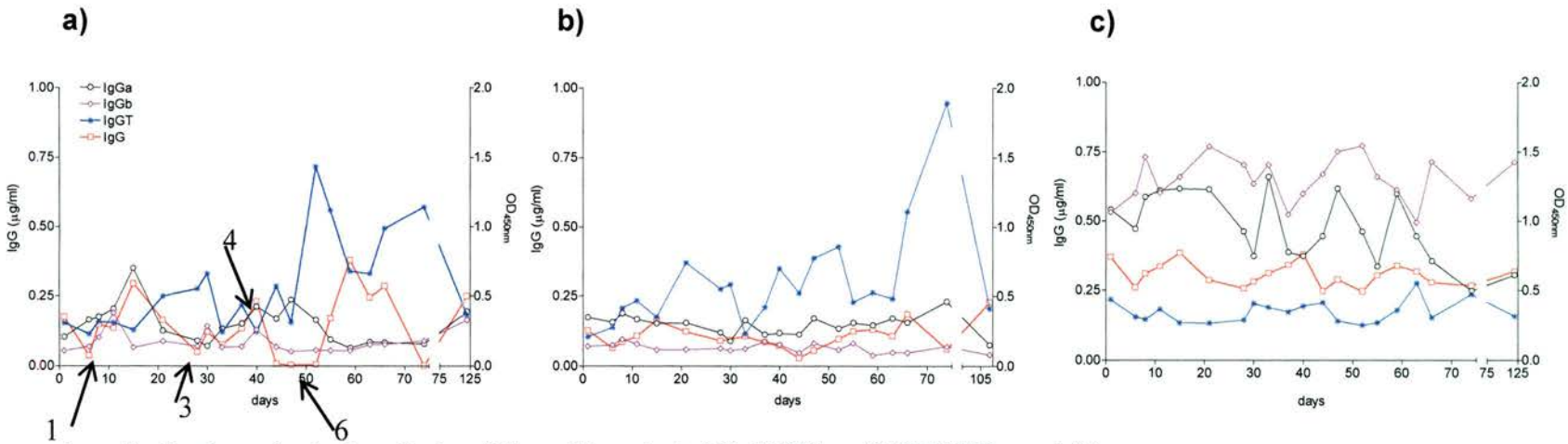
Fig.6.1



Longitudinal serological analysis of Horse A against a) BoNT/C b) BoNT/D c) SA

Numbered arrows refer to the soil samples used-please refer to Table 6.2. The blue arrow indicates the start of oral antibiotics.

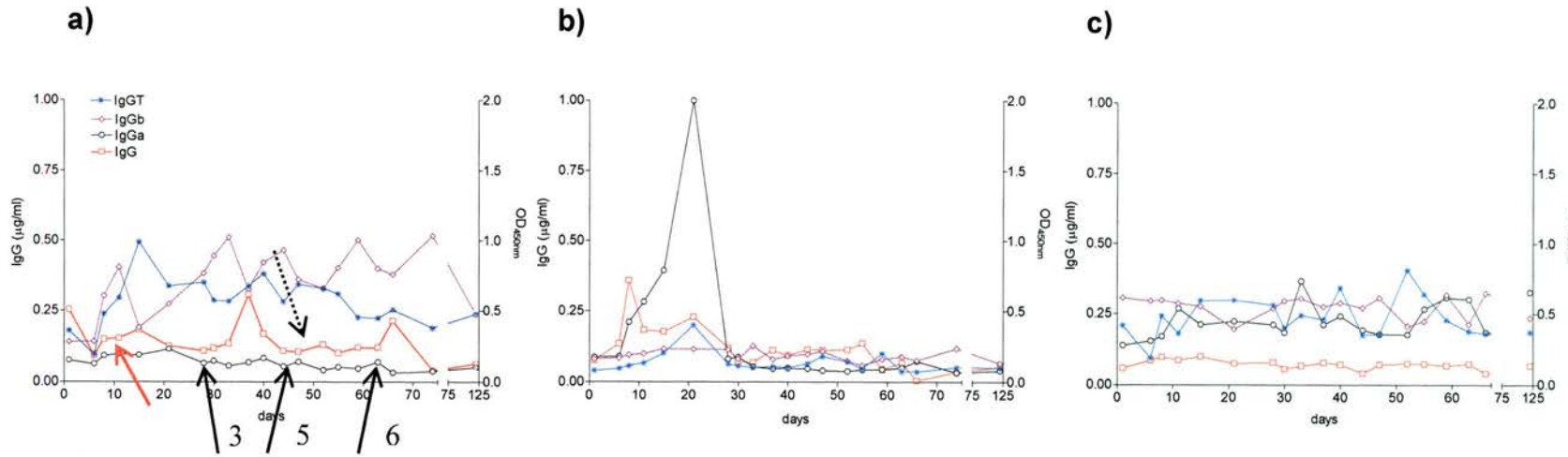
Fig. 6.2



Longitudinal serological analysis of Horse B against a) BoNT/C b) BoNT/D c) SA

IgG to BoNT/C fell following feeding of soil containing BoNTs A, B & E (4).

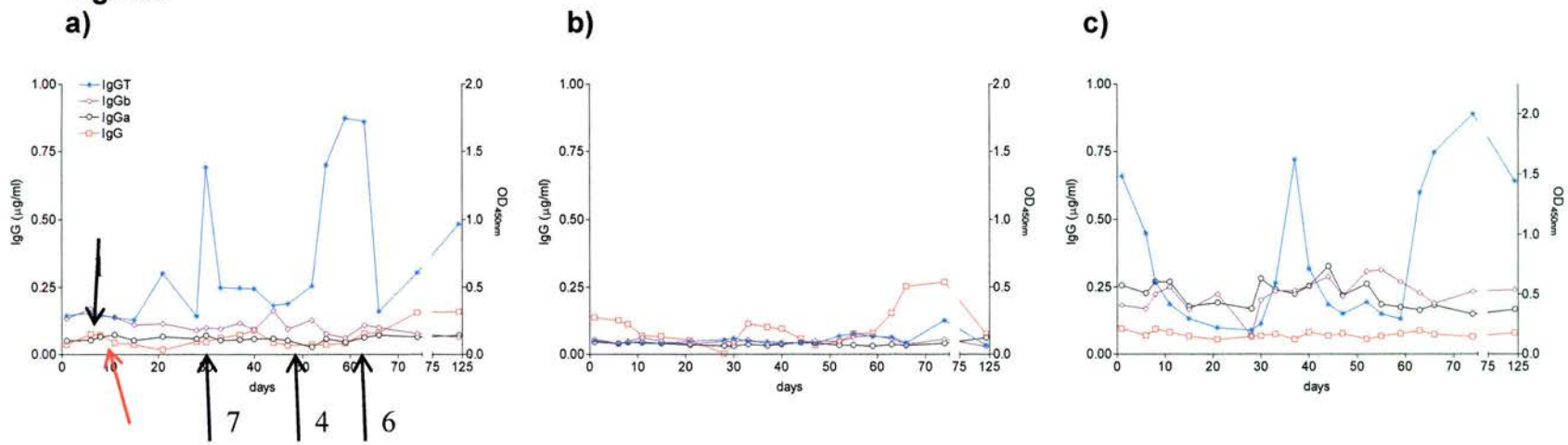
Fig.6.3



Longitudinal serological analysis of Horse C against a) BoNT/C b) BoNT/D c) SA

The red arrow indicates oral inulin and the dashed arrow indicates oral dextrin.

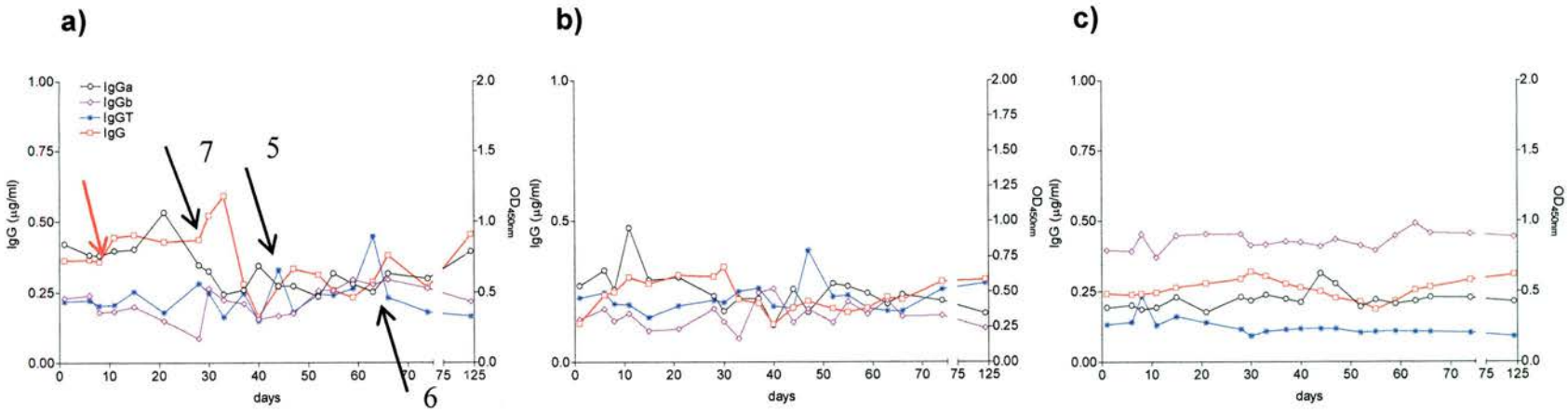
Fig. 6.4



Longitudinal serological analysis of Horse E against a) BoNT/C b) BoNT/D c) SA

The red arrow indicates oral inulin. Apricot kernels were also given with soil samples from arrow 4 onwards.

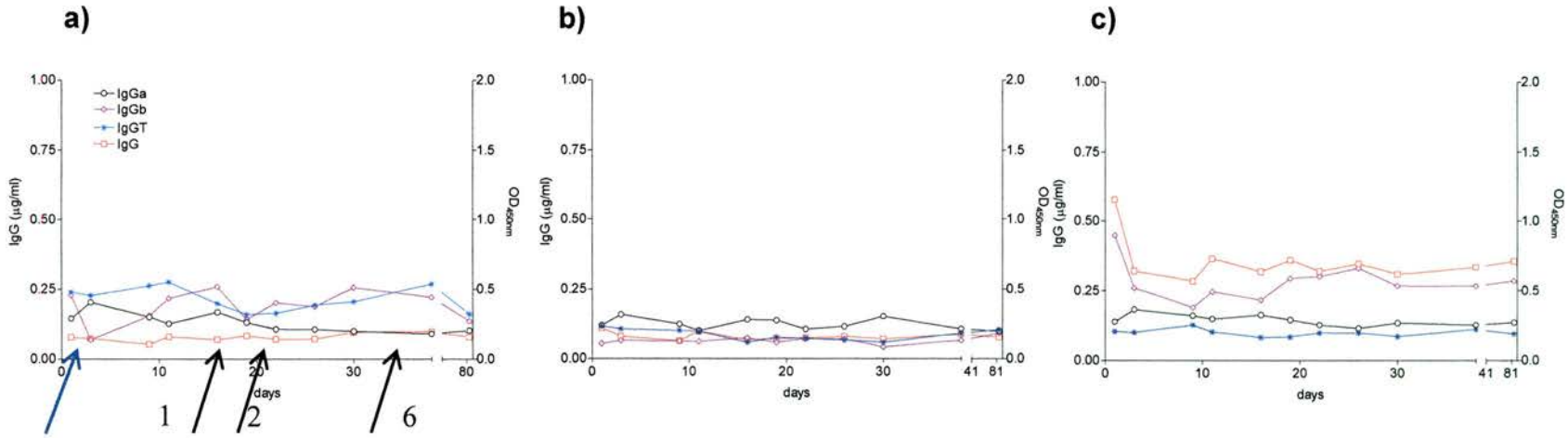
Fig.6.5



Longitudinal serological analysis of Horse F against a) BoNT/C b) BoNT/D c) SA

The red arrow indicates oral inulin.

Fig. 6.6



Longitudinal serological analysis of Horse H against a) BoNT/C b) BoNT/D c) SA

The blue arrow indicates oral antibiotics which were administered throughout this challenge.

Horse C

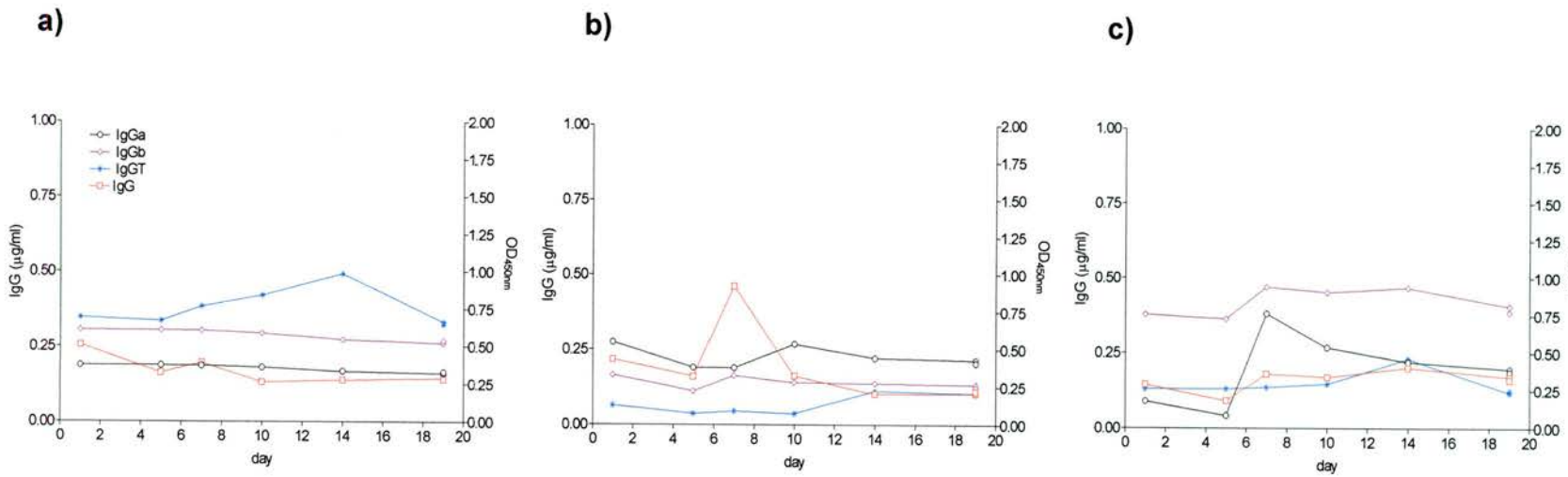
Horse C (Fig. 6.3) was given only oral inulin for the first and third weeks of the challenge, with no soil given until ~day 27 (soil sample 3). A peak of IgG to BoNT/D was seen in the second week and a slight increase in IgG to BoNT/C corresponds to an increase in IgGT and IgGb to BoNT/C. IgGa to BoNT/C demonstrated no dramatic fluctuations in OD level throughout the challenge period. A peak in IgGb and IgGT to BoNT/D corresponds to the peak of IgG. IgG to SA remains unchanged although small peaks of IgGa and IgGT are seen. A peak of IgG to BoNT/C is demonstrated following feeding of soil sample number 3, which was the sample containing BoNT/C before enrichment. A further peak of IgG to BoNT/C is seen after administration of soil sample number 6. Corresponding peaks of IgG to BoNT/D or SA were not demonstrated.

Horse E

Horse E (Fig.6.4) demonstrated large peaks of specific IgGT against BoNT/C and SA.

IgG levels to BoNT/C and BoNT/D were higher at the end of the challenge period than at the beginning, anti-BoNT/D rising after the end of administration of soil sample 4 (containing BoNTs A, B and E).

Fig. 6.7



Graph showing longitudinal serology of Horse G over the challenge period until euthanasia. IgG and IgG subclass levels to a) BoNT/C b) BoNT/D and c) SA.

Horse F

A rise in IgG to BoNT/C was demonstrated (Fig. 6.5), although corresponding peaks were not seen in IgG to BoNT/D or SA. IgG to all three antigens was at a higher level at the end of the challenge period than at the start, with the largest increases in IgG to BoNT/D.

Horse H

This horse showed little increase or decrease in specific IgG over the course of sampling (Fig. 6.6). Initially only oral antibiotics were given, followed by a mixture of soil 1 and antibiotics followed by soil 2 and soil 6, all with antibiotics.

6.3 *Analysis of Horse G*

Horse G was given soil mixture 1 three times daily over a period of 16 days and antibiotics on day 15 (erythromycin and potentiated sulphonamides via stomach tube). Colic signs were demonstrated later that day and the animal responded to analgesics. Intra-venous gentamicin was given on day 16 when the horse failed to eat concentrate feed containing potentiated sulphonamides. Colic signs consistent with intestinal ileus were demonstrated and the animal was finally euthanased on the basis of inability to provide adequate analgesia and the detection of small intestinal obstruction upon rectal examination.

