A case-control study to investigate risk factors for equine grass sickness with a particular reference to the role of *Clostridium botulinum.*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by Helen Elizabeth McCarthy.

2002

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Abstract

Equine grass sickness (EGS) is a disease of unknown aetiology. Clinical signs, pathology and diagnosis are reported in chapter 1. The epidemiology of the disease is reviewed in chapter 2 highlighting a number of horse-level and pasture-level risk factors for EGS.

The aim of this thesis was to test the hypothesis that EGS is associated with *C. botulinum* type C toxicosis. In addition, a number of horse-level and pasture-level risk factors were evaluated.

Chapter 3 provides serological evidence for an association between *C. botulinum* type C and EGS. Enzyme-linked immunnosorbent assays (ELISA's) were used to demonstrate that horses with EGS have significantly lower levels of antibodies to a) *C. botulinum* type C cell surface antigen, b) *C. novyi* type A cell surface antigen and c) *C. botulinum* type C toxin complex toxoid when compared to co-grazing control horses. The relationship between ELISA OD and age in control horses only has also been investigated. Basic model-based relationships provide a possible explanation of why younger horses are at greater risk from disease. For all three antigens, the point of equilibrium, where antibody production is equal to the rate of loss, is not reached until the horses are at least 7 years of age.

A matched case-control study identified age, the use of ivermectin as an anthelmintic, time on pasture and a recent change of feed type or quantity as risk factors for EGS (Chapter 4). Along with horse level risk factors, serology results remained highly significant when entered into the final multivariable models. The risk of EGS was found to decrease with increasing antibody levels. Analysis of faecal samples from EGS cases and premise matched control horses revealed that EGS was associated with an increased dry matter content of faecal material and an increased ammoniacal nitrogen content. Analysis of soil samples from EGS premises and premise controls revealed that EGS is associated with having EGS on the premise prior to the current outbreak, a pasture that had been disturbed and soil with higher total nitrogen content.

Chapter 5 explores space-time clustering of EGS cases in the UK. Strong evidence of space-time clustering is provided, particularly within 5km and 20 days of an arbitrary case, when individual EGS cases are investigated or individual outbreaks of EGS.

This thesis provides further evidence for the role of *C. botulinum* type C in EGS and has identified new horse-level and pasture-level risk factors for EGS. It is possible that alteration of the gastrointestinal environment assists in proliferation and toxin production by *C. botulinum* type C. In order to understand the disease more fully it is imperative that these relationships between horse-level and pasture level risk factors are more fully understood and their place on the causal pathway of disease is determined.

DECLARATION

I declare that apart from the advice and assistance acknowledged the work reported in this thesis is my own and has not been submitted for consideration for any other degree or qualification.

Helen E. McCarthy

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Acknowledgements

My grateful thanks go to Dr Chris Proudman, Dr Nigel French, Professor Barrie Edwards and Mr Keith Miller for initiating this project. The Home of Rest for Horses is also gratefully acknowledged for funding the project. I consider myself very fortunate to have had the opportunity to undertake such a challenging and topical PhD studentship. I also thank my supervisors, Dr Chris Proudman and Dr Nigel French for their constant encouragement and guidance.

My grateful thanks go to the many veterinary surgeons that referred suspected cases of grass sickness or submitted samples from such horses. Their collaboration was imperative to the success of this project.

I would also like to thank the veterinary surgeons that accompanied me on field visits for the purpose of blood sampling. These visits were sometimes very time consuming. I am therefore extremely grateful for their co-operation.

There are a number of people that I must thank for their assistance in the laboratory both in Edinburgh and Leahurst. These include; Professor Ian Poxton, Leonie Hunter, Mr Robert Brown, Dr Jacqui Matthews and Sam Dowdall. Dr K. Matthews is also acknowledged for the silver staining protocol (Chapter 3).

Thanks are also due to my colleagues and friends in the Epidemiology group at Leahurst. I would specifically like to thank Gina Pinchbeck for her guidance during the data analysis stage of the project.

Outside of academia, I must thank my housemates and friends Kieron Salmon, Shane McElroy, Yasemin Biddell and Graham (Bertie) Beckram.

My final thank-you goes to my parents for their continued support and encouragement throughout my postgraduate studentship.

List of abbreviations

µg	Microgram
µl	Microlitre
BoNT/C	*Clostridium botulinum* type C toxin
C	Centigrade
C.I.	Confidence Interval
EDTA	Ethylenediameteraacetic acid
EGS	Equine grass sickness
ELISA	Enzyme linked immunosorbent assay
Ig	Immunoglobulin
kDa	KiloDalton
M	Molar
MA	Milliamps
n	Number
NCM	Nitrocellulose membrane
nM	Nanometers
nM	Nanomolar
OD	Optical density
P	Probability
PAGE	Polyacrylamide gel electrophoresis
PBS.	Phosphate buffered saline
PBS-T	Phosphate buffered saline plus Tween
PBS-TF	Phosphate buffered saline plus Tween and foetal calf serum
PLLAH	Phillip Leverhulme large animal hospital
PPC	Positive power curve
SDS	Sodium dodecyl
TBS	Tris bufferred saline
TTBS	Tween Tris bufferred saline
v	Voltage

CHAPTER 1

EQUINE GRASS SICKNESS; CLINICAL SIGNS, PATHOLOGY AND DIAGNOSIS

Introduction

Grass sickness (EGS), or equine dysautonomia, is a largely fatal disease of all equidae. The disease is described as a polyneuropathy affecting the central, peripheral and enteric nervous system of the horse (Cotrell *et al.,* 1999). Even though the aetiological agent of this disease is unknown, the clinical signs, pathology, epidemiology (Chapter 2) diagnosis and treatment are well described.

Clinical signs

The clinical signs of EGS are characterised by dysfunction of the autonomic nervous system. Failure of normal alimentary function is one of the diseases' primary features and plays a key role in contributing to poor survival.

The disease is presented in four overlapping clinical categories; per-acute, acute, sub-acute and chronic (Doxey *et al.,* 1991a, Milne, 1997a, Edwards, 1987). All forms of grass sickness are generally regarded as representing differing degrees of the disease in the manifestation of one essential pathological condition (Greig 1928; Edwards, 1987). For the purpose of this review, the clinical signs of EGS will be described in two categories; acute and chronic. The per acute and acute forms of the disease are considered together due to the similarity in clinical presentation whilst the subacute form of EGS presents itself with a combination of clinical features of the acute and chronic form of the disease.

Generally, acute cases of EGS are defined as horses that yield gastric reflux on naso-gastric intubation (or spontaneously) and will survive for less than seven days without supportive fluid therapy. Chronic cases are defined as horses without gastric reflux and survive for longer than 7 days. In cases of acute grass sickness, clinical signs appear rapidly and the horses' condition can deteriorate

within a few hours to a few days. In cases of chronic grass sickness symptoms usually appear less severe and the horse can survive many weeks or even months. Acute cases of EGS usually present on initial examination with varying degrees of colic (Figure 1.1). These horses are tachycardic with a heart rate often accelerated to 70-80 beats per minute sometimes rising to 100 beats per minute (Edwards, 1987). The abdomen is often grossly distended and gut sounds are reduced or absent. Intestinal stasis in EGS prevents the constant passage of ingesta and fluid and gas accumulation follows stasis (Doxey *et al.*, 1991a).

The stomach may contain in excess of 20 litres of fluid whereas the normal amount is around 7 litres (Edwards, 1987). Spontaneous reflux of greenish, mucinous stomach contents is sometimes observed in acute cases of EGS (Edwards, 1987). Many cases will yield profuse gastric reflux on naso-gastric intubation. On rectal examination distended fluid filled loops of small intestine are evident. It has been stated that the increased luminal capacity of the gastric and small intestinal compartments causes visceral pain as well as systemic fluid and electrolyte imbalances (Cotrell *et al.*, 1999). The rectum is dry and tacky with small hard, mucous coated balls of faeces closely adhered to the mucosa (Edwards, 1987).

In cases of chronic EGS, the most striking feature is the dramatic loss of physical condition (Figure 1.2), accompanied by muscle atrophy and dehydration (Edwards, 1987). The abdomen is tucked up to an exaggerated degree giving the appearance of an emaciated greyhound and these cases often adopt a characteristic narrow-base stance (Figure 1.3). Defecation is infrequent and the faeces are scant, hard and dry. Excess stomach contents and spontaneous reflux

are not seen in chronic cases of EGS. Chronic cases often attempt to eat but swallowing is difficult. Tachycardia is frequently evident.

Even though the acute and chronic forms can be distinguished by the afore mentioned clinical signs, there are many signs that are common to both forms of the disease. Altered skeletal muscle excitability and tremor is observed, especially over the triceps, shoulders and stifle areas (Edwards, 1987). Afebrile, patchy sweating is seen behind the shoulders and the flanks (Figure 1.5). The animals are depressed (figure 1.4), dysphagic and rhinitis sicca and ptosis are often present. Hypersalivation is observed in cases of EGS and can vary from mild to excessive in nature (Figure 1.6 and 1.7). There is some doubt whether an absolute increase in salivary secretion occurs or whether excessive secretion is apparent and results from the accumulation of saliva in the mouth due to difficulty in swallowing and or failure of oesophageal peristalsis (Greig, 1942, Doxey *et al.*, 1991a). As a consequence of gut stasis a secondary, corrugated impaction of the colon is a characteristic of EGS found on rectal examination.

EGS can also exhibit pyrexia, elevated packed cell volume (PCV) and total protein (TP). An increased PCV and TP reflect the dehydrated state of these animals.

It can be concluded from the described clinical signs that significant injury to the nervous control of the alimentary system is life threatening, not only because of dysphagia but also because of the failure of the regulatory mechanisms in equine digestion involving water and electrolytes. Cotrell *et al.*, (1999) hypothesised that in acute cases of EGS lesions occur in the enteric nervous system of a susceptible horse and massive enteric neuronal damage occurs. Generalised smooth muscle atony, enhanced secretions and altered fluid fluxes are

followed by severe distension of the stomach and small intestine that leads to the characteristic ileus, colic and dehydration observed in EGS.

It should be highlighted here that not all cases of EGS, whether chronic or acute, show all the described clinical signs associated with the disease. There is a great variation in the presenting clinical signs of EGS and only a small proportion of the above described clinical signs associated with the disease may be present on first examination (Doxey *et al.*, 1991a, Edwards, 1987).

All photos: H.E. McCarthy

Figure 1.1: An acute case of EGS exhibiting signs of abdominal pain.

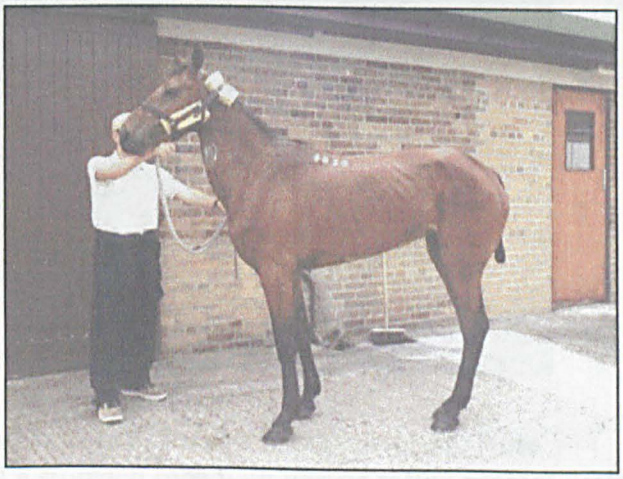


Figure 1.2: A chronic case of EGS of 4 weeks duration.

Figure 1.3: A chronic case of EGS; note the narrow-base stance

Figure 1.4: Depression in an acute case of EGS.

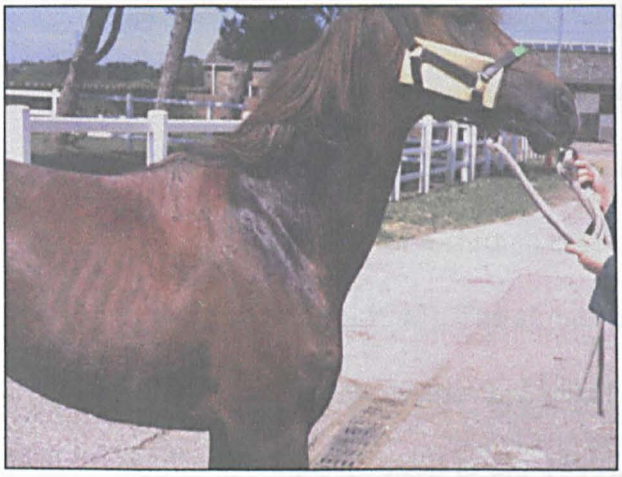


Figure 1.5: Patchy sweating in the neck region of a chronic EGS case

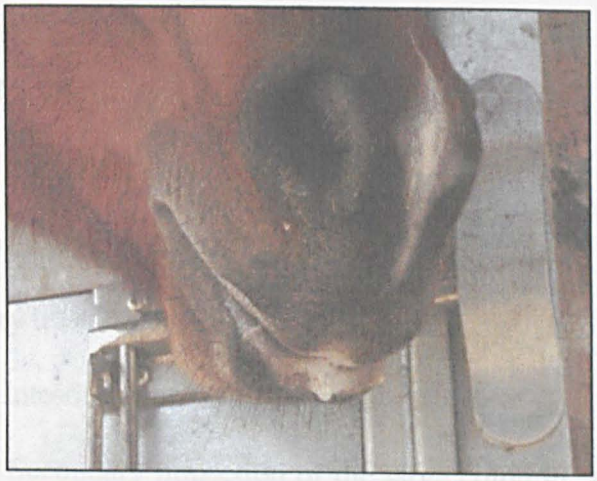


Figure 1.6: Mild hyper-salivation.

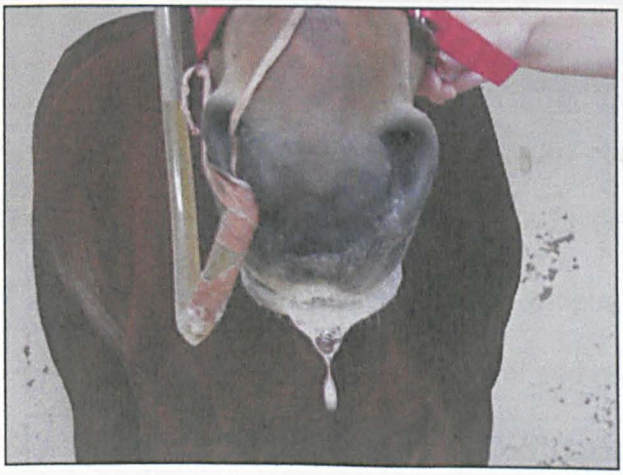


Figure 1.7: Excessive hypersalivation. Note presence of a stomach tube with gastric reflux present within.