6.3.1 *Longitudinal IgG levels*

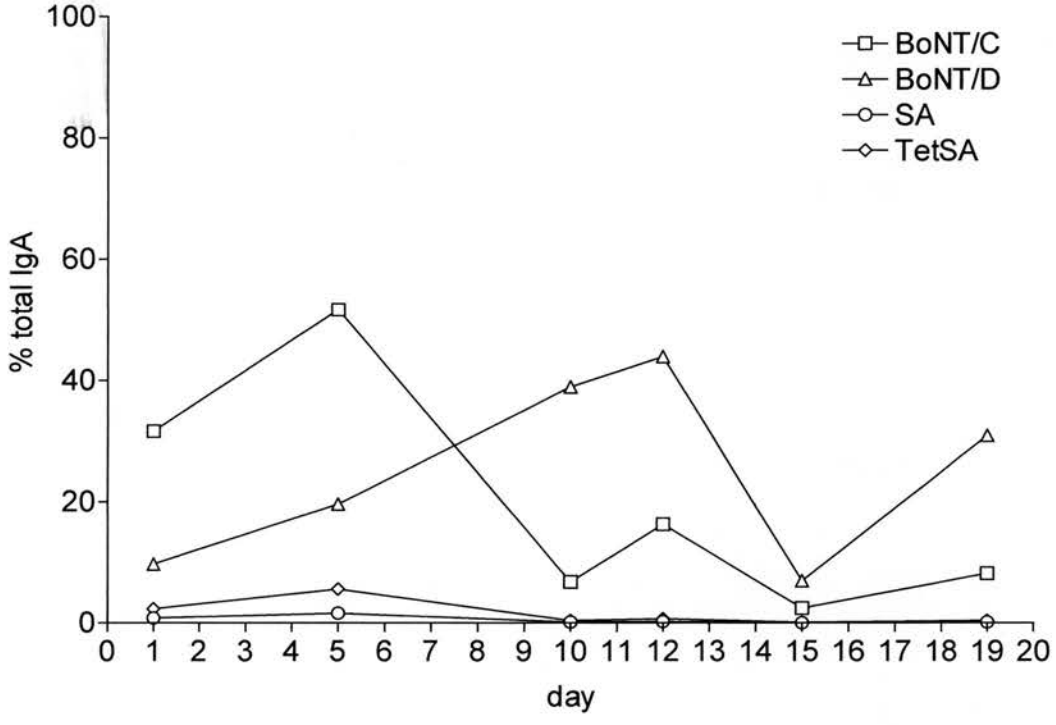
Longitudinal IgG levels can be seen in Fig. 6.7. A peak of IgG to BoNT/D is demonstrated at day 7 but apart from a rise in IgGT and IgGa to BoNT/C and SA respectively, nothing significant is seen.

6.3.2 *Mucosal IgA*

Post-mortem samples were collected for bacteriology and serology. Mucosal IgA was extracted and analysed as described in Chapters II and IV. Total IgA and total specific IgA are shown in Table 6.3. Longitudinal analysis of specific IgA (from faeces) is shown in Fig.6.8 and specific IgA detected in different GI locations is shown in Fig.6.9.

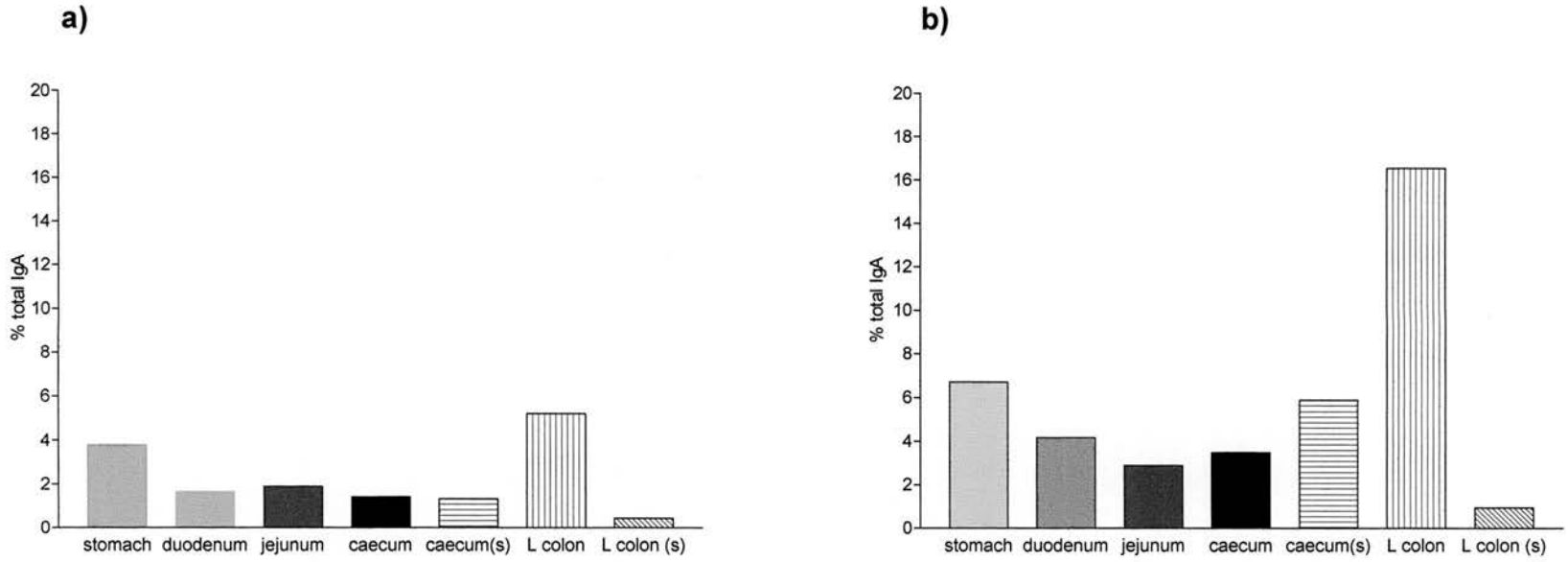
Specific IgA against both neurotoxins demonstrated peaks – IgA against BoNT/C after 5 days and to BoNT/D after 12 days. The TetSA assay was used as an indicator of soil exposure in all studies and IgA against this antigen hardly varied. There was little variation against SA. Samples contained a higher proportion of anti- BoNT/D IgA (see Fig. 6.9) than anti- BoNT/C IgA in all post-mortem samples.

Fig. 6.8



Longitudinal analysis of faecal IgA from Horse G. IgA is expressed as a percentage of total IgA recovered which is extremely variable from faecal samples. Interestingly, IgA to SA or to TetSA did not increase. IgA to TeTSA was used as an indicator of soil exposure on the presumption that if horses are encountering *C. botulinum,* they are also very likely to also be encountering *C. tetani.*

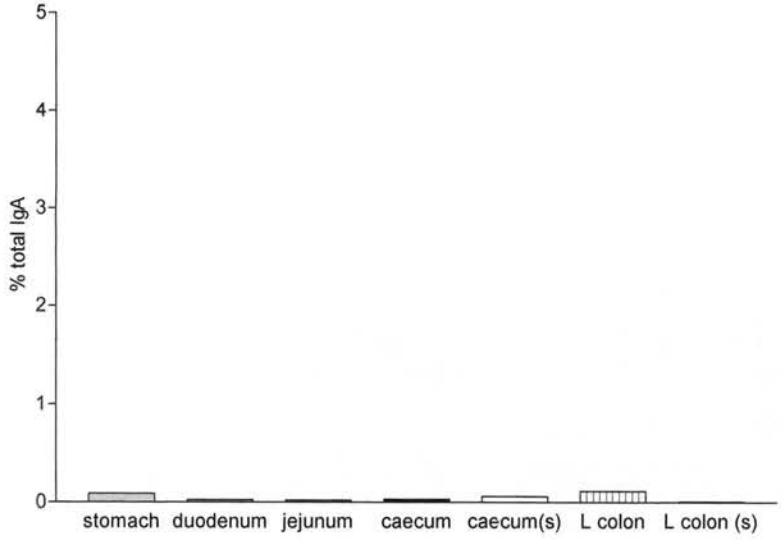
Fig. 6.9



Percentage of specific IgA extracted from GI samples from Horse G against a) BoNT/C b) BoNT/D c) SAd) TetSA
A relatively higher proportion of IgA against both BoNT/C and BoNT/D was found in the large colon when using the contents extraction technique.

Fig. 6.9

c)


% total IgA
0
1
2
3
4
5
stomach
duodenum
jejunum
caecum
caecum(s)
L colon
L colon (s)

d)

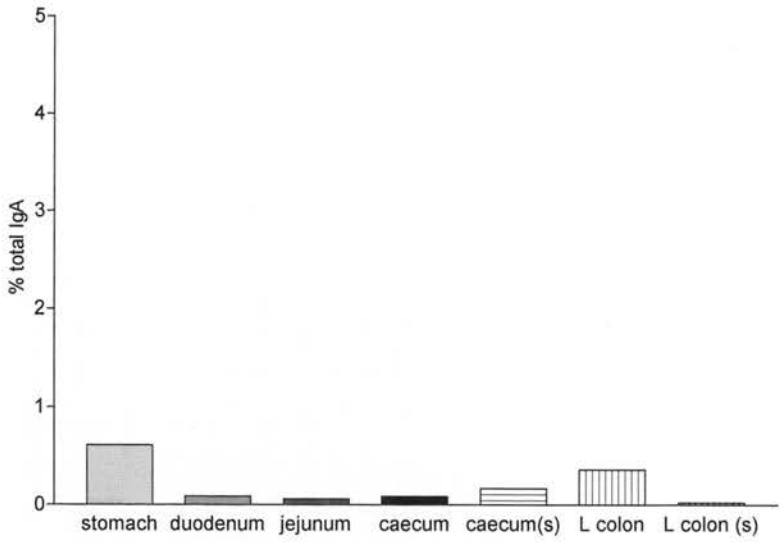

% total IgA
0
1
2
3
4
5
stomach
duodenum
jejunum
caecum
caecum(s)
L colon
L colon (s)

Table 6.3 Total IgA extracted from samples from experimental Horse G: a comparison of the two techniques. Specific IgA as a proportion of total IgA is shown in Fig. 6.8.

Sample	Total IgA µg/ml	BoNT/C IgA	BoNT/D IgA	SA IgA	TetSA IgA
Faeces 1	4.31	1.36	0.42	0.04	0.10
Faeces 2	3.13	1.62	0.61	0.05	0.18
Faeces 3	7.98	0.54	3.11	0.01	0.03
Faeces 4	3.33	0.55	1.46	0.01	0.02
Faeces 5	19.2	0.48	1.34	0.01	0.02
Faeces 6	4.32	0.35	1.34	0.01	0.02
Stomach	12.6	0.48	0.84	0.01	0.08
Duodenum	32.0	0.53	1.35	0.01	0.03
Jejunum	39.6	0.74	1.14	0.01	0.02
Caecum (c)	26.7	0.38	0.93	0.01	0.02
Caecum (s)	23.9	1.34	5.90	0.06	0.17
L colon (c)	7.65	0.40	1.27	0.01	0.03
L. colon (s)	90.6	0.41	0.89	0.01	0.02

c- extraction from contents s- saponin extraction. See Chapter II for details

An initial rise in IgA to BoNT/C is followed by a rise in IgA to BoNT/D. IgA to SA and TetSA remained the same.

It would seem however, that something did affect the specific IgG levels of some of the challenged horses. Horse C for example (Fig. 6.3) demonstrated fluctuations in IgG levels against BoNT/C that appear to correspond to doses of the different soils and was also given oral inulin as was Horse E, whose specific IgG remains unchanged but whose specific IgGT demonstrates large fluctuations against BoNT/C and SA but not against BoNT/D (Fig. 6.4). It is also interesting that IgG to BoNT/C dropped in Horse B after being given soil containing BoNTs A/B/E. Whether the trigger factor was missing, the dose insufficient or if the horses had too high an IgG level, and therefore protection, to the organism (s) is not clear, although it maybe that these animals are now too immunised against these clostridial antigens to be of any further use. Horse G was more interesting in that certain clinical signs were demonstrated but histopathology was incomplete at time of writing.

6.5 *General discussion*

The serology of grass sickness with regards to the toxicoinfection hypothesis is complex. The causative agent is unproven and exposure to the hypothesised organism and / or toxin(s) does not necessarily lead to disease / clinical illness. Rising levels of specific IgG to these antigens are not generally seen in chronically affected animals and there is currently no way of knowing if low levels in AGS cases are due to less exposure than their co-grazing counterparts or due to the disease process. Some researchers have shown a statistical link between low IgG levels to somatic antigens and to BoNT/C (Hunter et al, 2001; McCarthy et al, 2004) indicating naivety, immuno-incompetence or lack of exposure among affected horses. This could also be attributed to the disease process. This was not demonstrated in the horses investigated in Chapter IV where the only difference between specific IgG levels between cases and controls were against SA (Fig. 4.1c). The lower range of specific IgG of the collective cases was also only lower than the collective lower range of the controls against SA although no AGS case had a higher level of specific IgG against any of the antigens than the higher range of the co-grazers. Two animals had higher anti-BoNT/C IgG levels than the median of their respective herds (02 – 376 & 02 – 352) and it seems that among this data set that naivety is not the only factor in disease onset. Little is really understood about the role of IgG subclasses in disease and both these animals have relatively low levels of specific IgGb against SA: IgGb is the most abundant IgG subclass in serum (Sheoran et al, 2000) and thought to be involved in opsonisation (Sheoran et al, 1997).

McCarthy et al (2004) suggested that higher levels of IgG to SA may be protective by prevention of the growth of *C. botulinum* type C and subsequent toxin production but there is no evidence to suggest that this indicates anything other than IgG status against SA possibly just indicates exposure to the organism. The co-grazing controls were the only group studied to demonstrate a significant inverse relationship between anti-SA IgG and anti-BoNT/C IgG. No inverse correlation was observed in twelve months of sampling the healthy adult horses and although it is tempting to attribute low anti-BoNT/C levels to high anti-SA IgG levels, it may just indicate a higher level of exposure.

The work on CGS cases (Chapter V) does suggest that IgG levels to the organism at the start of the disease may influence disease outcome: significant differences were observed in initial specific IgG levels between horses that survived and those that did not. Taking into account the apparent lack of immune response to these antigens in chronic cases either inappropriate antigens are being used in assays or the animal experiences immunosuppression. One way forward, removing the bias of looking for involvement of *C. botulinum* type C/D, especially if inflammatory mechanisms or tissue necrosis might be involved would be to look for cytokine production in cases and controls. This would preferably be in mucosal tissue samples at the site of toxin production.

Fluctuations in longitudinal specific IgG studies had been demonstrated before (Hunter, 2001) but there is no published data on the specific IgG levels of clinically healthy horses. Studies were designed to try and supply data that could be used in comparisons between EGS cases and controls. Such studies are difficult to set up,

relying on goodwill and existing licensing. The pony study (beginning of Chapter III) showed increasing IgG levels across the sampling period but unfortunately that lack of further samples and sample from regular time points restricted the amount of statistical analysis that could be employed. The adult horse study (later in Chapter III) showed that specific IgG may have been affected by changes in management practice (although fluctuations were observed later on in the study that did not coincide with any change in practice) even though the premises were not associated with cases of GS. An anti-TetNT assay was used to show that the ELISA technique and analysis worked – showing classical IgG responses to a parentally administered antigen. This assay was also used in the study of CGS cases and demonstrated that these animals could produce an immune response and that specific IgG levels to this antigen were unaffected by the disease process, indicating that fluctuations to the other antigens used were not due to a non-specific immune response.

Perhaps the best supporting evidence for the involvement of *C. botulinum* type C/D in the aetiology of EGS is the detection of specific IgA in cases versus controls. Despite the problems of experimental techniques (discussed in Chapter IV) there is a clear increase in the amount of specific IgA recovered in the AGS samples than in the controls. Specific IgA is a good indicator of recent exposure and the results from the anti-TetSA (IgA) ELISA suggests that specific IgA is not just a result of soil exposure. It is also shown that some of the co-grazers also possessed a relatively significant amount of specific IgA and it would be expected that most horses in a herd would also be exposed at the same time possibly leading to subclinical disease. A histopathological study of clinically healthy co-grazers might be therefore useful but extremely difficult to set up.

Chapter VII

Investigations into canine and feline dysautonomia

7.1 *Introduction*

Feline dysautonomia (FD: Key-Gaskell syndrome) was first reported in 1982 (Key & Gaskell, 1982) and the disease was seen in practices throughout the UK during the 1980s. Incidence declined in the 1990s with the largest outbreak confined to a colony of experimental cats in 1993 (Nash et al, 1994, Symonds et al,, 1995). In 2001, an outbreak occurred in a colony of pet cats in Glasgow (Cave et al, 2001& 2003, Nunn et al, 2004) and a number have since been investigated. Feline dysautonomia has been reported throughout Europe (Edney et al, 1988) but more rarely in the US where it was mainly confined to cats recently imported from the UK (Canton et al, 1988, Guilford et al, 1988). Incidence in the UK was estimated at 1 in 20,000 to 1 in 50,000 in 2001 although it is thought that subclinical cases may occur (Cave et al, 2003) as the aetiology of at least 43% of cats showing decreased oesophageal motility is not resolved (Moses et al, 2000). The range of non-specific clinical signs, rarity of the disorder and lack of a definitive antemortem diagnosis may lead to the disease being under-diagnosed. No risk factors had been identified in the literature and the disease is reported in house-kept and free-roaming animals.