Gross Pathology

Gross pathological examination of EGS cases is by no means diagnostic but can reveal some typical characteristics of the disease. A post-mortem examination of an acute case of EGS may show gross erosion of the oesophageal lining (Gilmour, 1987). Liquid distension of both the stomach and small intestines are a feature of an acute case of EGS but no gross lesions are evident in these organs (Gilmour, 1987; Cotrell *et al.*, 1999). Dehydration of large colon and caecum contents are also found with an impacted mass of hard, dry, ingesta present (Gilmour, 1987; Cotrell *et al.*, 1999). Splenomegaly is also frequently observed in cases of EGS (Cotrell *et al.*, 1999).

The carcass of a chronic case of EGS characteristically varies from thin to emaciated (Greig, 1942). The alimentary tract at post-mortem examination may be grossly normal as gastric and small intestine distension is absent as they are relatively empty (Cotrell *et al.*, 1999). Secondary impaction of the large colon in chronic cases is variable (Cotrell *et al.*, 1999).

Neuropathology

Extensive and severe degeneration of neurones in the peripheral ganglia of the autonomic nervous system occurs in EGS (Doxey, 1992). Obel (1955) was the first to recognise neuronal degeneration of autonomic ganglia of EGS cases in Sweden. Neuronal degeneration is found in the vertebral and prevertebral ganglia and the alimentary mural plexuses (Obel, 1955). Barlow (1969) was the first to report changes in certain brain stem nuclei whilst Scholes *et al.*, (1993a) consistently observed severe depletion of intrinsic neurones in the ileum of EGS cases.

Peripheral nervous system

Damage to the paravertebral ganglia in cases of EGS constitutes damage to the stellate, cranial, caudal, cervical and thoracic sympathetic chain ganglia. Prevertebral damage includes the coeliaco-mesenteric, cranial and caudal mesenteric ganglia. Other affected ganglia include the dorsal root ganglia, ciliary ganglia and enteric nervous system (myenteric and submucosal plexuses) (Obel, 1955; Gilmour, 1987; Cotrell *et al*, 1999). Obel (1955) reported that the most marked changes of the autonomic nervous system are found in the cranial cervical ganglion, the stellate ganglion and the sympathetic trunk.

The characteristic neuronal changes of the sympathetic ganglia in cases of EGS include the loss of golgi structure, increased numbers of lyosomes and mitochondria, Nissl substance becomes marginated, nuclei are displaced peripherally and become crenated and neuronal axons show cytoskeleton destruction (Cotrell *et al.*, 1999).

Chandler & Brownlee (1967) reported that the predominant change in the sympathetic ganglia of grass sickness cases is vacuolation of cytoplasm. In acute cases, the majority of the ganglion cells are at first chromatolytic with small vacuoles occurring in the cytoplasm. In those nerve cells, where the cell damage continues to necrosis, these vacuoles become enlarged and the cell is completely destroyed. (Obel, 1955). In chronic cases sympathetic ganglia contain very few living ganglion cells but numerous vacuolated cell ghosts (Obel, 1955; Gilmour, 1987). Doxey, 1992 found no relationship between the severity of clinical signs in acute and sub acute cases of EGS and the amount of neuronal damage in the superior cervical, stellate and ceoliaco-mesenteric ganglia.

Perkins *et al.*, (2000) reported parasympathetic cardiac autonomic neuropathy in five horses with EGS. These findings indicate that the terminal cardiac parasympathetic neurones are involved in EGS.

The proportion of damaged cells is related to the duration of the clinical signs (Pogson *et al.*, 1992). Acute cases of grass sickness had large numbers of dead or damaged ganglion neurones. As the duration of illness increases so the proportion of dead and damaged cells declines. The coeliaco-mesenteric ganglia appeared to be affected differently from the right and left superior cervical ganglia and the stellate ganglia. The ceoliaco-mesenteric ganglia had a greater proportion of degenerate cells in both acute and sub-acute cases.

Enteric nervous system

Severe enteric neuropathy is observed in all cases of EGS. Obel (1955) first reported that chromatolytic changes were found in the enteric nervous system and more specifically found that most changes occurred in the Auerbachs (myenteric) plexus, but only slight changes in the Meissners (submucosal) plexus when studying acute cases of EGS.

Scholes *et al.*, (1993a) described enteric neuropathy in cases of EGS. Enteric neuropathy was observed in both myenteric (Figures 1.7 and 1.8) and submucosal ganglia in sections of the gastrointestinal tract. There appears to be a relationship between the extent of enteric neuronal damage and the severity of the clinical signs. Scholes *et al.*, (1993a) reported this damage to be widespread (stomach, duodenum, jejunum, large colon and caecum) in acute cases but localised to the distal small intestine, specifically the ileum, in the chronic cases. Changes varied from occasional vacuolation of nerve cell bodies to complete loss

of neurones. Morphological changes include chromatolysis resulting in cytoplasmic eosinophilia, cytoplasmic vacuolation, and pyknosis, karyorhexis or apparent loss of nuclei (Scholes *et al.,* 1993a). After birth the ability of neurones to divide is lost, and therefore the loss of enteric neurons is permanent and explains the intractable clinical course of the disease (Scholes *et al.,* 1993a).

With the damage being most severe in the ileum (Scholes *et al.,* 1993a), it is suggested that the putative toxin might be preferentially absorbed there.

It is highly likely that neuronal damage precedes alimentary dysfunction (Brownlee, 1959, Gilmour 1973, Wright & Hodson 1988).

Photos: H. E.. McCarthy

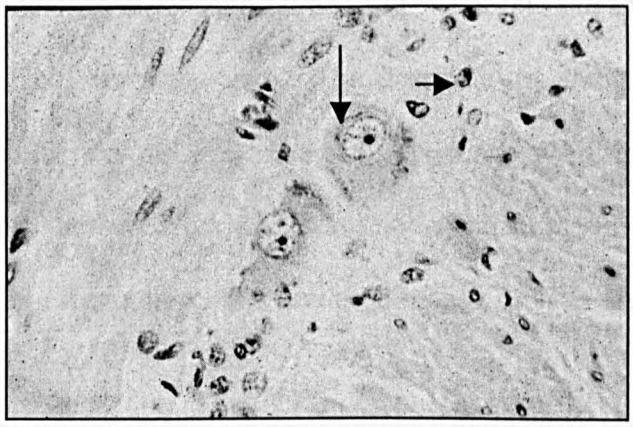


Figure 1.7: Section of normal equine ileum; intramyenteric plexus (x50). Unremarkable neurones are reported in this section. A neuron cell body is identified by the arrow. An apparently normal nucleus is present. Nissl substance is identified by the darker shading within the neuron cell body. Supporting cell nuclei are identified by the shorter arrow.

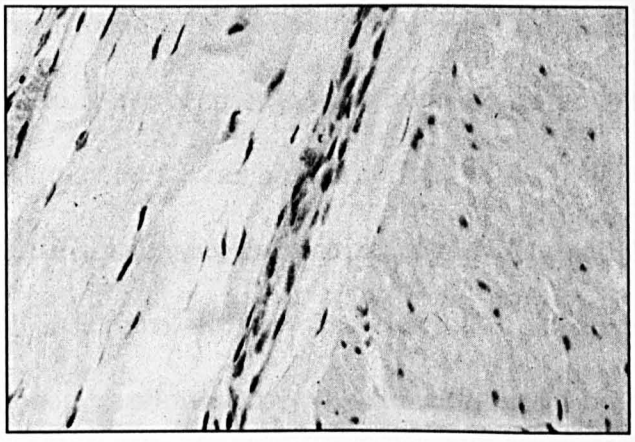


Figure 1.8: Section of ileum; intramyenetric plexus (x 50) from an acute case of EGS. There is a notable absence of intramyenteric neurones. Plentiful supporting cells are seen.

Central nervous system

Neuronal damage has been regularly observed in nuclei of the central nervous system (CNS) (Gilmour, 1973). Not all cranial nuclei are affected and many of the nuclei involved govern final motor pathways of the parasympathetic nervous system (Barlow, 1969). Gilmour (1973) reported neuronal damage in the dorsal root ganglia, intermedio-lateral nucleus, and ventral horn of the spinal cord in addition to nuclei of the brain stem.

The severity of CNS damage varies between affected horses and the significance of these neuronal lesions of the CNS is unclear (Barlow, 1969). The severity of these lesions appeared to be directly proportional to the duration of clinical illness.

Wright & Hodson (1988) suggested that the majority of lesions observed in the CNS are not severe. The possibility that the ganglionic changes in the autonomic nervous system are a secondary phenomenon lends weight to the concept that the CNS changes are non-specific as similar CNS lesions have been found in other non-related equine diseases such as laminitis (Wright & Hodson, 1988).

Degenerative neurological changes similar to those found in cases of EGS have been found in horses with 'mal seco' a disease almost identical to EGS in Argentina. It has been suggested from these findings that 'mal seco' and grass sickness may be the same disease (Uzal *et al.*, 1992). Dysautonomias have been reported in other species such as dogs (Wise and Lappin, 1990), cats, (Key–Gaskell syndrome, Key & Gaskell, 1982) and hares (Whitwell, 1991). These dysautonomias show remarkable similarities to those observed in EGS. Whitwell (1991) suggested that it would not be unrealistic to suggest that the neuronal

damage exhibited in the horse, cat and hare could arise following exposure to the same or very similar insult.

Pathophysiology

It has been hypothesised that the clinical signs of EGS arise from either a depression of parasympathetic activity and or stimulation of sympathetic activity. (Cotrell *et al.*, 1999). Greig (1928) and Obel (1955) considered that the clinical symptoms in grass sickness were compatible with sympathicotonia. This view is well confirmed by the changes observed in the sympathetic ganglia (Obel, 1955). In contrast, it has been suggested that the symptoms of EGS could arise from depression of the parasympathetic nervous system. However, certain structures, such as sweat glands, are only innervated by the sympathetic nervous system and if absolute depression of the parasympathetic nervous system occurred, such structures would not be activated (Greig, 1928).

Symptoms compatible with sympathicotonia include sweating, muscle tremors, tachycardia and increased plasma concentrations of catelcholamines (adrenaline and noradrenaline), ACTH and cortisol. Horses suffering from EGS have significantly higher levels than control horses and horses suffering from colic (Hodson *et al.*, 1984, Hodson *et al.*, 1986). It is possible that these large increases in plasma catelcholamines, ACTH and cortisol concentrations in horses with EGS may be due to a primary dysfunction, stress, or over activity of the sympatho-adrenal system (Hodson *et al.*, 1984; Hodson *et al.*, 1986). The observed increase in sympathoadrenal activity in both acute and sub acute forms of the disease may either be the cause or effect of EGS (Hodson *et al.*, 1984). It has already been stated that this hyperactivation of the sympatho-adrenal system

may be triggered by stress but it is uncertain whether the stress is a result of the severity of the disease or if it plays a role in the aetiology of the disease (Hodson 1984; Hodson *et al.*, 1986).

Hodson *et al.*, (1984) showed a significant difference in plasma histamine levels in early EGS cases and other groups of non EGS horses studied. The value was apparently particularly high in peracute but low in chronic EGS. A similar pattern was also found for ileum histamine levels. The low plasma histamine levels in the chronic EGS case suggest the animal had recovered from the stresses of a more severe form of the disease or that the disease was milder throughout its course. It has been suggested that the high circulating plasma levels of histamine, together with catelcholamines may be responsible for the tachycardia observed in cases of EGS (Hodson *et al.*, 1989).

Marked changes to the regulatory peptide system of the bowel have been found in cases of EGS (Hodson *et al.*, 1982; Hodson & Wright 1987). A reduction in the number of nerve fibres imunnostained for vasoactive intestinal peptide (VIP), substance P and encephalin were observed in nerve endings in the myenteric and submucosal plexuses of the ileum (Hodson & Wright, 1987). VIP is an important inhibitory transmitter found in myenteric neurones (Hodson & Wright, 1987). It relaxes intestinal smooth muscle, acts as a vasodilator and has a direct stimulatory effect on secretion by enterocytes (Vallant, 1991). Substance P also acts as a vasodilator, stimulates contraction of intestinal smooth muscle and inhibits absorption (Vallant, 1991). Enkephalin appears to have indirect inhibitory actions on both intestinal muscle and enterocytes, both of which are actions that are effected via submucosal and myenteric neurons. Massive release of VIP and substance P would affect a range of gastrointestinal functions including secretion,

blood flow and motility (Bishop *et al.*, 1984). However, because all three neuropeptides are depleted in grass sickness, it is not possible to relate the gastrointestinal dysfunction with loss of these neuropeptides (Vallant, 1991). Cotrell *et al.*, (1999) proposed that massive release of VIP and substance P from enteric neurones may activate secretion and contribute to the large quantities of fluid in the stomach and small intestine seen in the acute form of the disease and, when depleted, lack of secretion in the later stages

Diagnosis

One of the major problems facing clinicians is the ability to diagnose EGS in the living animal. The need for a simple ante-mortem diagnostic technique arises because at present, there is no simple and quick *in vitro* laboratory method.

Post-mortem diagnosis was once the only definitive way of diagnosing the disease by examining sympathetic ganglia for the already described typical nerve cell changes. The sympathetic ganglia commonly examined in a post-mortem diagnosis include the cranial mesenteric ganglia, the ceoliaco-mesenteric ganglia and the thoracic sympathetic chain. Due to recent work by Scholes *et al.*, (1993b), the ileum is also histologically examined at post-mortem.

Over the years, several methods of ante-mortem diagnosis have been evaluated or discussed. These include; the use of clinical signs, abdominal paracentesis, barium swallow, phenylephrine eye drops, rectal biopsy and plasma catelcholamine levels. None of these methods in the list provides a suitable and specific method of diagnosing EGS in the living horse. However, the current, validated method of ante-mortem diagnosis that is now widely accepted is that of

an exploratory laporotomy and ileal biopsy. This is the only definitive method of diagnosing EGS ante-mortem.

Clinical signs

Doxey *et al.*, (1991a) assessed the diagnostic value of clinical signs presented in cases of EGS. Only a proportion of the clinical signs associated with grass sickness may be present at first examination and there appears to be no single pathognomic sign for the disease (Doxey *et al.*, 1991a). As a result diagnosis of the disease using clinical signs is non-specific.

Abdominal paracentesis

Abdominal paracentesis enables the diagnosis of a strangulating obstruction to be eliminated (Edwards 1997). Milne *et al.*, (1994) evaluated the use of peritoneal fluid as a diagnostic aid in EGS. They compared peritoneal fluid from cases of grass sickness, medical colic, surgical colic and normal horses. It was concluded that peritoneal fluid which is not blood stained but has a high specific gravity and total protein concentration, but without marked increase in alkaline phosphates activity, provides supporting evidence for a diagnosis of EGS. It must be noted however, that acute EGS cases often don't have any peritoneal fluid due to dehydration.