Canine dysautonomia (CD) was first reported in the UK in 1983 (Roschlitz & Bennet 1983) and has been reported in Norway (Presthus & Bjerkas, 1987; Schulze et al,

1997) although incidence in canines is much lower than in felines in Europe (O'Brien & Johnson 2002). Increasing numbers of cases of CD have been reported in the US since 1988 (Wise & Lappin, 1991; Longshore et al, 1996; Berghaus et al, 2001; Harkin et al, 2002) and several risk factors for the development of the disease have been identified. These include age (a higher susceptibility found in dogs under two years of age), those animals living in a rural environment and those allowed to roam freely and scavenge (Berghaus et al, 2001; Harkin et al, 2002). An association has also been made with climatic conditions, incidence being higher in early spring (Berghaus et al, 2001).

7.1.2 *Clinical signs.*

As with equids, a range of clinical signs can be observed in cats and dogs and there are some similarities and some key differences between EGS and canine and feline dysautonomia. Clinical signs in cats and dogs include those described previously in horses and also include vomiting/regurgitation, prolapsed third eyelids and dysuria and major clinical signs are shown in Table 7.1. A scoring system for FD was proposed by Sharp et al, (1990) based on clinical signs to aid clinicians in diagnosis. Clinical features are divided into groups based on whether they are uncommon except in dysautonomia (Group A-see Table 7.2) or less commonly observed in dysautonomia and/or seen in other conditions (Group B). Seen together, the features would be suggestive of feline dysautonomia. The system also enables grading of cases according to clinical severity.

Jamieson et al, (2002) has proposed a similar scoring system for CD based on clinical signs and histopathological confirmation of 25 cases, the details of which can be seen in Table 7.3.

Table 7.1. Some major clinical signs of canine and feline dysautonomia

Clinical Sign	FD	CD
Dry mucous membranes	+++	+++
Decreased anal sphincter tone	+++	+++
Dilated pupils, poor pupillary light reflexes	+++	++*
Prolapsed third eyelids	+++	+*
Anorexia	+++	++*
Dysuria, urinary incontinence	+/-*	++*
Regurgitation/vomiting	+++	+++
Bradycardia	+	+†
Constipation	+++	+/-
Diarrhoea	+/-	++
Abdominal pain	+	++†
Depression, lethargy	+++	+

+/- present in 10-49% cases + present in 50-59% cases ++present in 60-70% cases +++ present 71-100% *reported more in UK cases than US. † reported less in the UK than in the US.

Adapted from Wise & Lappin 1990; Sharp1990; Cave et al, 2001;

Feline Dysautonomia website:

(http://www.gla.ac.uk/faculties/vet/sah/dysautonomia/)

Table.7.2 Clinical Grading System for Feline Dysautonomia

Clinical Feature		Score
Group A		
1	Dry, crusty nose	2
2	Reduced tear secretion	2
3	Myadriasis or reduced pupillary light response	2
4	Bradycardia (rate <120 beats/min)	2
5	Regurgitation & oesophageal dysfunction*	2
Group B		
6	Constipation	1
7	*Proprioceptive deficits*	1
8	Dry oral mucosa	1
9	Prolapsed membrana nictitans	1
10	Dysuria or bladder atony	1
11	Anal areflexia	1

Total Score	Clinical grade	Clinical Diagnosis
1-4	1	Inconclusive
5-8	2	Probable
9-12	3	Positive
13-16	4	Positive

taken from Sharp *et al* 1990.
*demonstrated using radiography.

Chapter VII

Investigations into canine and feline dysautonomia

7.1 *Introduction*

Feline dysautonomia (FD: Key-Gaskell syndrome) was first reported in 1982 (Key & Gaskell, 1982) and the disease was seen in practices throughout the UK during the 1980s. Incidence declined in the 1990s with the largest outbreak confined to a colony of experimental cats in 1993 (Nash et al, 1994, Symonds et al,, 1995). In 2001, an outbreak occurred in a colony of pet cats in Glasgow (Cave et al, 2001& 2003, Nunn et al, 2004) and a number have since been investigated. Feline dysautonomia has been reported throughout Europe (Edney et al, 1988) but more rarely in the US where it was mainly confined to cats recently imported from the UK (Canton et al, 1988, Guilford et al, 1988). Incidence in the UK was estimated at 1 in 20,000 to 1 in 50,000 in 2001 although it is thought that subclinical cases may occur (Cave et al, 2003) as the aetiology of at least 43% of cats showing decreased oesophageal motility is not resolved (Moses et al, 2000). The range of non-specific clinical signs, rarity of the disorder and lack of a definitive antemortem diagnosis may lead to the disease being under-diagnosed. No risk factors had been identified in the literature and the disease is reported in house-kept and free-roaming animals.

Canine dysautonomia (CD) was first reported in the UK in 1983 (Roschlitz & Bennet 1983) and has been reported in Norway (Presthus & Bjerkas, 1987; Schulze et al,

1997) although incidence in canines is much lower than in felines in Europe (O'Brien & Johnson 2002). Increasing numbers of cases of CD have been reported in the US since 1988 (Wise & Lappin, 1991; Longshore et al, 1996; Berghaus et al, 2001; Harkin et al, 2002) and several risk factors for the development of the disease have been identified. These include age (a higher susceptibility found in dogs under two years of age), those animals living in a rural environment and those allowed to roam freely and scavenge (Berghaus et al, 2001; Harkin et al, 2002). An association has also been made with climatic conditions, incidence being higher in early spring (Berghaus et al, 2001).

7.1.2 *Clinical signs.*

As with equids, a range of clinical signs can be observed in cats and dogs and there are some similarities and some key differences between EGS and canine and feline dysautonomia. Clinical signs in cats and dogs include those described previously in horses and also include vomiting/regurgitation, prolapsed third eyelids and dysuria and major clinical signs are shown in Table 7.1. A scoring system for FD was proposed by Sharp et al, (1990) based on clinical signs to aid clinicians in diagnosis. Clinical features are divided into groups based on whether they are uncommon except in dysautonomia (Group A-see Table 7.2) or less commonly observed in dysautonomia and/or seen in other conditions (Group B). Seen together, the features would be suggestive of feline dysautonomia. The system also enables grading of cases according to clinical severity.

Jamieson et al, (2002) has proposed a similar scoring system for CD based on clinical signs and histopathological confirmation of 25 cases, the details of which can be seen in Table 7.3.

Table 7.1. Some major clinical signs of canine and feline dysautonomia

Clinical Sign	FD	CD
Dry mucous membranes	+++	+++
Decreased anal sphincter tone	+++	+++
Dilated pupils, poor pupillary light reflexes	+++	++*
Prolapsed third eyelids	+++	+*
Anorexia	+++	++*
Dysuria, urinary incontinence	+/-*	++*
Regurgitation/vomiting	+++	+++
Bradycardia	+	+†
Constipation	+++	+/-
Diarrhoea	+/-	++
Abdominal pain	+	++†
Depression, lethargy	+++	+

+/- present in 10-49% cases + present in 50-59% cases ++present in 60-70% cases +++ present 71-100% *reported more in UK cases than US. † reported less in the UK than in the US.

Adapted from Wise & Lappin 1990; Sharp1990; Cave et al, 2001;

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3	Myadriasis or reduced pupillary light response	2
4	Bradycardia (rate <120 beats/min)	2
5	Regurgitation & oesophageal dysfunction*	2
Group B		
6	Constipation	1
7	*Proprioceptive deficits*	1
8	Dry oral mucosa	1
9	Prolapsed membrana nictitans	1
10	Dysuria or bladder atony	1
11	Anal areflexia	1

Total Score	Clinical grade	Clinical Diagnosis
1-4	1	Inconclusive
5-8	2	Probable
9-12	3	Positive
13-16	4	Positive

taken from Sharp *et al* 1990.
*demonstrated using radiography.

Table 7.3 Clinical grading system for canine dysautonomia

Clinical feature	score
Bladder atony or dysuria	3
Dilated pupils or reduced pupillary light reflex	3
Decreased tear production	3
Dry nasal mucous membranes	3
Anal areflexia	3
Regurgitation/retching with megaoesophagus or poor Oesophageal motility*	3
Third eyelid prolapse	2
Intestinal hypomotility	2
Dysphagia	2
Syncope	2
Vomiting	1
Diarrhoea	1
Constipation/faecal tenesmus	1
Abdominal pain	1
Maximum	30

*as demonstrated by radiography

Total Score	Clinical Diagnosis
1-10	Inconclusive
11-20	Probable
21-30	Positive

Fixed pupil dilation is often a feature of feline dysautonomia but is rarely observed in EGS, horses are often tachycardic whilst cats and dogs with the disease are more usually bradycardic.

7.1.3 *Pathology.*

Gross pathological changes in cats or dogs are not consistent and can include mega-oesophagus, signs of aspiration pneumonia, cachexia, a full distended bladder and colonic impaction (Symonds et al, 1995; Longshore et al, 1996; Berghaus et al, 2001; O'Brien & Johnson, 2002).

Histopathological examination is still the only definitive way to diagnose primary dysautonomias. Histologic findings are similar among different species with sometimes drastic reduction in neuronal density. Abnormal-appearing neurons demonstrate nuclear eccentricity and ragged, eosinophilic cytoplasm lacking Nissl substance.

7.2 Materials and Methods

7.2.1 *Samples: Canine Serological Study*

Sera from 41 histopathologically confirmed cases of canine dysautonomia were donated by Dr K. Harkin of Kansas State University, along with 32 control samples from healthy dogs. The control group had an age range of (13-0.5 y, median 7.5 y) and the case group (12.0-0.17y, median 2.0y). The ages of four case animals were unknown.

7.2.2 *Samples: Investigation into an outbreak of FD in a colony of pet cats.*

Of a colony of eight house-kept cats, six were clinically affected. None of the cats had access to the outdoors. The signalment of cases is shown in Table 7.4, clinical signs and outcome are summarised in Table 7.5. The index case (A1) was admitted to Glasgow University Veterinary School five days after the advent of clinical signs. The remaining clinically affected cats (B1, B2, C1) were admitted after 5 -8 days of clinical signs. Two cats died (A1, A2) and one was euthanased *in extremis* (A3). Only two cats (A1 and A3) went to post-mortem and dysautonomia was diagnosed histopathologically. Signs persisted in the surviving cats. B3 and C2 were asymptomatic but fluoroscopy 96 days after the beginning of the outbreak, showed subjective evidence of reduced oesophageal function. Both cases may represent a sub-clinical form of the disease. The cats were fed a single brand of tinned food from a communal bowl and a single brand of dry food from two communal hoppers. The batch of dry food at the time of the outbreak had been in use for 6 days prior to the index case. On average it took 10 days for the cats to consume all dried food contained in the hoppers. Fresh tins of food were opened daily. Feeding practices had not changed within the preceding 12 months.

Seven further serum samples were received from isolated cases of FD after the initial outbreak, these were included in the serological specific IgG analysis. Of these, four were classed as positive based on clinical signs and three classed as probable. Two of the positive cases and one of the probable cases were subsequently confirmed as positive by histopathology.

Blood samples (1ml) from clinical cases were collected in sterile vacutainers with no additives and allowed to clot overnight at 4°C. Serum was collected after centrifuging at 1000 g for 15 min and stored at –20°C until assayed. Food and faecal samples were collected into sterile universal containers and frozen at -70°C until processing.

Table 7.4: Signalment and genetic relationship of cats within the household

Group	Cat	Relationship	Breed	Age (years)	Sex
A	1	Same parents	Birman	5	M (N)
	2	Same litter		5	F (E)
	3			5	F (E)
B	1	Same parents	Birman	9	M (N)
	2	Different litters		14	F (N)
	3	Shared queen with B1 & B2 but different tom & litter		14	F (N)
C	1	Same parents	Colourpoint	11	M (N)
	2	Same litter		11	M (N)

M = male F = female N = neutered E = entire

7.2.3 *Samples: IgA and Toxin Detection Controls*

Eleven adult cats (age >24 months) were used as controls. Cats were from the same geographical area (Glasgow) and had unrestricted access to the outdoors. Cats were

Table 7.5. Major clinical signs and outcome of affected cats

Cat	Clinical sign: Lethargy	Regurgitation/ Vomiting	Appetite	Dysphagia	Constipation	Urinary retention	Bilateral pupil dilatation	Outcome	Histopathological confirmation
A1	+	+	Anorexic	+	+	+	+	Died	+
A2	+	+	Anorexic	+	+	+	+	Died	-
A3	+	+	Anorexic	+	+	+	+	Euthanased	+
B1	+	+	Reduced	-	-	-	+	Survived	-
B2	+	+	Anorexic	-	-	+	+	Survived	-
B3	-	+	Normal	-	-	-	-	Survived	-
C1	-	+	Normal	-	-	-	-	Survived	-
C2	-	-	Normal	-	-	-	-	Survived	-

4 = present X = absent

in good health according to owners (staff from Glasgow Veterinary School). Diet history for these cats is unknown. Fresh faecal samples were collected from litter trays into sterile universals and frozen at -70°C until processing.

7.2.4 *Primary serological study*

Samples were obtained from GUVS Feline Centre where samples are tested for FeLV and T4. Samples for serology from healthy controls were classified into two groups; indoor cats (n = 30) and those having access outdoors (n = 29) and were randomly selected. The mean age (and range) of the indoor and outdoor populations were 16.4 (69) months and 32 (102) months, respectively.

7.2.5 *Serological study; matched case-control.*

Serum samples (n = 116) were obtained as above. Samples were chosen on the basis of information given at the time of sampling. Specifically; age, breed, health status, free-roaming or house-kept, single or multicat household. The age range (years) of the whole group n = 112 (15.0-0.3, median 2.46), of the free-roaming group only n = 64 (14.0-0.67, median 2.96) and of the house-kept cats n = 48 (15.0-0.33, median 2.00). There was no significant difference between the age of the two groups (P = 0.44, Mann-Whitney U test).

7.2.6 *Sample collection and storage.*

Faecal samples (2g) from cases and controls were collected immediately after defaecation in sterile universal containers and frozen at –70°C until processed. Cases were sampled 14 weeks after the onset of clinical signs in the index case.

Ileum and its contents were obtained from Case A3 at necropsy. Briefly, the ileum was occluded proximally and distally using nylon suture, removed, and frozen at –20°C until assayed.

A sample of dry food fed to cases at the start of the outbreak was stored at –70°C.

Unopened cans of tinned food fed to cases were stored at room temperature.

7.2.7 *Sample preparation: Preparation of faeces with protease inhibitors prior to IgA ELISAs.*

All reagents were stored at –20°C and kept on ice during the procedure. Two parts protease inhibitor solution (soybean trypsin inhibitor 1mg/ml in PBS, 50mM EDTA containing 0.05% Tween 20) were added to one part weighed sample (~1g). Phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich, 0.1M in ethanol) was added to a final concentration of 1mM. The mixture was vortex mixed for 30-60 sec and then centrifuged at 3800g for 10min.The supernate was removed and PMSF was added to a final concentration of 1% (v/v). After mixing well it was allowed to stand for 15min on ice. Heat inactivated foetal calf serum (FCS) was added to a final

concentration of 4% (v/v) and centrifuged at 15700g for 5min. The supernatant was removed and stored at –70°C.

7.2.8 *Sample preparation: Preparation of ileal tissue with saponin prior to IgA ELISAs.*

Antibody extraction from ileum was achieved with saponin as described by Berquist (2000). One gram of frozen tissue was aseptically removed and thawed for 8 hours at 4°C in PBS containing 2% saponin (w/v), soybean protease trypsin inhibitor (1mg/ml), EDTA; 0.05M, Tween 20 (0.05%), PMSF (2mM), sodium azide (0.2mg/ml) and 4% FCS (v/v). A 2ml volume was added per gram of tissue. After thawing, samples were agitated using sterile forceps and then vortexed for 30s.

Samples were centrifuged at 15,000g for 5 min and the supernatants collected and stored at –70°C until used.

7.2.9 *Preparation of faeces and food samples prior to ELISA for BoNT/C and bacteriology.*

Non-enrichment: weighed samples (~1g) were added to 10ml of phosphate buffered saline, pH7.2, containing 0.2% gelatin, (PBS-G). Samples were incubated overnight at 4°C.

Enrichment: Samples (~1-2g) were added to 15ml of pre-reduced NZ-CASE-CMB medium (Hunter et al,, 1999) and incubated anaerobically at 30°C for 5 days.

After vortexing thoroughly, samples were centrifuged at 3,800g for 20min. The supernate was collected and stored at –20°C.

7.3 ELISA

7.3.1 *EDTA extraction of* C. botulinum *surface antigens.*

Clostridium cultures (20ml) were prepared in Fastidious Anaerobe Broth (Lab M) and incubated anaerobically at 30°C for 18h. The strain used was a toxin-negative *C. botulinum* type C (NCTC 3732). EDTA extracts were prepared as described by (Poxton 1984) and stored at –20°C. Extract protein concentration was determined as described by Lowry et al, (1951).

7.3.2 *ELISA detection of anti-BoNT/C toxoid IgA and anti-*C. botulinum *surface antigen IgA from faeces and ileal tissue.*

Microwell plates (Nunc, Fisher Scientific, Loughborough, UK) were coated with BoNT/C toxoid (Metabiologicals, Wisconsin, USA) at 5µg/ml or surface antigens at 30µg/ml diluted in coating buffer (0.05M sodium carbonate buffer, pH9.6, 0.02% w/v sodium azide) and incubated overnight at 4°C or at room temperature respectively. The plates were washed four times with ELISA wash buffer (1 x PBS BR14a tablet/l Oxoid, Basingstoke, UK, 15mM/l NaCl, 2mM/l KCL, Tween 20 0.05%) pH7.3. Plates were blocked with PBS containing 3% teleostean gelatin (Sigma, Poole, Dorset: PBS-TG) and 0.02% sodium azide (200µl/well) and

incubated, with shaking, for 4h at 37°C before being washed four times with ELISA wash buffer. They were stored in sealed polythene bags at –20°C until use. Samples were diluted 1 in 4 with PBS-TG before the addition of 100µl/well in quadruplicate. They were incubated overnight at room temperature and then washed four times in ELISA wash buffer.