Barium swallow

Greet & Whitwell (1986) stated that barium swallow offered a valuable aid to the ante-mortem diagnosis of chronic EGS. Barium swallow and image intensification radiography demonstrates oesophageal dysfunction. In a study, 25

horses suspected to have grass sickness were given a barium swallow. Using image intensification the passage of the barium bolus was followed radiographically from the pharynx to the stomach. As previously described, muscle atony from the pharynx to the rectum is characteristic of EGS. All 18 horses in which grass sickness was later confirmed at post-mortem examination showed defective oesophageal motility. In some of these cases contrast material passed fairly rapidly to the thoracic inlet where it pooled and in others, the barium descended the cervical oesophagus very slowly without peristaltic movement. It is suggested that the radiological abnormalities seen in horses with EGS results from neurological impairment to the oesophagus. Pooling of contrast at the level of the thoracic inlet and marked oesophageal dilation are indicative of neuromuscular impairment of oesophageal motility but are not always present and are not necessarily specific to grass sickness (Greet and Whitwell 1986).

Phenylephrine eye drops

More recently, the effect of an ocular administration of the alpha-1 adrenergic agonist phenylephrine was evaluated as a diagnostic aid in EGS (Hahn & Mayhew, 2000). It has been hypothesised that ptosis, or downward displacement of the upper eyelid, may be due to sympathetic denervation and that EGS cases would respond to a topical administration a of sympathetic agonist (Hahn & Mayhew, 2000). Hahn & Mayhew (2000) used 23 cases of grass sickness and 12 control horses and administered 0.5ml of 0.5% Phenylephrine to one eye in each horse. The angle of the eyelashes to the cornea were evaluated 30 minutes later by comparing the treated and control eyes. The angles of the eyelashes to a line drawn between the medial canthi of the eyes were measured

and the differences analysed. A significant difference ($P<0.001$) between the horses with grass sickness and the control horses was observed and the authors concluded that the use of phenylephrine eye drops is a useful technique in the diagnosis of grass sickness. There was however, a 25 per cent overlap between the highest responding control animals and the lowest responding grass sickness case.

Rectal biopsy and plasma catelcholamine levels.

The detection of loss of gut peptides on immunocytochemical examination of rectal biopsy tissue was discussed by Pinsent (1989). Unfortunately, there is much less loss from the rectum than from the ileum and large colon. Another suggested option (Pinsent, 1989) is the measurement of plasma catelcholamine levels because these are higher in horses with EGS than normal horses. However, the assay is apparently difficult and time consuming.

Exploratory laporotomy and Ileal biopsy

An exploratory laporotomy is often justified in suspected cases of EGS to eliminate a physical obstruction of the gastrointestinal tract that could be corrected surgically (Edwards, 1987). At laporotomy, subsequently confirmed cases of EGS show severe secondary impaction of the large colon, colonic malposition, fluid distension of the small intestine and grossly abnormal motility with vigorous incoordinate contractions and severe spasm in exteriorised small intestine or, flaccid intestine which shows no evidence of motility (Edwards, 1987).

The ante-mortem diagnostic technique based on an ileal biopsy was developed by Scholes *et al.*, (1993b). Severe enteric neuronal degeneration in the ileum had been described in parallel with sympathetic ganglionopathy, which

suggested that histological examination of ileal biopsies might provide a means of diagnosing EGS anti-mortem.

A diagnosis from an ileal biopsy can be obtained within 24-36 hours. Under general anaesthesia, a full thickness elipse, 15mm x 10mm is taken midway between the mesenteric and antimesenteric borders of the ileum 5cm distal to the origin of the ileocaecal fold (Edwards, 1987). The biopsy is immediately place in Bouin's solution or 10% formal saline, fixed, wax embedded, and sections are subsequently stained with haematoxylin and eosin.

In order to evaluate the diagnostic value of an ileal biopsy, Scholes *et al* (1993b) compared ileal sections from 18 horses with EGS, 15 horses with other alimentary disease and 3 horses without gastrointestinal disease. In the ileal biopsies, severe neuronal degeneration and loss were observed in the horses with EGS only. Numerous, apparently normal enteric neurons were found in horses with anterior enteritis, spontaneous ileus, ileal impaction and in viable intestine from horses with strangulation obstruction colic.

The results of this study suggested that the demonstration of severe ileal ganglionopathy without significant inflammatory or other morphological changes, in association with general gastrointestinal stasis in horses over one year of age is diagnostic for grass sickness. The ileal biopsy can eliminate the need for further diagnostic investigation and minimising the distress these horses suffer. However, Milne *et al.*, (1994) suggested that under certain circumstances, subjecting a chronic case of EGS was unwise because the prognosis might be adversely affected and the cost considerable.

Treatment & Recovery

Greig (1942) was the first to report that recovery of EGS cases is rare. More recently reports have stated that there is no effective or useful treatment for grass sickness (Edwards, 1987, Pinsent 1989).

Clinical relief is available following gastric decompression. This will relieve the visceral pain following gut distension. Almost continuous fluid therapy is also indicated but death will occur as soon as this is discontinued (Edwards, 1987). On humane grounds euthanasia, without delay is the only logical course to take (Pinsent, 1989)

The view that prognosis is hopeless in treating cases of chronic grass sickness has been challenged (Milne, 1997b). Case selection is paramount in avoiding unnecessary suffering and the delay of euthanasia. Criteria for selecting cases for treatment include no gastric reflux, no dyspahgia and the horses must have survived for at least seven days. Apparently, a diet of high density carbohydrate, fat and protein which is palatable and easily swallowed should be fed along with frequent grooming, short walks in hand and constant human contact (Milne, 1997b).

A clinical trial has also been carried out to determine the effect of cisapride on rate of passage of digesta and clinical parameters in horses with chronic grass sickness (Milne *et al.*, 1996). Cisapride is a prokinetic agent used in the treatment of equine postoperative ileus. The drug facilitates acetylcholine release from the postganglionic cholinergic nerves of the myenteric plexus of the gut (Lee *et al.*, 1984). It is suggested that because some morphologically normal neurones remain in the myenteric plexus of the gut in chronic grass sickness, cisapride might be of therapeutic benefit in this disease. The results of the study

concluded that, cisapride administered intra-muscularly or orally has a beneficial effect on gut motility in cases of chronic EGS. Selection of appropriate cases is emphasised and additional management factors should not be ignored.

It is claimed that following the above methods of treatment, a recovery rate of 70% can be achieved in selected chronic cases (Milne, 1997a). However, in those cases that do recover, it is often where no definitive anti-mortem diagnosis has been made. These cases are then suspected to have been misdiagnosed.

It is reported that all surviving horses (27, 4 subsequently died) were capable of ridden work or breeding activity but many horses suffer residual problems such as excessive sweating and swallowing difficulty. Doxey *et al.*, (1995) also reported that horses recovering from EGS showed marked and often erratic changes in behaviour and appetite.

Milne *et al.*, (1994) evaluated the use of clinical measurements to predict the outcome in chronic cases of EGS. Dyspahgia and colic were significantly more severe, the appetite was poorer and the gut sounds were more severely reduced in non-survivors. Ponies were significantly less likely to survive than cobs and none of the survivors had severe rhinitis. It was concluded that the degree of dyspahgia and the audibility of gut sounds were useful prognostic indicators. This however, was expected because they may be directly related to the degree of neuronal damage (Milne *et al.*, 1994).

Conclusion

This review shows that the clinical signs exhibited by EGS cases are well described. How the clinical signs arise however, has yet to be elucidated.

Sympathicotonia, or stimulation of the sympathetic nervous system, is a relatively common hypothesis but this hypothesis will not be proven, or otherwise, until the causal agent of this disease is uncovered and the pathophysiology of this disease more fully understood.

The neuropathology of EGS is also well understood has enabled a valuable method of ante-mortem diagnosis of EGS cases but there however, a need for a more simple and quick method of diagnosing the disease to prevent further suffering of these already distressed cases. In an ideal world, a simple and quick diagnosis from a blood sample would be perfect.

It can be concluded that even though we know a lot about EGS, understanding the pathophysiology, identifying a more simple diagnostic method and improving the treatment of EGS cases will not be advanced much further until the aetiological agent and causal pathway of this distressing disease are identified. Until this happens, epidemiology can suggest rational disease prevention strategies by identifying risk factors for this disease of unknown aetiology.

Project Aims and Objectives

A CASE-CONTROL STUDY TO INVESTIGATE RISK FACTORS FOR EQUINE GRASS SICKNESS WITH A PARTICULAR REFERENCE TO THE ROLE OF *CLOSTRIDIUM BOTULINUM.*

This study aims to investigate the hypothesis that equine grass sickness is associated with toxicoinfection with *Clostridium botulinum* type C. A case-control study was conducted to test this hypothesis. The aim was to measure exposure to *Clostridium botulinum* type C and its C1 neurotoxin in 60 histologically confirmed cases of grass sickness, and 120 co-grazing, matched controls.

In addition to serological evidence of exposure to *Clostridium botulinum* type C, horse-level risk factors were to be evaluated. Examples of potential horse-level risk factors include; age, breed, duration on pasture and change of pasture, feed supplementation, anthelmintic use and faecal nitrogen content.

This study also aims to test the hypothesis that pasture level factors are important in grass sickness. This secondary hypothesis assumes that pasture-level variables are critical in stimulating toxin production and in compromising normal resistance in certain individuals. The aim is to evaluate pasture-level risk factors by comparing 60 pastures that had given rise to cases of equine grass sickness, and 120 control premises. Examples of possible pasture-level risk factors include; geographical location, meteorological conditions, pasture type, pasture and soil nitrogen content, soil pH, fertilisation.

Using information and data collected throughout the study period, relationships between potential horse-level and pasture level risk factors and grass sickness are to examined using univariable and multivariable techniques. A proposed causal pathway demonstrating the potential role of *Clostridium*

botulinum type C, horse-level and pasture-level risk factors are illustrated in figure 1.10.

In addition, the location and time of diagnosis for each case of grass sickness will be mapped and used to detect and describe the degree of space-time clustering of equine grass sickness cases in England and Wales. Information from this study will potentially assist in the rational formulation of disease diagnosis, treatment and prevention strategies for grass sickness.

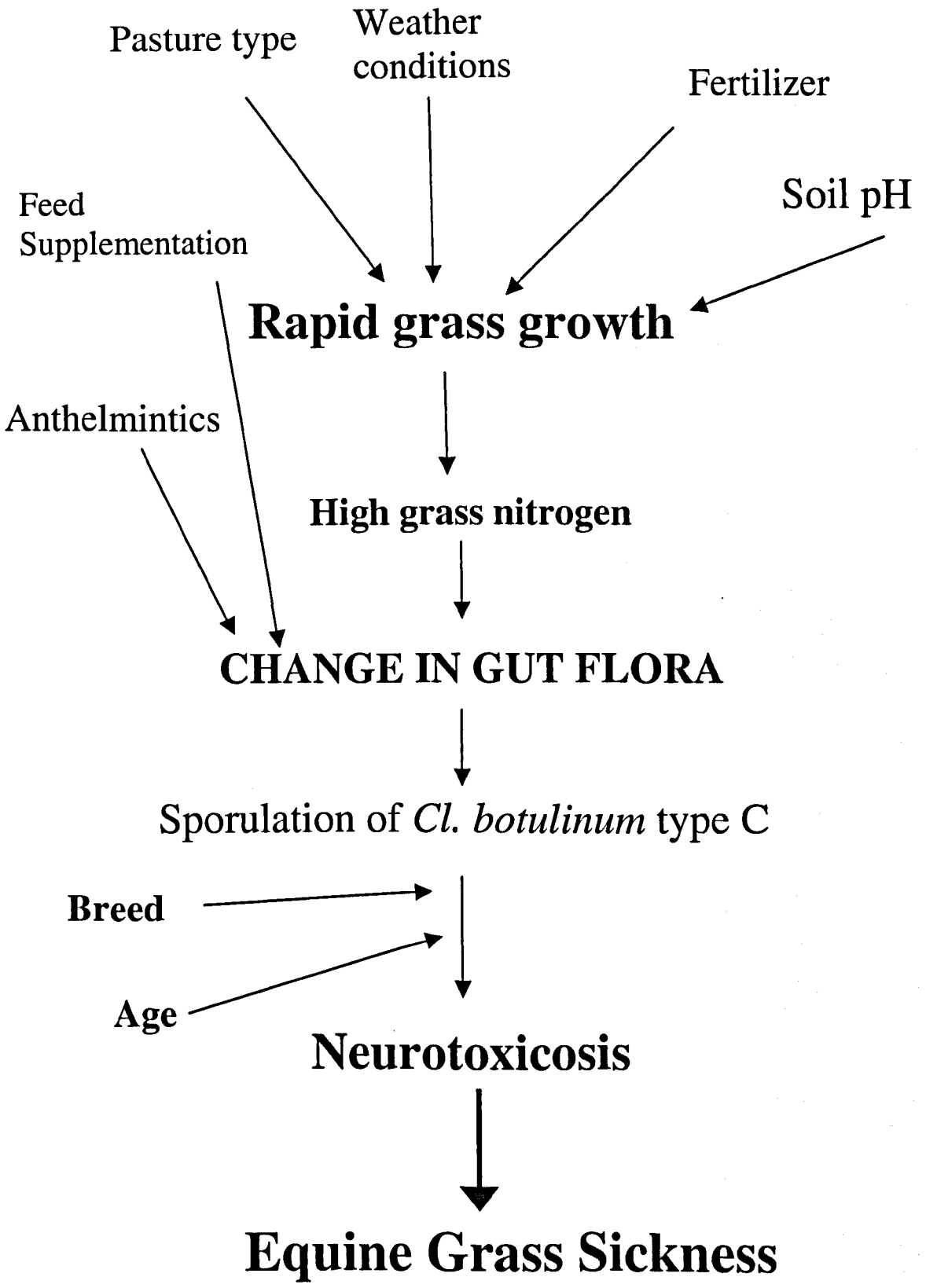


Figure 1.10: Proposed causal pathway by which pasture and other variables lead to toxicoinfectious botulism resulting in clinical grass sickness.

CHAPTER 2

THE EPIDEMIOLOGY OF EQUINE GRASS SICKNESS - A LITERATURE REVIEW (1909-1999)

The Veterinary Record, Volume 149, Number 10. September 8, 2001

SUMMARY

The epidemiology of equine grass sickness is reviewed in this chapter. The geographical spread of the disease between 1909 and 1999 is described with particular reference to the occurrence of the disease in England and Wales. Experimental investigations that attempted to identify a causal agent are also summarised. The epidemiological techniques used to investigate grass sickness vary from clinical observations, to more advanced methods such as case-control studies using logistic regression analyses. A number of risk factors for grass sickness have been consistently reported (age, time of year and recent movement of pasture/premise). A number of possible risk factors have been reported for which findings remain inconsistent (weather, pasture type, breed, supplementary feeding and anthelmintic use). More focused epidemiological investigations are required to identify more fully those horses and premises that may be at a higher than normal risk of disease.

INTRODUCTION

Grass sickness is a largely fatal disease of horses, ponies and donkeys that was first recognised early in the twentieth century. The disease is a dysautonomia, characterised by severe and extensive lesions in neurons of the autonomic and enteric nervous systems (Pollin & Griffiths, 1992). Clinical signs include sweating, muscle fasiculations, tachycardia (Greig, 1942) and clinical biochemistry reveals elevated plasma catelcholamines (Hodson *et al.*, 1984), both are compatible with over-activity of the sympatho-adrenal system. The only

definitive way of diagnosing grass sickness is histologically from an ileal biopsy taken at surgery or from sympathetic ganglia and/or ileal tissue at post mortem examination (Scholes *et al.*, 1993b).