Rabbit anti-cat IgA (Nordic Immunologicals, Tilberg, Netherlands) was diluted 1 in 400 with PBS-TG and 100µl added to each well. Plates were incubated for 3h at 37°C with shaking. Plates were then washed four times with ELISA wash buffer. Anti-rabbit IgA conjugated to alkaline phosphatase (Sigma) was diluted 1in 2000 with PBS-TG. Plates were incubated for 3h at 37°C with shaking. Plates were then washed four times with ELISA wash buffer.

Alkaline phosphatase substrate tablets (Sigma 104-105 phosphatase tablets, p-nitrophenyl phosphate 5mg) were dissolved in 5ml substrate solvent (0.05 mol/l sodium carbonate solution, pH 9.8, with 1mmol/l magnesium chloride) to give a concentration of 1mg/ml. Plates were then incubated at room temperature for 30min and read with an Anthos 2001 plate reader at 405 nm referenced at 620nm.

7.3.3 *ELISA detection of BoNT/C toxin from cat food, faeces and ileum.*

A polyvalent guinea pig antiserum (CAMR, Porton Down, UK.) raised against purified BoNT/C was used as a capture antibody. Optimal dilutions of the reagents were determined using a checkerboard titration. Microwell plates (Nunc) were coated

(100µl/well) with the antiserum diluted 1 in 10,000 in 0.05M sodium carbonate buffer, pH9.6, 0.02% w/v sodium azide and incubated overnight at 4°C. Plates were washed three times with ELISA wash buffer.

Plates were post-coated (PBS-TG: 200µl/well) for 2h at 37°C in a shallow water bath, before washing three times in ELISA wash buffer.

Samples were added (100µl/well) neat or diluted 1 in 2 and 1 in 4 for direct samples and 1 in 5, 1 in 25 for enriched samples in PBS-TG and were incubated for 3h at 37°C in a shallow water bath before washing 3 times in ELISA wash buffer. The guinea-pig anti-BoNT/C horse radish peroxidase conjugate (CAMR) was diluted 1 in 300 in PBS-TG (100µl/well) and incubated for 3h at 37°C in a shallow water bath. After washing 3 times in PBS-T, substrate solution (3,3',5,5'-tetramethyl-benzidine dihydrochloride tablets (Sigma) dissolved in phosphate citrate buffer pH5.0 with 2µl 30% hydrogen peroxide/10 ml added, 100 µl/ml) was added, 100µl well. The reaction was allowed to develop at room temperature for up to 60 min and was stopped by addition of 50µl of 2M H_2SO_4 to each well.. A standard curve of purified BoNT/C (from CAMR) was run on each plate.

The limits of detection of this assay were 0.8ng/ml and a standard curve of purified neurotoxin of concentrations ranging from 200ng to 0.8 ng were included on each plate.

7.3.4 *ELISA to detect IgG to* Clostridium botulinum *type C toxoid. (primary serological study)*

Coating and blocking of plates

Surface antigens were diluted to 30µg/ml in 0.05M sodium carbonate buffer (0.05M sodium carbonate buffer, pH9.6, 0.02% w/v sodium azide) and the C1 and type D toxoid complex to 5µg/ml and added to Nunc Polysorb plates (100µl/well). After overnight incubation at room temperature and 4°C respectively, plates were washed four times in ELISA wash buffer and the toxin plates blocked for 90 min with PBS-TG (200µl/well) at 37°C. After washing four times plates were stored at –20°C in sealed plastic bags until used.

Plates were blocked with 200µl of PBS-ST (phosphate buffered saline, 5% foetal bovine serum, 0.1% Tween 20, 0.02% sodium azide) for 2 hours, with shaking, at 37°C.

Serum samples were diluted 1/50 in PBS-ST and 100µl were added in duplicate for each sample. PBS-ST only was added to blanks and to negative controls. Plates were then incubated for 90 mins at 37°C with shaking.

Detecting antibody, rabbit anti-cat IgG (Abcam) was diluted 1/2000 in PBS-ST and 100ul added to all wells. Incubation was for 90mins at 37°C.

Anti-rabbit IgG conjugated to alkaline phosphatase was diluted 1/2000nin PBS-ST and 100ul added to all wells. Incubation was for 90mins at 37°C.

Substrate was used as previously mentioned.

ODs were calculated by combining the OD from the blank wells to that of the antigen control wells and subtracting this from the average value of the duplicated sample wells.

After initial assays, a sample was identified that gave an OD of 1.0 and this was used to normalise all further assays.

7.3.5 *ELISA for the Detection of Specific IgG*

7.3.5.1 *Standard curve of canine and feline IgG*

Standard curves of IgG were constructed using a Bethyl Laboratories Feline and Canine IgG Quantication kits. The reference serum was diluted according to their instructions in PBS containing 3% teleostean gelatin (PBS-TG).Serum was added in duplicate wells (100 µl/well). All incubations were carried out at 37°C for 90min and washed six times between each stage with ELISA wash buffer (1 x PBS BR14a tablet/l Oxoid, Basingstoke, UK, 15mM/l NaCl, 2mM/l KCL, Tween 20 0.05%) pH7.3. Conjugate was diluted to 1/10000 and 100 µl added to each well before incubation and subsequent washing. Colour development was with TMB added to phosphate-citrate buffer (100 µl/well) and the colour allowed to develop for 30 min. Development was stopped by addition of 100 µl/well of 2M H_2SO_4. Plates were read on an Anthos 2001 plate reader at 450nm, referenced at 620nm.

7.3.5.2 *ELISA for the detection of total specific IgG and specific IgG*

IgG assays were all carried out using HRP conjugates from Bethyl Laboratories i.e. goat anti-cat IgG using the same reagents and incubation conditions as used for the generation of standard curves. Optimum dilutions of sample and conjugate were established using checkerboard titrations and dog and cat sera was diluted 1/500 and 1/200 respectively and respective conjugates at 1/10,000. Each sample was assayed in duplicate on two separate occasions. Positive controls are not available and initial assays were used to identify suitable controls (median responders). These controls were run on each plate and readings used to normalize intraplate variation, all samples were run on the same day.

Controls and analysis

Blank wells, consisting of coating buffer were included on each plate. Negative controls, consisting of antigen diluent in place of sample, were included on the plates to control for nonspecific binding of antibody to the antigen. All statistical analysis was performed on Sigmaplot or on Prism V3.03.

All univariable and multivariable logistic regression analyses was carried out by Tom Cave, formerly of GUVS.

7.4. *Bacteriological isolation of organisms from faeces and food.*

Tenfold dilutions (10^{-1} to 10^{-4}) were made from the non-enriched and enriched samples in pre-reduced nutrient broth at 24 and 48h. These were plated onto Fastidious Anaerobe Agar (FAA, Lab M, Bury, UK) containing 5% egg yolk

emulsion (Oxoid) and 10μg/ml gentamicin. Plates were incubated for up to 5 days and examined by eye for lecithinase and lipase activity. Likely colonies were re-streaked for purity and also incubated aerobically on Columbia blood agar (Oxoid CBA base with 5 % defibrinated horse blood) to check for aerobic growth. Colonies were also examined by Gram stain and phase contrast microscopy.

7.5 *Detection of toxin genes by PCR in clinical samples, and cat food.*

7.5.1 *DNA Extraction*

1g samples of faeces/ileal contents or of cat food were incubated anaerobically at 30°C in 15ml of pre-reduced NZ-CASE/CMB media. After 3 days, 1.5ml of the enrichment was transferred to a sterile eppendorf and the DNA extraction carried out using a Nucleospin Tissue Kit following the manufacturer's instructions.
Known toxigenic strains were used as positive controls.
DNA was diluted 1/5, 1/10 and 1/100 in molecular grade water (Sigma) and kept at –20°C until use.

7.5.2 *Primer sets*

Primer sets are described in Table 7.6. PCR for toxins C1 consisted of a nested PCR reaction. PCR for all the other toxins consisted of a single PCR reaction.

Table 7.6 Primer sets are described in Table. PCR for toxins C1, D and novyi consisted of a nested PCR reaction. PCR for the C2 toxin consisted of a single PCR reaction.

Gene Sequences for Toxin Primers

	Upstream		Downstream	
Toxin type	Name	Sequence (5'-3')	Name	Sequence (5'-3')
C1*	CS-11	AAACCTCCTCGAGTTACAAGCCC	CS-22	CTAGACAAGGTAACAACTGGGTTA
C2†	C2C1F†	AAGGAAGATAAAACAAAAAT	C2C1R	CCTAATGATACAAATGAAAA
C2††	C2C2F	GCAGAAGTTTCAGGTAGTTTACAAC	C2C2R	CGCATTCTATAACGACCTTCTGGA
D**	DS-11	GTGATCCTGTTAATGACAATG	DS-22	TCCTTGCAATGTAAGGGATGC
α	NovAF	GGTGCGATTCAAGAGGCCACA	NovAR	CGCTCCTAGCAGTCCCGAAAT

Gene Sequences for Toxin Primers (Nested)

	Upstream		Downstream	
Toxin type	Name	Sequence (5'-3')	Name	Sequence (5'-3')
C1*	NTCS-11	GAAAATCTACCCTCTCCTACATCA	NTCS-22	AATAAGGTCTATAGTTGGACCTCC

* Williamson et al (1999) ** Takeshi et al, (1996)

† Fujii et al, (1996) †† Kimura et al (1998)

7.5.3 *Cycling conditions*

Cycling conditions for primer Sets; DS11/DS22, CS11/CS22

Number of cycles	Temperature	Time
1	80°C	5min
30	95 °C	1min
	55 °C	1min
	72 °C	2min
1	72 °C	10min

Conditions for type C nested primers remained the same except annealing temperatures were altered to 52°C and 54°C respectively.

Cycling conditions for primer Set; C2C1F/C2C1R and C2C2F,C2C2R

Number of cycles	Temperature	Time
1	94 °C	3 min
35	94 °C	45sec
	55 °C	2min
	72 °C	1min
1	72 °C	5min

Cycling conditions for primer Set; NovAF/NovAR

Number of cycles	Temperature	Time
1	94 °C	3mins
30	95 °C	1min
	48 °C	1min
	72 °C	1min
1	72 °C	5mins

Conditions for *C. novyi* type A nested primers remained the same except annealing temperature was altered to 55°C.

7.5.4 *PCR reaction mixtures*

A 100µl reaction was used consisting of 5µl of template DNA, 10µl PCR buffer (10mM), 3µl magnesium chloride ($MgCl_2$;50mM), deoxynucleotide triphosphate (1.6µl dNTP;12.5mM), 2µl of each primer (5µM), 2.5 Units (U) *Taq* polymerase and 71.6µl molecular grade water. Negative controls consisted of above mixture but contained no template DNA and 76.6µl water.

A master mix of these reagents was prepared and aliquoted into capped thermowell PCR tubes and reactions were carried out in a programmable thermocycler (Genius, Techne) PCR machine.

7.5.5 *PCR product visualisation*

3µl of PCR product was added to 2µl molecular grade water and 2µl loading buffer and mixed. The solution was run on a 1% agarose gel containing 1µl/ml ethidium bromide. Bands were compared to a 100bp ladder and gels were photographed using an imager.

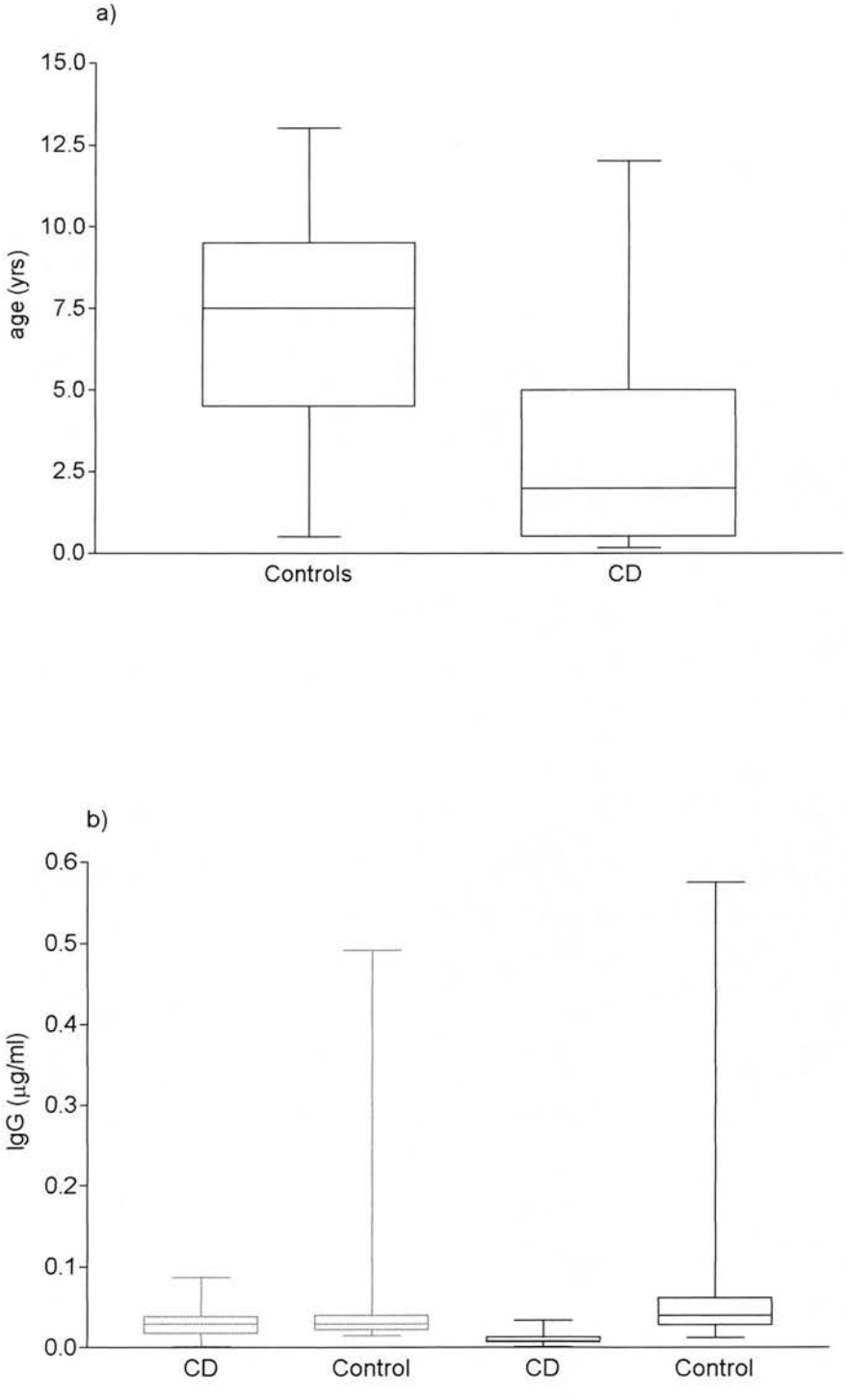
7.6 Results

7.6.1 *Canine Serological Study*

Results of this study are shown in Fig.7.1 The ages and antibody levels of the control dogs were not normally (Gaussian) distributed even after transformation, consequently, non-parametric tests (Mann-Whitney U tests and Spearmen's correlation) were used on these samples.

Specific IgG against BoNT/C toxoid was not significantly different between the two groups (P = 0.42) although a significant difference was demonstrated between specific IgG against surface antigens (P = <0.0001) (as shown in Fig. 7.1). The ages of the two groups was also significantly different, with the control dogs being significantly older (P = <0.0001). No correlation between age and antibody acquisition was demonstrated in the control dogs to either antigen, however, a correlation between antibody titre against surface antigen with age was seen when the groups were combined (n = 69, Spearman's R = 0.3467, P= 0.0035). No correlation was observed between anti-BoNT/C titre and anti-SA titre in controls (n = 32, Spearman's r = 0.25, P = 0.17), cases (n = 41, Spearman's R = 0.07, P = 0.68) or when groups were combined (n = 73, Spearman's r = 0.14, P = 0.24)

Fig.7.1



Graphs showing a) age differences between controls and cases of CD b) between specific IgG levels of controls and CD cases. Red plots depict anti-BoNT/C IgG levels and black plots depict anti-SA IgG levels.

7.7 *Investigation into feline dysautonomia outbreak*

7.7.1 *ELISA to detect IgA antibodies to BoNT/C toxoid and C. botulinum surface antigens from faeces and ileal tissue.*

Specific IgA antibody to BoNT/C and surface antigens was detected mainly in affected cats and a significant difference was observed in specific IgA levels in healthy controls and Key-Gaskell Syndrome (FD) cats. The lowest value seen in affected cats (Fig. 7.2 a & b) is from the ileum of case A3; this sample was taken during the outbreak (week 3) and not 14 weeks afterwards like the others.