The causal agent of grass sickness is unknown. Associations between the disease and a number of infective or toxic agents have been investigated. Until recently, none was convincingly associated with disease. The search for a single causal agent has recently made way for epidemiological research looking at a multifactorial aetiology.

This paper reviews over 80 years of epidemiological research on grass sickness, summarises current knowledge of the disease and identifies gaps in our knowledge that could be addressed epidemiologically.

GEOGRAPHICAL SPREAD OF GRASS SICKNESS

There are a number of reports of the first occurrence of grass sickness in several regions of the UK since 1900. The 'erratic' occurrence of grass sickness is one of the main features of this disease (Guthrie, 1940). The distribution of disease varies from year to year and often only one animal in a grazing group is affected. Greig (1942) speculated that; 'there is little doubt that these are not chance occurrences but that there is some fundamental law, as yet not understood, which governs their manifestation'.

Scotland

The first reported outbreak of grass sickness occurred in 1909, in army horses at Barry Camp near Broughty Ferry, Angus (Tocher *et al.*, 1923). It is possible that a number of sporadic cases occurred before this date but the clinical syndrome had not been recognised or described (McKay, 1958). These early cases were diagnosed by veterinary surgeons as 'obstinate cases of impaction' of the large intestine (Guthrie, 1940).

Between 1911 and 1922 outbreaks of grass sickness occurred in the county of Angus and also in the east of Perthshire with the distribution of cases being irregular. In 1913 in Angus, many horses died of the disease and the disease then gradually spread to other parts of Scotland. Until 1918 grass sickness was apparently confined to the east of the River Tay. After this date it appeared to the west of the Tay but in then spread westwards and northwards to Perth and Aberdeen (Tocher *et al.*, 1923, Greig, 1942). The disease also spread south to parts of the Lothian's, Berwick, Wigtown, Dumfries, Kirkcudbright, Ayrshire and Lanark but the West Highlands remained relatively free (Guthrie, 1940). By 1928, grass sickness had been described in every county of north east Scotland but it was still the south eastern part of the country which had the highest number of recorded cases (Pool, 1928).

Grass sickness was first reported in Orkney in 1938 (Greig, 1942). Thirteen cases were recorded between 1938 and 1940. This was the first reported record of its occurrence on any of the Scottish islands (Greig, 1942).

In 1923, an effort was made to plot the geographical distribution of grass sickness in the east of Scotland (Tocher *et al.*, 1923). Following the outbreak at Barry camp in 1909, cases next occurred at Broughty Ferry in 1911. In the following two years a further eight farms were affected in the area immediately north of Barry Camp and Dundee. The disease became more widely distributed by 1917 moving out towards Blairgowrie, Kirriemuir and Forfar (Figure 2.1). There was high mortality from the disease in 1919 near Dundee and Coupar Angus and during 1919 and 1922 its spread was mapped in Morayshire, Banffshire and Aberdeenshire. The first case in these areas was seen around the Elgin area. In Morayshire, during this period 128 of 888 horses (14.4%) died. Tocher reported at the time that no theory could explain the spread of disease and it was undecided whether the distribution of cases resembled that of a contagious epidemic disease or a disease arising from the spread of a sporing organism.

England

Since 1911, grass sickness has not only been recorded in every county in Scotland, but also most counties of England and Wales. There is some discrepancy in the dates of when these grass sickness cases first occurred but reports emphasise the fact that grass sickness has occurred in England and Wales for almost as long as it has occurred in Scotland. Guthrie (1940) described the occurrence of grass sickness in many counties of England & Wales (figure 2.2) as well as dates of when the disease first occurred (Table 2.1).

From 1911, the cases recorded in England were initially confined to the northern counties of Northumberland and Cumberland (Greig, 1942). A report by the Agricultural Research Council in 1939 reported that in the previous few years several hundred cases had occurred in England (Greig, 1942) outbreaks having been recorded in the Severn Valley, Essex and the east and north Ridings of Yorkshire.

Begg (1936) reported the occurrence of grass sickness in Hampshire, and Wylie (1936) reported a case in Essex. During the October and November of 1939 two suspected cases were reported in Wiltshire and a third in Hampshire (Wallace, 1940). A three-year-old shire mare from the Midlands was clinically diagnosed with grass sickness in May 1941. It was thought that in the same part of the country the deaths of two horses with acute abdominal pain, may have been due to the disease (Lee, 1941). There was further evidence of the spread of the disease in 1945 with the report of two cases in Northamptonshire (Ellis & Cooper, 1945).

Three cases occurred on the same premises in Leigh, Lancashire in 1963 (Greer & Bardgett, 1963), but in the same report it was mentioned that the staff at the University of Liverpool veterinary field station had seen only two other cases in the previous ten years. In 1971 two cases were reported at a Thoroughbred stud in Surrey (Limont 1971).

Figure 2.1: Maps showing the origin and spread of grass sickness in Scotland (fatal cases). (After Tocher *et al.*, 1923). Maps not to scale.