The ELISA OD value for faecal IgA antibody BoNT/C toxoid IgA from free-roaming control cats (median 0.23, range 0.6 – 0.03) was significantly lower than that for surviving cases (median 1.5, range 1.7 – 1.3) ($p<0.001$). This analysis excludes the ileum sample from A3 this analysis as the time of sampling was much sooner. If included in the final analysis results are still significant (median 1.45, range 1.7-0.7) ($p<0.001$). Similarly, IgA against EDTA extracted surface antigens was much lower in controls (median 0.23 (range 0.85-0.06) than in affected, surviving cats (median 0.92 (range 1.35- 0.81) ($p<0.001$). Again, if the ileal sample from A3 is included, results are still significant (median 0.865, range 1.35-0.34) ($p< 0.05$).

7.7.2 *Bacteriological isolation of organisms from faeces and food.*

Microbiologically, faeces samples did not yield any colonies with morphology similar to *C. botulinum* Group III organisms and there was no growth anaerobically from the tinned cat food. Lecithinase and lipase positive colonies were observed on FAA agar with gram

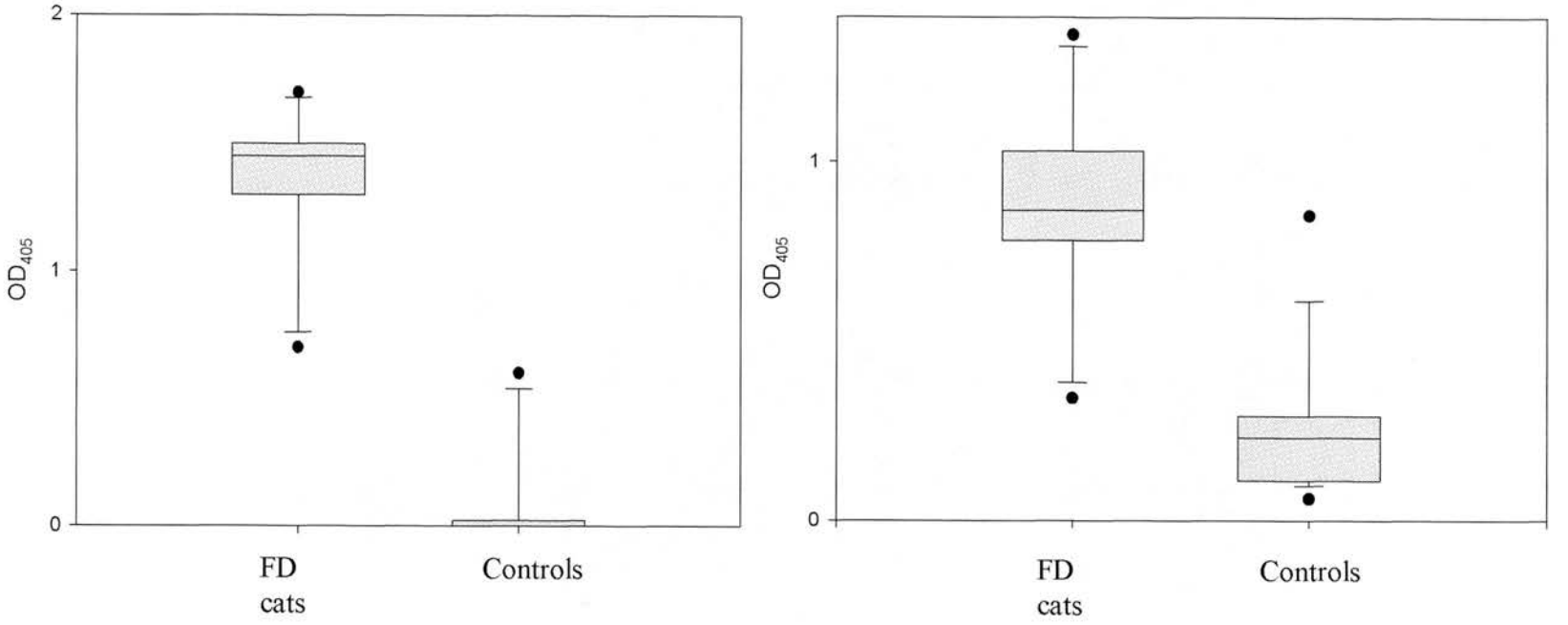


Fig.7.2. Boxplots showing IgA against a) BoNT/C toxoid complex b) against EDTA extracted surface antigens of a toxin negative *C. botulinum* type C strain. The affected cats were sampled fourteen weeks after the onset of clinical signs (all were faecal extracts with the exception of the lowest value which was from ileal contents from a necropsied animal in week three of the outbreak). The control group (n = 11) consisted of samples from healthy, free-roaming cats. The affected group of cats had significantly higher levels of faecal IgA than the control group (P = <0.001) in both assays.

positive rods visible under microscope but attempts to subculture the organism into pure culture were unsuccessful despite several attempts.

7.7.3 *PCR detection of botulinum neurotoxins from faeces and food.*

No faecal sample was positive for any of the PCR reactions carried out from clinical samples. The dried cat food did show a weak band, positive for BoNT/D but this was not repeatable either from this, or subsequent enrichments.

7.7.4 *ELISA detection for BoNT/C toxin from cat food, faeces and ileum.*

Table 7.6 summarises these results and shows amounts of toxin detected using the direct detection method. Toxin was detected in faeces via direct detection in samples from three of the six clinical cases. Toxin was also found in faeces via direct detection in samples from one of the two sub-clinical cases (C1). The index case (A1) had the highest faecal toxin concentration. Toxin was not detected in control faeces or from the cat food using this method.

Following enrichment, toxin was detected in faeces in all samples from clinical cases with the exception of B3. Toxin remained undetectable in control faeces. Toxin was detected in dry cat food but not in tinned cat food.

Table 7.6. Toxin detection in food and faeces of affected cats.

Case	Direct (ng/g)	Enriched
A1	15.6	+
A2	ND	+
A3	2.1	+
B1	ND	+
B2	0.8	+
B3	ND	ND
C1	ND	+
C2	1.6	+
Dried food	ND	+
Tinned food	ND	ND

ND = none detected, + = Present.

7.8 *Feline serological studies*

7.8.1 *Initial serological study*

Using one-way ANOVA (Kruskal-Wallis test, followed by a Dunn's post-test to compare groups) to compare the mean ages of the three FD cats were found to be significantly older than both the indoor-kept and free-roaming controls ($P = <0.01$, $P = 0.001$, respectively). Free-roaming controls were not significantly older than the indoor control group ($P = >0.05$). A comparison of specific IgG levels can be seen in Fig. 7.3.

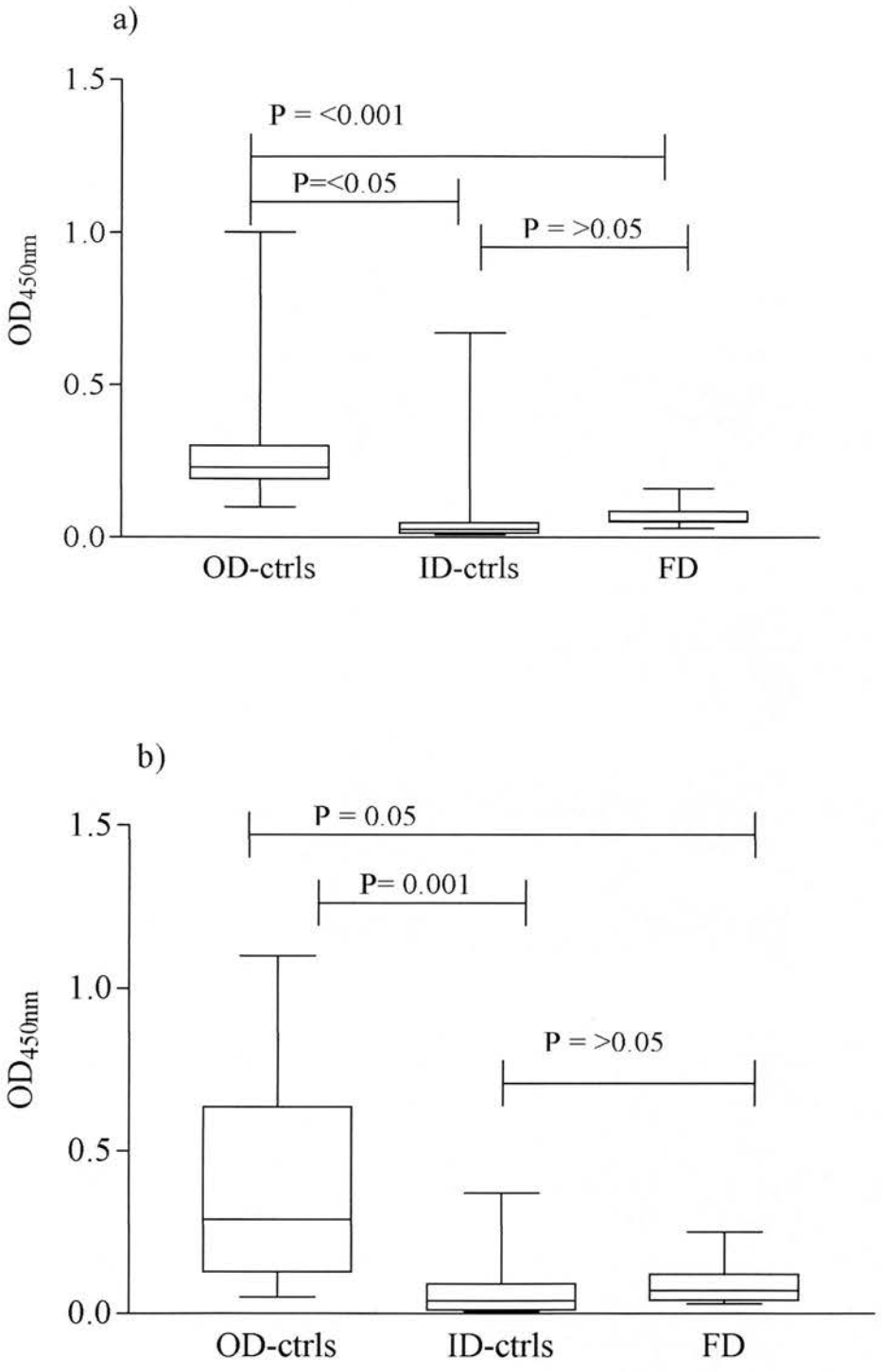


Fig. 7.3. Median comparisons of serum IgG of specific antigen of outdoor (OD) and indoor (ID) control groups and cases against a) BoNT/C toxoid and b) EDTA extracted surface antigens.

Using the same test, free-roaming controls had a significantly higher IgG titre against BoNT/C toxoid than both indoor-kept and FD cats (P = <0.0001, P = <0.05 respectively). Indoor-kept cats did not have a significantly lower IgG titre than FD cats (P = >0.05). There was no significant difference between anti-BoNT/C toxoid IgG of indoor controls and FD cats (P<0.005).

Free-roaming controls had a significantly higher IgG level against SA than both indoor-kept controls and FD cats (P = <0.001, P = 0.05). There was no significant difference between indoor-kept cats and FD cats (P = >0.05).

Correlations between age and antibody levels and between antibody levels specific for different antigens were calculated using Prism software. The program uses Pearson's calculation unless the sample distribution is not normally distributed (which it tests automatically) whereby Spearman's rank correlation is performed. Results of the correlations are shown in Table 7.7. Confidence intervals were set at 95%.

7.8.2 *Matched case-control study*

Specific IgG detection in cases versus matched-controls

Descriptive statistics for the control groups and case group are outlined in Table 7.8. No significant differences between specific IgG levels of Indoor-kept and Free-roaming controls were demonstrated (Fig.7.4). Between indoor and free-roaming control groups, the only difference approaching statistical significance was between IgG levels against BoNT/C (P = 0.08, MW). When comparing indoor and outdoor controls groups against the FD group, the only significant differences observed

Table 7.7. Correlation between age and antibody levels.

Group	antigen	test	
Free-roaming controls	BoNT/C toxoid	Pearson's	P = 0.003 r^2 = 0.28 CI= 0.201-0.75
	SA	Pearson's	P = 0.620 r^2 = 0.009 CI= -0.279- - 0.448
Indoor-kept Controls	BoNT/C toxoid	Spearman's	P = 0.5 R = 0.13 CI = -0.25- -0.475
	SA	Spearman's	P = 0.246 r^2 = 0.19 CI = -0.137- - 0.478
Combined controls	BoNT/C toxoid	Spearman's	P = <0.0001 r^2 = 0.38 CI = 0.283-0.681
	SA	Spearman's	P = 0.003 r^2 = 0.384 CI = 0.135-0.588

Table 7.7 cont'd. Correlation between antibody titres to different antigens (anti-BoNT/C versus anti-SA)

Group	test	
Free-roaming Controls	Pearson's	P = 0.04 r^2 = 0.144 CI = 0.014- 0.655
Indoor-kept Controls	Spearman's	P = <0.0001 r^2 = 0.893 CI = 0.782-0.948
Combined controls	Spearman's	P = <0.0001 r^2 = 0.821 CI = 0.711 – 0.892

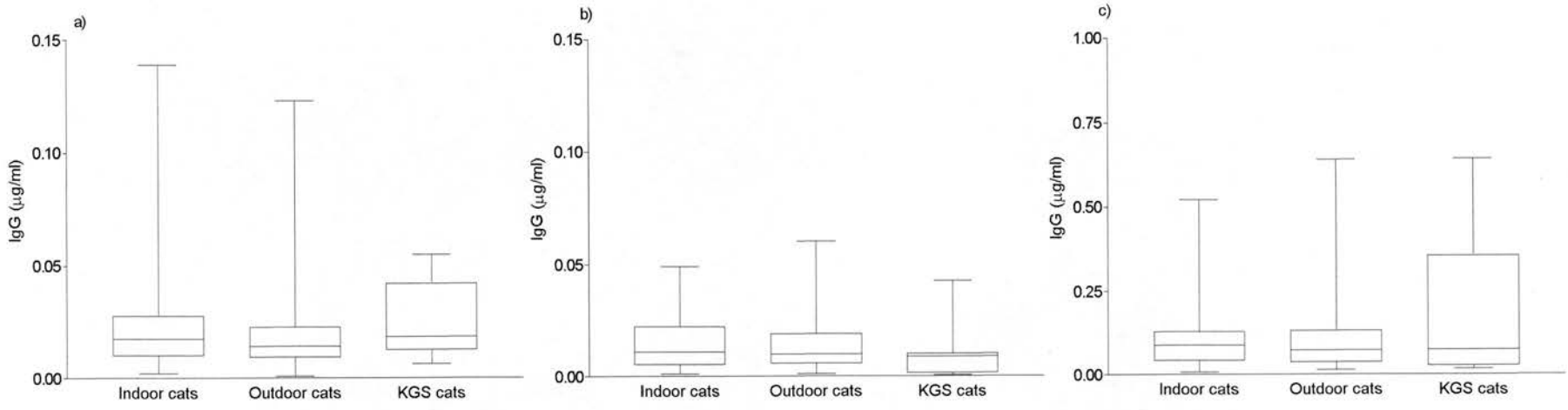


Fig. 7.4. Boxplots demonstrating levels of specific antibody against a) BoNT/C b) BoNT/D c) SA

were between IgG levels against BoNT/D; P = 0.01 and P = 0.04 versus indoor-kept and free-roaming controls respectively.

Table 7.8. Median and range of specific IgG levels in groups of cats ug/ml serum.

Group	BoNT/C	BoNT/D	SA
Combined controls	0.015 0.139-0.009	0.049 0.049 -0.001	0.085 0.640-0.008
Free-roaming controls	0.014 0.123-0.010	0.010 0.061 -0.001	0.074 0.640-0.014
Indoor-kept controls	0.017 0.139-0.002	0.011 0.049-0.001	0.088 0.521-0.008
In-contact controls	0.050 0.150-0.014	0.020 0.040-0.010	0.390 0.644-0.3581
FD group (incl. In-contacts)	0.015 0.150-0.007	0.002 0.043-0.001	0.043 0.644-0.0185

Using the Kruskal-Wallis and Dunn's post-test, no significant difference was demonstrated between the groups for any antigen (Fig.7.5).

When comparing control groups with FD cats, four animals are excluded from the next analysis (cases B2, B3, C1 and C2). They are excluded on the basis of scoring by clinical signs based on Sharp et al (1990) whereby they scored 4, 0, 2 & 1 respectively, leading to inconclusive/negative diagnosis. By excluding them from the next analysis a suitable number of 'in-contact' controls can be examined.

Kruskal-Wallis tests demonstrated that control cats had significantly higher levels of specific IgG than cases against BoNT/D (P = <0.01) but not BoNT/C (P = >0.05 in both cases). In contact animals demonstrated a significantly higher specific titre than controls against BoNT/C and SA but not BoNT/D (P =< 0.05,

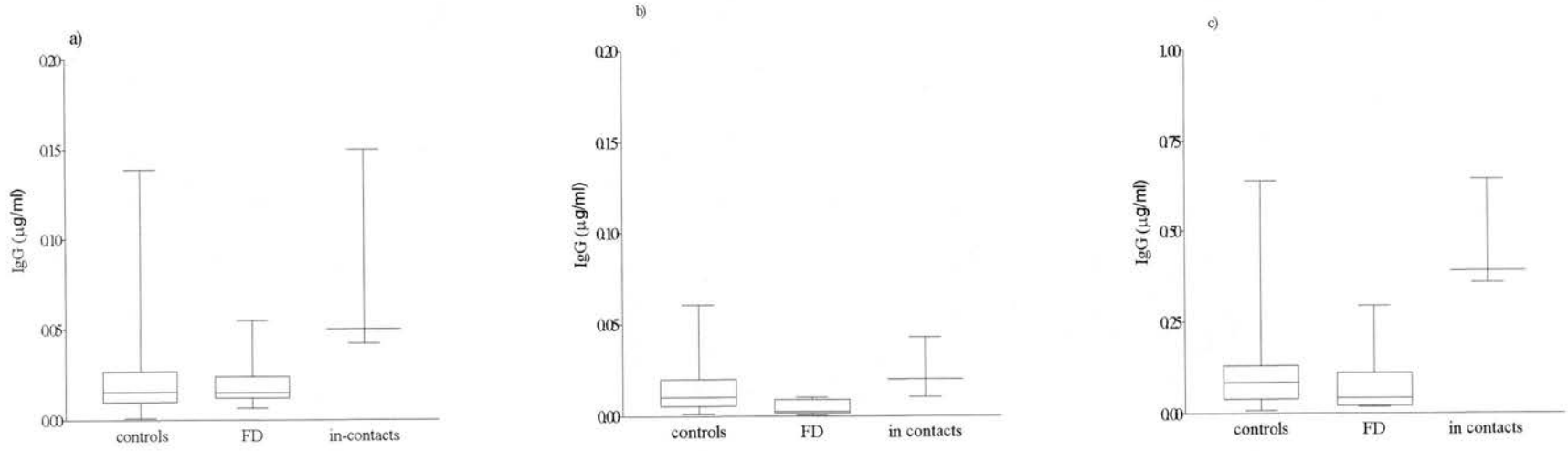


Fig. 7.5 Comparison of specific IgG levels between combined controls, FD cases and in-contact cats against a) BoNT/C, b) BoNT/D and c) surface antigens. Control cats demonstrated significantly higher levels of specific IgG than cases against BoNT/D (P = <0.01) but not BoNT/C (P = >0.05 in both cases). In contact animals demonstrated a significantly higher specific titre than controls against BoNT/C and SA but not BoNT/D. (P =< 0.05, P = < 0.05, P = <0.01 respectively.). In-contact animals possessed significantly higher IgG titres than cases against all three antigens (P =< 0.05, P = <0.01 and P =< 0.01).

P = < 0.05, P = <0.01 respectively). In-contact animals possessed significantly higher IgG levels than cases against all three antigens (P =< 0.05, P = <0.01 and P =< 0.01).

7.8.3 *Multivariable modelling of canine and feline dysautonomia.*

Canine

Univariable logistic regression (Tom Cave, Vale Vets) was used to identify predictor variables for CD status and low levels of antibody levels to surface antigens and young age were both strongly correlated with a positive CD status. Multivariable logistic regression was then used to control for the effect of age and SA antibody titre to look for an association between CD status and low BoNT/C antibody titre. A low antibody titre to BoNT/C was not associated with CD status in this data group. When linear regression was employed to look for predictors for BoNT/C levels and a positive correlation was seen between this and SA antibody levels. Therefore, levels against SA and BoNT/C are correlated and a low SA antibody titre is associated with CD. However, in this data set, there is no association between CD status and anti-BoNT/C titre that cannot be explained by them both being associated with young age.

Feline

The effect of different variables (FD status, age, breed, sex, sick versus healthy cats, free-roaming versus indoor and single versus multicat household) upon serological levels for BoNT/C, BoNT/D and SA were explored using Student's t-tests and linear regression. Continuous variables were transformed to fit a normal distribution and

multivariable linear regression was then used to control for confounding variables (model entry p = <0.25; model retention p = < 0.05).

Anti-SA IgG

Multivariable modelling demonstrated a significant association with increasing age and pedigree versus domestic cats.

Anti-BoNT/C IgG

Multivariable modelling demonstrated a significant association with increasing age, healthy versus sick and male versus female cats.

Anti-BoNT/D IgG

Multivariable modelling demonstrated a significant association only with FD status- anti BoNT/D IgG titre was significantly lower in cats with a diagnosis of FD than in control cats (geometric mean 3.70×10^3 versus 10.8×10^3; p = 0.006).

Based on this model, low systemic immunity to BoNT/D is associated with FD.

7.8.4 *Longitudinal sampling of affected cats.*

Graphs showing changes in specific IgG are shown in Fig.7.6

Antibodies against BoNT/C toxoid

All cases had an antibody titre above the median value of the combined control groups with the exception of the most acutely affected cases, A1 and A2. The second

least clinically affected animal (C2-clinical score of 1) had the highest antibody titre of the group when first sampled at Day 14 of the outbreak. Over the first 7 days of the outbreak, marked increases in titre were observed in case B1 and a marked decrease in C1.

Antibodies against BoNT/D toxoid

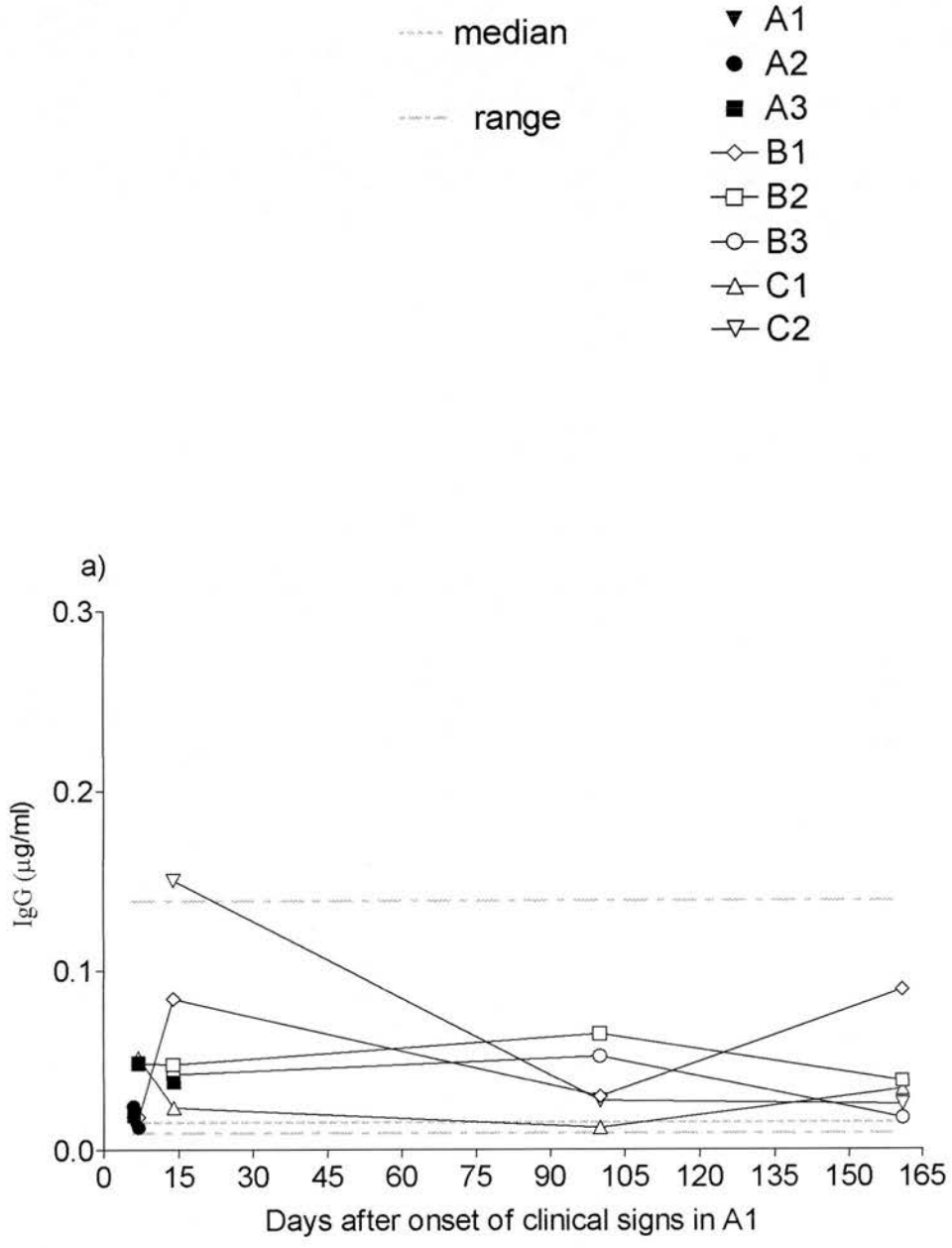
All cases assayed during the second week of the outbreak had levels below that of the median of the combined control groups with the exception of C1 (clinical score 2) which had a comparable titre to the highest range of the control group. All surviving cases had a titre above that of the median at day 14 of the outbreak. B1 showed a massive increase in titre when sampled at Day 163 of the outbreak and this animal was still demonstrating clinical signs such as depression and regurgitation.

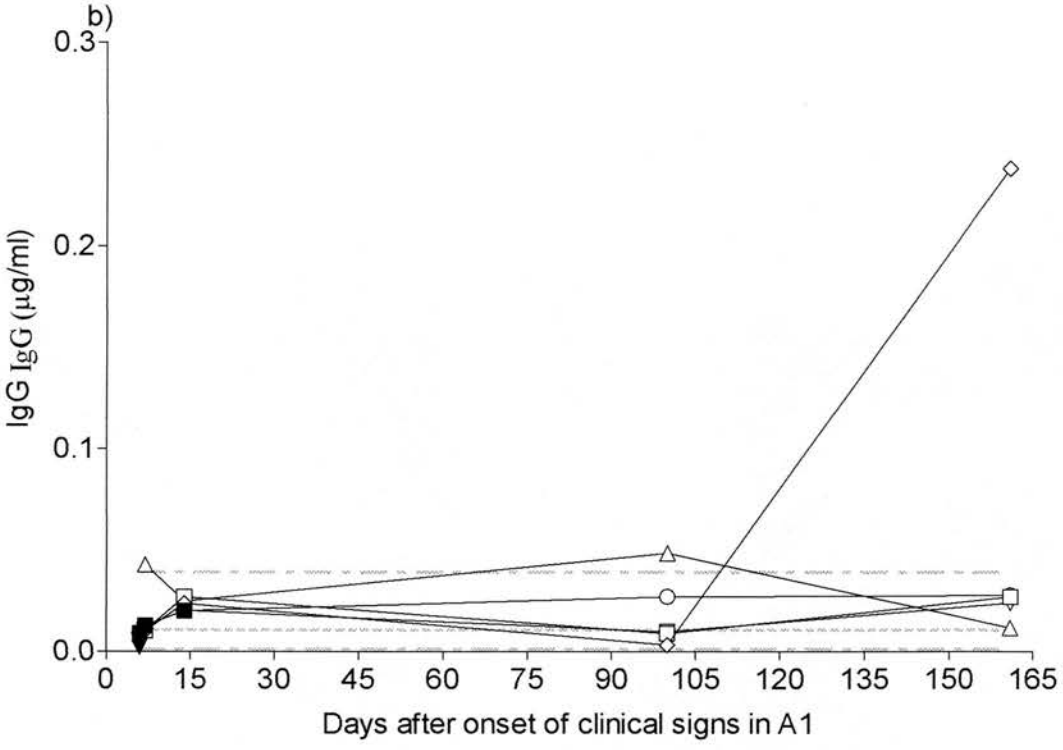
Antibodies against EDTA extracted surface antigens.

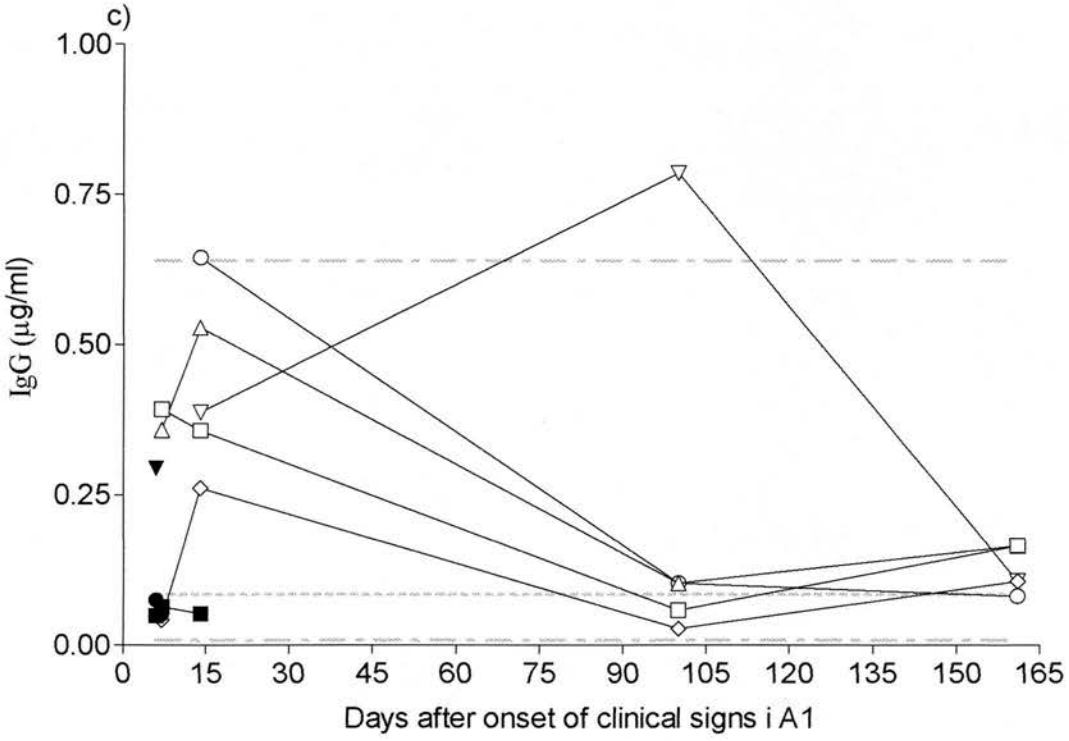
Index case, A1, demonstrated an initial titre above the median of the combined control groups although A1 and A2 were below the median. B1 and C1 showed and increase in titre between weeks 1 and 2 although B2 decreased. C2 had the highest titre against surface antigens of any cat sampled.

Fig.7.6 Longitudinal sampling of specific IgG against

a) BoNT/C toxoid, b) BoNT/D toxoid c) EDTA extracted surface antigens for 8 FD affected cats. Median and ranges were calculated by combining the indoor-kept and outdoor control groups.




b)
0.3
0.2
0.1
0.0
IgG IgG (µg/ml)
0
15
30
45
60
75
90
105
120
135
150
165
Days after onset of clinical signs in A1


c)
1.00
0.75
0.50
0.25
0.00
IgG (µg/ml)
0
15
30
45
60
75
90
105
120
135
150
165
Days after onset of clinical signs i A1

7.9 *Discussion*

There is no record in published literature of potential causes of feline and canine dysautonomias being investigated. Incidence of the disease is extremely rare in both species and there are problems obtaining longitudinal serum samples due to the general acuteness of the disease in these species and the difficulty in obtaining serum from such ill animals. There is also difficulty in persuading owners to let their animals go to postmortem and obtaining samples from suitable controls.

Samples from canine cases were all histopathologically confirmed and were samples from a previously published study (Harkin et al, 2002). It was unfortunate that the control group was unsatisfactory due to age and further control samples from the same area would be useful. A suitable control group would require samples from clinically healthy animals below two years of age, preferably also lifestyle matched and would probably thus require licensing and a considerable time commitment to involved parties. Given that the control group did not have a significant higher titre against BoNT/C toxoid than the case group, despite being significantly older may indicate that the toxin is rarely encountered. No correlation was demonstrated between anti-BoNT/C and anti-SA levels in any combination of groups again suggesting that the toxin may be rarely encountered and that anti-SA levels could be a result of cross-reactivity between *C. novyi* type A surface antigens and other group III clostridial antigens. It is reasonable to suggest also, that in a healthy gut, antibody to surface antigens may be protective as the organisms do not get a chance to proliferate and produce toxin. There is evidence of dietary change increasing the

number of clostridia in the canine gut as in cats (Sparkes et al, 1998; Baillon et al, 2004) but there is no record of recent dietary change with this group of dogs. Comparatively few bacteriological studies of the canine and feline GI tract are published. Most studies focus on the changes caused by the addition of fructo-oligosaccharides (Sparkes et al, 1998) or probiotics (Baillon, et al, 2004). Clostridia are not identified to the species level so it is unknown whether any Group III clostridia are found in the GI tract of healthy animals. However, dogs are susceptible to clostridial overgrowth (Baillon et al, 2004) so environments in the canine gut may be able to support *C. botulinum* type C/D at certain times. Greetham et al, (2002) used a cultural and genotypic approach to examine bacterial populations in healthy, adult dogs although it is not clear whether these animals were laboratory animals and only faecal samples were collected. No Group III clostridia were identified in this study. Papasouliotis et al (1998) found that healthy cats carried higher numbers of strict anaerobes than humans, with clostridial species the most frequently isolated. Both Greetham et al, (2002) and Baillon et al, (2004) reported substantial differences in the numbers of clostridia detected using molecular versus traditional culture methods.

Evidence suggests that canine dysautonomia may be linked with scavenging behaviour in dogs in the US. There is no record of dysautonomia being investigated or found in wildlife as here in the UK (Whitwell, 1991) and the cases of grass sickness that have been reported are mainly restricted to horses recently imported from the UK. However, *C. botulinum* type C has been implicated in numerous cases of forage poisoning in various species including horses (Schoenbaum et al, 2000)

cattle (Galey et al, 2000) and in wild, bighorn sheep (Swift et al, 2000). Harkin et al, (2002) reported an outbreak of canine dysautonomia in a litter of 5-week-old puppies whose dam had been euthanased for suspected dysautonomia. The dam was two years old and the only livestock she had had access to after giving birth were pheasants. The puppies had no contact with any other animals other than their dam or the owner. Two other dogs on the premises, one sibling to the dam and an older dog, were unaffected. Unfortunately, no bacteriology was carried out during this outbreak and one can only conjecture as to whether the dam was shedding spores or whether toxin was contained in her milk. One puppy though, remained clinically healthy. Testing ileal or other intestinal samples for the presence of botulinum neurotoxins or neurotoxin genes would provide further evidence of the involvement of *C. botulinum* type C/D in canine dysautonomia. However, the rarity of the disease in the UK would make such a study impracticable.

The investigation into the outbreak of FD in the group of pet cats led to much circumstantial evidence that lends support to the toxicoinfection hypothesis. All but one animal (the least clinically affected) had detectable levels of BoNT/C via antigen capture ELISA. It is possible that this cat would have been positive for the toxin if the mouse bioassay had been available as it is ~100 times more sensitive than the ELISA. This cat was positive for specific IgA, as were all the outbreak cats, which indicates recent exposure.

Detection of toxin and isolation of the organism itself is extremely difficult:

C. botulinum is highly fastidious and the toxin is unstably encoded on a bacteriophage which is easily lost upon subculture. Presence of toxin after enrichment does not definitively mean that toxin was present *in vivo* and absence of toxin does not mean the organism was not there. Indicative colonies were observed from an enrichment of the dried cat food but it proved impossible to isolate them. Correspondence with the cat food manufacturer confirmed that the cat food is heated to only 80^0C which would allow the survival of clostridial spores. Unfortunately the batch number of the particular bag was not recorded and it is impossible to say whether the bag was contaminated before, during or after manufacture.

Taq polymerase can be inhibited by polysaccharides and various other materials found in faecal and other enrichment cultures. Adequate DNA cleanup has been a major hurdle in the use of molecular techniques to investigate dysautonomias. Since this work was carried out, advances have been made in this area by other members of the research group. DNA extraction now uses a combination of CTAB (to bind polysaccharides) and a bead beater to disrupt spores and Gram-positive cells.

These results demonstrate similar evidence of an association between feline dysautonomia and a toxico-infection with *C. botulinum* type C as has been reported in equine grass sickness. Hunter and others (1999) detected toxin in healthy controls leading to the hypothesis that *C. botulinum* type C/D may be present in the GI tract of healthy horses. In cats, toxin was only detected in samples from affected cats, none was detected in healthy controls. It is interesting to note that the samples containing the highest concentration of toxin were from the three cats most acutely

affected (A1, A2 and A3) and the one sample that was negative using both protocols came from the least affected cat (B3). Toxin has been detected before in high percentages of dysautonomic cats (Hunter, 2001). Given the very different diets and GI physiology of equines and felines it is possible that indoor-kept cats are not normally exposed to *C. botulinum* type C/D via canned food. The dried food was positive for toxin after enrichment. It may be that cereal in dried food harbours *C. botulinum* (and other clostridia) and provides an environment in the gut that permits their proliferation.

Local mucosal immunity may be important in protection against the disease, especially in free-roaming cats that may be exposed to *C. botulinum* type C/D through hunting. However, in house-kept cats that are not naturally exposed to the organism, specific IgA may be a good indicator of exposure. It was evident from these results that affected cats had some levels of specific IgA and if healthy controls possessed specific IgA, that it was below the levels of detection of the assay used. The control group were all free-roaming but only 3 of these showed a (much lower) positive signal to BoNT/C toxoid compared to all of the affected cats. However this indicates that certain cats are exposed to the toxin at certain times. No positive control for specific IgA is available and some concentration of IgA in samples from affected cats may have been seen due to dehydration although they were not clinically dehydrated at the time. Also, due to availability of reagents at the time, the assay was not quantitative. The numbers used in the study were small and a matched case-control study, matching age, lifestyle and diet would be useful.

It is not known whether gut stasis is caused by production of the toxin or is a consequence of it. However, detection of specific IgA in gut samples from acute cases of grass sickness (Hunter 2001), indicate that there has been exposure to the organism before the onset of clinical signs and that gut stasis may be an effect rather than a cause of the disease.

Generally, dysautonomias occur in younger animals (Doxey et al,, 1991; Berghaus et al, 2001; Harkin et al, 2002) and a young age is a recognised risk factor in both horses and dogs. A correlation between BoNT specific antibodies and age has been demonstrated in horses (McCarthy et al, 2004).

The cats involved in the Glasgow outbreak were all adult although the three most acutely affected cats (A1, A2 & A3) were also the youngest in the colony. There is some anecdotal evidence of a genetic susceptibility in felines (Symonds et al,, 1995) and these three animals were also littermates.

The idea that indoor-kept cats may be more susceptible due to naivety was an attractive one although the disease is reported in free-roaming cats also. Previous serological work in horses had shown significantly lower specific antibody levels in sick horses compared with controls (Hunter et al,, 2001) although age-matching of controls had not been possible.

Initial serological results were promising, free-roaming animals having a higher titre against BoNT/C and SA than indoor-kept and FD animals. Although the FD group

had significantly higher IgG titre against BoNT/C this might have been partially explained by recent exposure or because outbreak cats were not sampled until at least seven days after onset of clinical signs in the index case. Multiple comparisons of groups means are inadvisable due (Motulsky 1996) due to the increased likelihood of finding significant differences so one-way ANOVA was also employed (Kruskal-Wallis, non-parametric test followed by a Dunn's post-test for multiple comparisons). This test demonstrated significant differences between free-roaming controls, indoor controls and FD cats for both BoNT/C toxoid and surface antigens for each antigen. However, significant differences were not observed between indoor controls and FD cats for either antigen.

Correlation was performed to check for a positive correlation between antibody acquisition and age. The only positive correlation demonstrated in individual control groups was between free-roaming controls and anti-BoNT/C IgG. However, when the two control groups were combined, a positive correlation was seen against both antigens.

Correlation between antibody levels (anti-BoNT/C versus anti-SA) is not always observed in horses which may be due to greater exposure to neurotoxin-negative Group III strains than to neurotoxin positive strains or because a good antibody titre against SA indicates a good immune response to the organism, helping prevent *C. botulinum* type C/D growth in the gut and therefore prevents toxin production. In co-grazing horses, an inverse correlation between SA and anti-BoNT/C antibody has been demonstrated. Although a negative correlation was not observed in the free-

roaming controls, a much smaller correlation was demonstrated than in indoor-kept controls. A strong positive correlation was observed between anti-BoNT/C and anti-SA IgG levels in the indoor-kept control groups, possibly due to lower levels of anti-SA antibodies (due to less exposure to the organism) allowing more toxin production in this group of animals.

Due to the differences in age of the case and control groups and the chance of obtaining age-matched controls, it was decided to repeat the study. By this time, a BoNT/D toxoid had also become available and a commercial ELISA kit to quantify feline IgG.

Multivariable analysis was employed to control for confounding variables-most importantly age and lifestyle, i.e. free-roaming versus indoor-kept cats. The only significant finding was the association between low anti-BoNT/D titre and FD.

A recognised clinical scoring system was used to exclude certain cases although this is arguably subjective. Definitive diagnosis can only be by histopathological examination of ganglia and the excluded animals could have had FD and therefore not be counted as 'healthy' in-contact animals. The presence of specific IgA in all cases and toxin in most demonstrates recent exposure. However, these animals survived although its impossible to say whether this is due to less exposure to the toxic insult or due to a better immune response that limited neuronal damage.
The three most acutely affected cats demonstrated initial specific IgG levels below or equivalent to the median IgG of the control cats against BoNT/C and BoNT/D. B1,

the only surviving cat not excluded on the basis of clinical scoring, showed a low initial titre against all three antigens that rose sharply when sampled on day 14. C1 demonstrated a drop in specific IgG against BoNT/C and BoNT/D over the same time period although it demonstrated a rise in anti-SA. A drop in specific IgG is seen in some horses, thought to relate to the IgG being used up against the antigen. The initial levels of the surviving cats may have limited the disease/prevented it and the dramatic rise in titre produced by B1 may have been too late to prevent the neuronal damage that caused the presenting clinical signs. Chronic GS cases often demonstrate fluctuations of specific IgG and it remains unclear as to whether the animal is being re-exposed to the antigens due to compromised gut motility or whether the neuronal damage leads to problems in cytokine production. Sampling at 30 days in the case of the cats would have been useful to see whether the levels had increased again. It is interesting to note that the levels of the cats at 164 days were roughly approximate to their initial levels-particularly anti-toxoid levels. The obvious exception to this is the anti-BoNT/D titre of case B1. Numbers of cases are too small to draw conclusions in this longitudinal analysis but due to the rarity of such studies, interesting in themselves.

In conclusion, similar supportive but circumstantial evidence indicating *C. botulinum* type C/D in the aetiology of feline dysautonomias was demonstrated during these investigations.

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Association between Key-Gaskell syndrome and infection by *Clostridium botulinum* type C/D

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There is growing evidence that equine dysautonomia is a toxicoinfection with *Clostridium botulinum* type C. The possibility that feline dysautonomia has the same aetiology was investigated by attempting to detect botulinum type C neurotoxin in the food, faeces and the contents of the ileum of affected cats, and by serology. The toxin was detected directly in four of eight affected cats and after enrichment in seven of them, and in their dried food. No toxin was detected in healthy control cats or in their tinned food. Recent exposure to the organism was assessed by the detection of immunoglobulin A (IgA) in the faeces of healthy control cats and affected cats. The levels of IgA antibodies to the toxin and to surface antigens of *C botulinum* type C in the faeces of the affected cats 14 weeks after the outbreak were significantly higher than in the faeces of the control cats.

FELINE dysautonomia is a disease characterised by extensive degeneration of the autonomic nervous system (Key and Gaskell 1982), and dysautonomia has also been reported in horses, dogs, rabbits and hares. Most of the cases of feline, equine and leporine dysautonomia have been reported in the UK (Whitwell 1991, Whitwell and Needham 1996), whereas most cases of canine dysautonomia have been reported in the USA (Berghaus and others 2001).

In horses with grass sickness, C1 neurotoxin from *Clostridium botulinum* type C (BoNT/C) has been detected in 74 per cent of acute cases and 67 per cent of both subacute and chronic cases, but in only 10 per cent of control horses (Hunter and others 1999). During an outbreak of dysautonomia in cats, the same toxin was detected indirectly from ileal samples from 11 of the 16 affected cats, and after enrichment from nine of them. The levels of specific immunoglobulin A (IgA) to both EDTA-extracted *C botulinum* surface antigens and a BoNT/C-toxoid complex have also been found to be significantly higher in horses with grass sickness than in control horses (Hunter 2001).

In November 2001, there was an outbreak of feline dysautonomia in a closed non-breeding colony of eight pet cats (Cave and others 2001) which was investigated to test the hypothesis that the aetiology of feline dysautonomia might be similar to that of equine grass sickness, and that it is caused by toxicoinfection by *C botulinum* type C/D. The procedures used to investigate equine grass sickness were applied: the detection of the toxin in food, faeces and gut contents by ELISA; the isolation of *C botulinum* from food and faeces; and the detection of specific IgA antibodies in the faeces and ileal contents to clostridial surface antigens and BoNT/C.

MATERIALS AND METHODS

Cases

Six of a colony of eight house-kept cats, none of which had access to the outdoors, were clinically affected. The characteristics of the cases are shown in Table 1, and the clinical signs and outcomes are summarised in Table 2. The index case (A1) was admitted to Glasgow University Veterinary School five days after first showing clinical signs, and cats B1, B2 and C1 were admitted five to eight days after first showing clinical signs. Cats A1 and A2 died and A3 was euthanased in extremis. Only A1 and A3 were examined postmortem, and dysautonomia was diagnosed histopathologically. Clinical signs persisted in the surviving cats (B1, B2 and C1). Cats B3 and C2 showed no clinical signs, but a fluoroscopic examination 96 days after the beginning of the outbreak showed subjective evidence of reduced oesophageal function; they may have suffered a subclinical form of the disease. The cats were fed a single brand of tinned food from a communal bowl and a single brand of dry food from two communal hoppers. At the time of the outbreak the batch of dry food had been in use for six days before the index case. On average it took 10 days for the cats to consume all the dried food contained in the hoppers. Fresh tins of food were opened daily. The cats' food and pattern of feeding had not changed during the preceding 12 months.

Control cats

Eleven cats over two years old were used as controls. They were from the same geographical area (Glasgow) and had unrestricted access to the outdoors. They were owned by staff from Glasgow Veterinary School, who considered that they were in good health; their dietary history was unknown.

Sample collection and storage

Faecal samples of 2 g from the cases and controls were collected in sterile universal containers immediately after defecation and frozen at –70°C until processed. The cases were sampled 14 weeks after the onset of clinical signs in the index case.

The ileum and its contents were obtained from case A3 postmortem; the ileum was tied off proximally and distally with nylon suture, removed and frozen at –20°C until assayed.

A sample of the dry food fed to the cases at the start of the outbreak was stored at –70°C, and unopened cans of the tinned food fed to them were stored at room temperature.

Sample preparation

Preparation of faeces with protease inhibitors for the IgA ELISAs All reagents were stored at –20°C and kept on ice during the procedure. Two parts protease inhibitor solution (soybean trypsin inhibitor 1 mg/ml in phosphate-buffered saline [PBS], 50mM EDTA containing 0·05 per cent Tween 20) were added to one part weighed sample (approximately 1 g). Phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich), 0·1M in ethanol, was added to a final concentration of 1mM. The mixture was vortexed for 30 to 60 seconds and then centrifuged at 3800 *g* for 10 minutes. The supernatant was removed and PMSF was added to a final concentration of 1 per cent (v/v). After mixing well, the mixture was allowed to stand for 15 minutes on ice. Heat-inactivated fetal calf serum (FCS)

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was added to a final concentration of 4 per cent (v/v) and the mixture was centrifuged at 15,700 *g* for five minutes. The supernatant was removed and stored at –70°C.

Preparation of ileal tissue with saponin for the IgA ELISAs Antibody was extracted from the ileum with saponin as described by Bergquist and others (2000); 1 g of frozen tissue was removed aseptically and thawed for eight hours at 4°C in 2 ml PBS containing 2 per cent saponin (w/v), 1 mg/ml soybean protease trypsin inhibitor, 50mM EDTA, 0·05 per cent Tween 20, 2mM PMSF, 0·2 mg/ml sodium azide and 4 per cent FCS (v/v). After thawing, the samples were agitated with sterile forceps and then vortexed for 30 seconds. The samples were centrifuged at 15,000 *g* for five minutes and the supernatants were collected and stored at –70°C until used.

Preparation of faeces and food samples for the ELISA for BoNT/C and bacteriology For non-enriched samples, approximately 1 g of the sample was added to 10 ml of PBS, pH 7·2, containing 0·2 per cent gelatin (PBS-G), and the samples were incubated overnight at 4°C.

For enriched samples, approximately 1 to 2 g was added to 15 ml of prereduced NZ-CASE-CMB medium (Hunter and others 1999) and incubated anaerobically at 30°C for five days. After vortexing thoroughly, the samples were centrifuged at 3800 *g* for 20 minutes and the supernatant was collected and stored at –20°C.

EDTA extraction of *C botulinum* surface antigens Cultures of *C botulinum* (20 ml) were prepared in Fastidious Anaerobe Broth (Lab M) and incubated anaerobically at 30°C for 18 hours. The strain used was a toxin-negative *C botulinum* type C (NCTC 3732). EDTA extracts were prepared as described by Poxton (1984) and stored at –20°C, and their protein concentration was determined as described by Lowry and others (1951).

ELISA detection of anti-BoNT/C toxoid IgA and anti-*C botulinum* surface antigen IgA from faeces and ileal tissue

Microwell plates (Nunc; Fisher Scientific) were coated with BoNT/C toxoid (Metabiologicals) at 5 µg/ml or surface antigens at 30 µg/ml diluted in coating buffer (0·05M sodium carbonate buffer, pH 9·6, 0·02 per cent w/v sodium azide) and incubated overnight at 4°C or at room temperature, respectively. The plates were washed four times with ELISA wash buffer (one PBS BR14a tablet/litre [Oxoid], 15mM sodium chloride, 2mM potassium chloride, 0·05 per cent Tween 20, pH 7·3). The plates were blocked with PBS containing 3 per cent teleostean gelatin (Sigma) (PBS-TG) and 0·02 per cent sodium azide (200 µl per well) and incubated while being shaken for four hours at 37°C, before being washed four times with ELISA wash buffer. They were stored in sealed polythene bags at –20°C until used.

TABLE 1: Characteristics and genetic relationships of the eight affected cats

Group	Cat	Relationship	Breed	Age (years)	Sex
A	1	Same parents, same litter	Birman	5	M (N)
	2			5	F (E)
	3			5	F (E)
B	1	Same parents, different litters	Birman	9	M (N)
	2			14	F (N)
	3	Shared queen with B1 and B2 but different tom and litter		14	F (N)
C	1	Same parents, same litter	Colourpoint	11	M (N)
	2			11	M (N)

M Male, F Female, N Neutered, E Entire

Samples were diluted 1 in 4 with PBS-TG before the addition of 100 µl/well in quadruplicate. They were incubated overnight at room temperature and then washed four times in ELISA wash buffer.

Rabbit anti-cat IgA (Nordic Immunologicals) was diluted 1 in 400 with PBS-TG and 100 µl was added to each well. The plates were incubated for three hours at 37°C while being shaken, and were then washed four times with ELISA wash buffer.

Anti-rabbit IgA conjugated to alkaline phosphatase (Sigma) was diluted 1 in 2000 with PBS-TG and 100 µl was added to each well. The plates were incubated for three hours at 37°C while being shaken, and were then washed four times with ELISA wash buffer.

Alkaline phosphatase substrate tablets (104-105 phosphatase tablets, p-nitrophenyl phosphate 5 mg/ml; Sigma) were diluted 1 in 10,000 with substrate solvent (0·05M sodium carbonate solution, pH 9·8, with 1mM magnesium chloride) to give a concentration of 1 mg/ml and 100 µl of this solution was added to each well. The plates were then incubated at room temperature for 30 minutes and read with an Anthos plate reader at 405 nm, referenced at 620 nm.

Bacteriological isolation of organisms from faeces and food

Tenfold dilutions (10^{-1} to 10^{-4}) were made from the non-enriched and enriched samples in pre-reduced nutrient broth after 24 and 48 hours, and plated on to fastidious anaerobe agar (FAA) (Lab M) containing 5 per cent egg yolk emulsion (Oxoid) and 10 µg/ml gentamicin. The plates were incubated for up to five days and examined for lecithinase and lipase activity. Likely colonies were re-streaked for purity and also incubated aerobically on Columbia blood agar (Oxoid CBA base with 5 per cent defibrinated horse blood) to check for aerobic growth. The colonies were also examined by Gram stain and phase-contrast microscopy.

ELISA detection of BoNT/C toxin from cat food, faeces and ileum

A polyvalent guinea pig antiserum (CAMR) raised against purified BoNT/C was used as a capture antibody. Optimal dilutions of the reagents were determined by using a checker-

TABLE 2: Major clinical signs shown by the eight affected cats and the outcome of the disease

Cat	Lethargy	Regurgitation/ vomiting	Appetite	Dysphagia	Constipation	Urinary retention	Bilateral pupillary dilatation	Outcome	Histopathological confirmation
A1	+	+	Anorexic	+	+	+	+	Died	+
A2	+	+	Anorexic	+	+	+	+	Died	–
A3	+	+	Anorexic	+	+	+	+	Euthanased	+
B1	+	+	Reduced	–	–	–	+	Survived	–
B2	+	+	Anorexic	–	–	+	+	Survived	–
B3	–	+	Normal	–	–	–	–	Survived	–
C1	–	+	Normal	–	–	–	–	Survived	–
C2	–	–	Normal	–	–	–	–	Survived	–

+ Present, – Absent

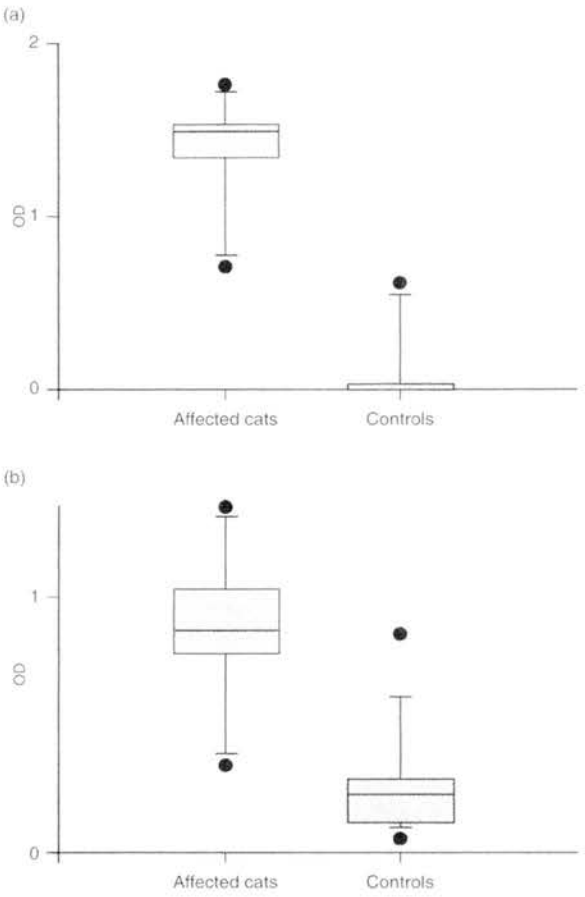

FIG 1: Box and whiskers plots of faecal immunoglobulin A (IgA) expressed in terms of the optical density (OD) in the ELISA against (a) botulinum type C neurotoxin toxoid complex and (b) against EDTA-extracted surface antigens of a toxin-negative *C botulinum* type C strain. The affected cats were sampled 14 weeks after the onset of clinical signs (with the exception of the lowest value, which was from an ileal sample from a cat which was euthanased in the third week of the outbreak). The control group consisted of 11 healthy, free-roaming cats. The affected group of cats had significantly higher levels of faecal IgA than the control group (P<0·001 and P<0·005, respectively) in both assays

board titration. Microwell plates (Nunc) were coated with 100 µl per well of the antiserum diluted 1 in 10,000 in 0·05M sodium carbonate buffer, pH 9·6, 0·02 per cent w/v sodium azide, and incubated overnight at 4°C. The plates were then washed three times with ELISA wash buffer.

They were then postcoated with PBS-TG (200 µl per well) for two hours at 37°C in a shallow water bath, and washed three times in ELISA wash buffer.

The non-enriched samples were added (100 µl per well) either neat or diluted 1 in 2 and 1 in 4 in PBS-TG, and the enriched samples (100 µl per well) were added neat or diluted 1 in 5 and 1 in 25 in PBS-TG, and incubated for three hours at 37°C in a shallow water bath, and then washed three times in ELISA wash buffer. The guinea pig anti-BoNT/C horseradish peroxidase conjugate (CAMR) was diluted 1 in 300 in PBS-TG, added to the plates (100 µl per well) and incubated for three hours in a shallow water bath. After washing three times in PBS containing 0·05 per cent Tween 20, substrate solution (3,3', 5,5'-tetramethyl-benzidine dihydrochloride tablets [Sigma] dissolved in phosphate citrate buffer, pH 5·0, with 2 µl of 30 per cent hydrogen peroxide per 10 ml) was added at 100 µl per well. The reaction was allowed to develop at room temperature for up to 60 minutes and was stopped by the addition of 50 µl of 2M sulphuric acid to each well. A standard curve of purified BoNT/C (CAMR) was run on each plate. The limits of detection of this assay were 0·8 ng/ml and a standard curve of purified neurotoxin with concentrations ranging from 200 ng to 0·8 ng was included on each plate.

Controls and statistical analysis

No positive control sera were available, but positive signals were identified in the initial assays and these were used as controls and to normalise the results in later assays. Negative controls consisted of coated wells with no sample but with conjugate, and coated wells with sample but no conjugate.

The results were analysed by using SPSS software (SPSS Science) and the Mann-Whitney U test. Significance was set at the 5 per cent level.

TABLE 3: Detection of the botulinum type C neurotoxin in the food and faeces of the affected cats

Case	Directly (ng/kg)	After enrichment
A1	15·6	+
A2	ND	+
A3	2·1	+
B1	ND	+
B2	0·8	+
B3	ND	ND
C1	ND	+
C2	1·6	+
Dried food	ND	+

ND Not detected, + Present

RESULTS

ELISA to detect IgA antibodies to BoNT/C toxoid and *C botulinum* surface antigens from faeces and ileal tissue

Specific IgA antibody to BoNT/C and surface antigens was detected mainly in the affected cats which had significantly higher mean specific IgA levels than the healthy control cats. The lowest value in the affected cats (Fig 1) was observed in the ileum of case A3, in a sample taken during the outbreak (week 3) and not 14 weeks afterwards like the others.

The ELISA values for IgA antibody to BoNT/C toxoid in the free-roaming control cats (median 0·23, range 0·6 to 0·03) were significantly lower than in the surviving cases (median 1·5, range 1·7 to 1·3) [P<0·001]). This analysis excludes the ileum sample from A3, but if it is included the results are still significant (median 1·45, range 1·7 to 0·7 [P<0·001]). Similarly, the levels of IgA against EDTA-extracted surface antigens were much lower in the control cats (median 0·23, range 0·85 to 0·06) than in the surviving affected cats (median 0·92, range 1·35 to 0·81 [P<0·001]). Again, if the ileal sample from A3 is included, the results are still significant (median 0·87, range 1·35 to 0·34 [P<0·05]).

Bacteriological isolation of organisms from faeces and food

On the basis of the colony morphology the faecal samples did not yield any likely colonies, and there was no growth anaerobically from the tinned cat food. Likely colonies did appear in the cultures of the dry food, but subculture was unsuccessful despite several attempts.

ELISA detection of BoNT/C toxin in cat food, faeces and ileum

Toxin was detected without enrichment in faecal samples from three of the six clinical cases, and one of the two subclinical cases (C1). The index case (A1) had the highest faecal concentration of toxin. No toxin was detected in the faeces of the control cats or in the cat food by this method. After enrichment, toxin was detected in faecal samples from all the clinical cases except B3, but none was detected in faeces from the control cats. Toxin was detected in the dry cat food but not in the tinned cat food. Table 3 summarises these results and shows the amounts of toxin detected by the direct detection method.

DISCUSSION

There is evidence that equine dysautonomia, or grass sickness, is caused by a toxicoinfection with *C botulinum* group III phenotype (serotype C/D) (Hunter and others 1999, Hunter 2001).

C botulinum type C mainly affects animals and can produce three toxins: C1 (BoNT/C) is a bacteriophage-encoded neurotoxin that inhibits the release of neurotransmitters by specifically proteolysing syntaxin (Schiavo and others 1995) and SNAP-25 (Foran and others 1996) at cholinergic nerve terminals; C2 and C3 toxins both have ADP-ribosylating activity, and C2 can disrupt the ultrastructure of the cytoskeleton (Mauss and others 1990). *C botulinum* type D is very similar to *C botulinum* type C and is distinguishable only by the bacteriophage-encoded type D neurotoxin. Mosaic toxins consisting of type C and type D subunits also occur (Morrishi and others 1989). Of these toxins, only BoNT/C has been shown to be cytotoxic in vitro (Kurokawa and others 1987) and could be the cause of the pathology observed in dysautonomias.

It is very difficult to isolate the organism and to detect the toxin. *C botulinum* is highly fastidious and the toxin is encoded on an unstable bacteriophage which is easily lost upon subculture. The presence of toxin after enrichment does not necessarily mean that toxin was present in vivo and the absence of toxin does not necessarily mean that the organism was not there. These results provide evidence that there is an association between feline dysautonomia and a toxicoinfection with *C botulinum* type C similar to that reported in equine grass sickness. Hunter and others (1999) detected toxin in healthy horses leading to the hypothesis that *C botulinum* type C/D may be present in the gastrointestinal tract of healthy horses. In these cats, the toxin was detected only in samples from the affected cats, and the samples containing the highest concentrations of toxin were from the three most acutely affected cats (A1, A2 and A3); the one negative sample came from the least affected cat (B3). The toxin assay is highly specific and does not detect BoNT/D or any of the other botulinum neurotoxins.

Given the very different diets and gastrointestinal physiology of horses and cats it is possible that cats kept indoors are not normally exposed to *C botulinum* type C/D in their canned food. The dried food was positive for toxin after enrichment, and it is possible that contamination with cereal or soil in dried food may harbour *C botulinum* (and other clostridia) and provide an environment in the gut that allows them to proliferate. Dry food is heated only to 80°C during processing, theoretically allowing *C botulinum* spores to survive. Since the investigation, the dried and tinned foods of different brands fed to three other cats with Key-Gaskell syndrome have been assayed for toxin and all have been negative. These cats were allowed access to the outdoors, and Berghaus and others (2001) and Harkin and others (2002) have shown that free-roaming dogs, known to forage, are more likely to develop dysautonomia.

Local mucosal immunity may be important in protecting animals against the disease, especially free-roaming cats that may be exposed to *C botulinum* type C/D through hunting. However, in house-kept cats that are not naturally exposed to the organism, specific IgA may be a good indicator of their exposure. The affected cats had detectable levels of specific IgA whereas the healthy controls did not. The control cats were free-roaming but only three of them showed a positive signal to BoNT/C toxoid, compared with all of the affected cats. No dietary history was available for the control cats. No positive control for specific IgA is available and the concentration of IgA in the samples from the affected cats may have been increased by dehydration, although they were not clinically dehydrated when sampled. The assay used was not quantitative, and there were only a small number of cats; a case-control study would be useful, particularly of animals fed similar diets.

It is not known whether the gut stasis is caused directly by the production of the toxin. However, the detection of specific IgA in gut samples from acute cases of grass sickness (Hunter 2001) indicates that the horses had been exposed to the organism before the clinical signs developed, and that the gut stasis may be an effect rather than a cause of the disease. The levels of specific serum IgG have been found to be significantly lower in horses with grass sickness than in healthy controls (Hunter and Poxton 2001), suggesting that weakly immune animals may be more susceptible to grass sickness.

The results of this investigation provide strong circumstantial evidence of an association between feline dysautonomia and *C botulinum* type C. The toxin and specific IgA were detected in samples from the affected cats, and the toxin was also detected in samples of the dried food fed to the cats. Only a small number of samples from the affected and control cats were available, but the results warrant further investigation of any new cases of feline dysautonomia.

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Stabilisation of scoliosis in two koi *(Cyprinus carpio)*

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Two koi *(Cyprinus carpio)* from the same pond developed similar lesions of scoliosis. Radiographic examinations showed that their spines had become malaligned as a result of vertebral compression fractures involving T14 to T16. The vertebrae in both fish were stabilised with screws, k-wire and polymethylmethacrylate. They both appeared to improve after surgery, but they began to decline and died within three months. A postmortem examination revealed multi-organ inflammation that was not associated with the surgical implants.

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SCOLIOSIS, or 'bent-back', in koi (*Cyprinus carpio*) is being reported more often. Many causes have been suggested, including vitamin C deficiency (John and others 1979, Halver and Hardy 1994); electrocution as a result of either lightning strike (Barlow 1993), faulty submersible pumps (Johnson 1997) or electrofishing (Sharber and Carothers 1988); tryptophan deficiency (Halver and Shanks 1960, Kloppel and Post 1975, Poston and Rumsey 1983, Walton and others 1984, Post 1993); trauma; organophosphates (Couch and others 1977, Alam and Maughan 1993, Waddington 1995); and bacterial cold water disease (Noga 1996). Although some fish continue to do well with a noticeable curvature of the spine, others become debilitated and intervention becomes necessary. This paper describes the progression of the condition and the surgical stabilisation of scoliosis in two koi.

CASE HISTORY 1

In July 2001, an approximately two-year-old, 56 cm, 1314 g, female doitsu sanke koi developed a 3 cm chevron-shaped ulcer, dorsal to its right pectoral fin. The fish shared a 56,775 litre pond, with a maximum depth of 137 cm, with 30 other koi and 10 goldfish (*Carassius auratus*). The water quality had been good, but in the previous three months some of the fish had developed ulcerative skin disease, and when this koi began separating itself from its school the ulcer was first noticed. The koi had been treated by the owner with a series of three injections of 5·7 mg/kg amikacin sulphate (Amiglyde-V, 50 mg/ml; Fort Dodge) administered intracoelomically every 48 hours. When the injections were given, the ulcer was cleansed with 10 per cent povidone iodine solution (Betadine; Purdue Frederick) and then treated with a topical triple antibiotic ointment (Neosporin; Warner Lambert). All the fish in the pond were provided with paste food containing 0·5 mg/g enrofloxacin (Baytril 2·27 per cent; Bayer Corporation) at a dose of 10 mg enrofloxacin/kg/day. When there was no improvement, the paste food was instead impregnated with 2 mg/g trimethoprim-sulphadiazine (Tribrissen; Schering Plough) and fed to provide a dose of 30 mg/kg/day, upon a veterinarian's recommendation.

Clinical and radiographic findings

The ulcer was hyperaemic but healing, and there was mild scoliosis, with a left lateral deviation of the tail at the level of the anal fin.

A biopsy taken from the tip of the right pectoral fin had no evidence of microscopic changes. A skin scrape taken from the ulcerative lesion contained a few unidentified non pathogenic protozoal organisms and one free-living nematode. The fish was anaesthetised in 200 mg/litre of buffered tricane methanesulphonate (MS222) (Finquel; Argent Chemical Laboratories) for the purpose of radiographic examination (Love and Lewbart 1997) and maintained by delivering the anaesthetic-containing water through a syringe over its gills as needed. Dorsoventral radiographs revealed a right laterodorsal angulation involving the 14th, 15th and 16th trunk vertebrae (T14 to T16) at the level of the caudal swimbladder. The vertebrae at this site appeared to be osteopenic and the intervertebral spaces were not well delineated (Fig 1). Lateral views revealed a dorsolateral subluxation of the spinal column just caudal to the last rib, involving T14 and T15. The lateral angulation was considered to be due to a combination of a fracture and a subluxation. Several callused, mid body fractures were apparent in the caudal ribs. The koi recovered uneventfully in fresh dechlorinated water; it resumed swimming with the group of fish and the ulcer started to heal; it was treated with trimethoprim sulphadiazine for two more weeks, but no further treatment was considered necessary.