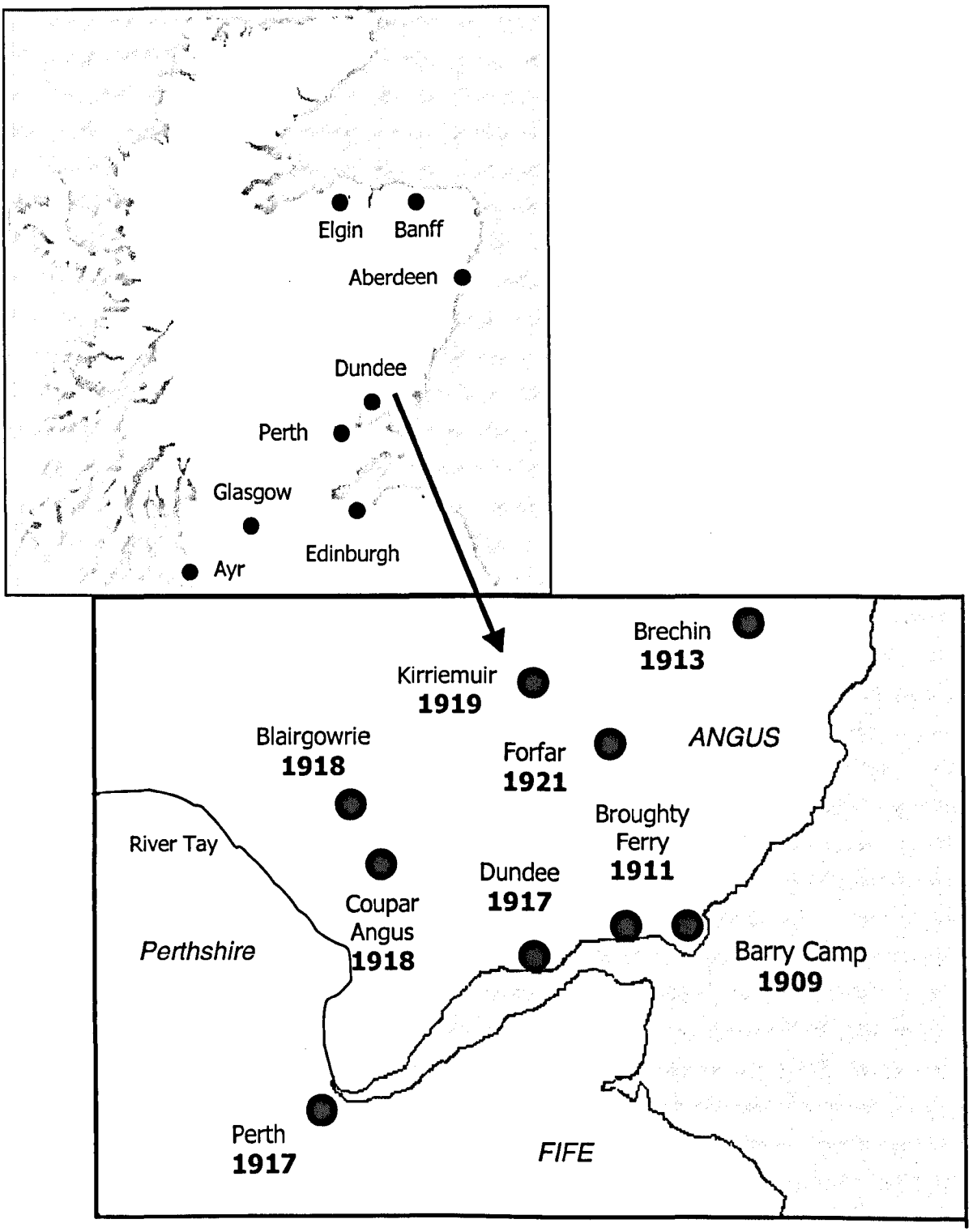
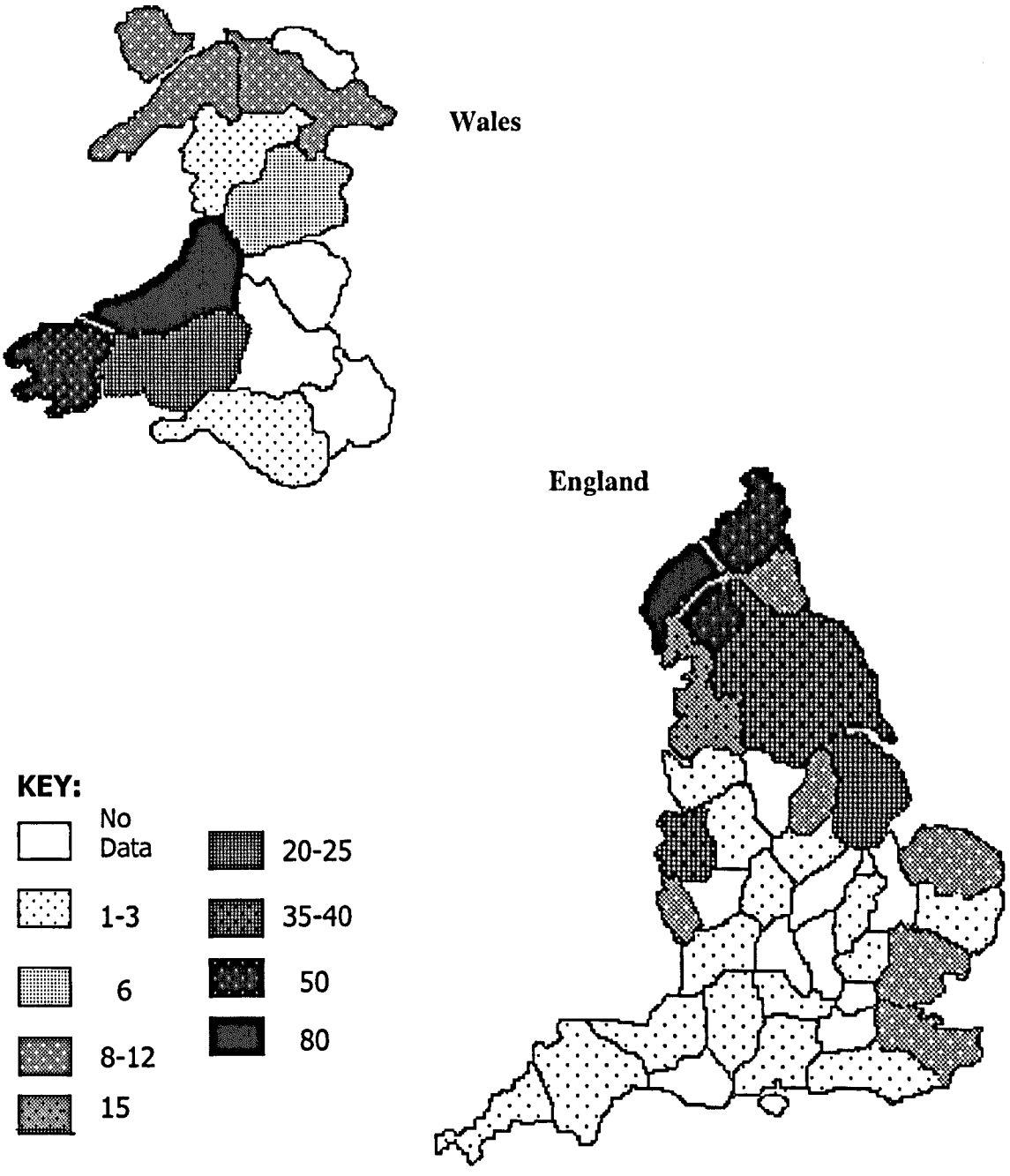


Figure 2.2: Number of Grass Sickness Cases in England & Wales. Approximate Number Of Cases Per Year, Pre 1974 County Boundaries (After Guthrie, 1940



Maps not to scale

Table 2.1: First Appearances of Grass Sickness in England and Wales as reported by Guthrie (1940).

County (pre 1974 county boundaries)	Date and location GS first recognised
Cumberland	Brampton, 1915
Northumberland	Alnwick, 1915
Westmoreland (Cumbria)	Kendal, 1935
Pembroke	Pembroke, 1925
North Riding (Yorkshire)	Harem, 1915
Shropshire	Severn Valley, 1930
Carmarthen	Kidwelly and Pembrey, 1897
West Riding (Yorkshire)	Blackburn, 1900
Lancashire	Liverpool, 1919
Kent	Eltham, 1915
Denbighshire	Denbigh, 1939
Caernarfon & Anglesey	Anglesey, 1936

Wales

Grass sickness is believed to have occurred first in Wales in 1897 (Guthrie, 1940). However, other reports claim that the disease did not occur until the early 1920's (Lloyd, 1934, Thomas, 1943). The first case apparently occurred at St Dogmeals, Pembrokeshire in 1922 and grass sickness was also reported in Carmarthenshire in 1928 (Lloyd, 1934). In 1934 the disease occurred almost

exclusively to the south of the river Teifi, and was most prevalent in the St Dogmeals, Moylgrove and Eglwyswrw districts. By 1940 grass sickness was described as 'rife' in southwest Wales, it was widespread throughout the counties of Carmarthen, Cardigan and Pembrokeshire (Guthrie, 1940, Thomas, 1943). It was reported that acute cases were more common in the spring with chronic cases being more common in autumn. On one occasion six cases were witnessed on one farm during one visit (Thomas, 1943).

Northern Ireland

In June 1941, a three-year old gelding from Belfast was clinically diagnosed with the chronic form of the disease. The horse was diagnosed by a veterinary surgeon who had just returned from Scotland where he had seen a number of cases of the disease (McFarlane, 1941). A tentative diagnosis of grass sickness was made on a second horse in Northern Ireland in the same year (McColl, 1941).

Ireland

Grass sickness is widely believed not to occur in Ireland but there is evidence to suggest an extremely low incidence. Mullaney (1957) described a six-year old hunter with clinical signs and post mortem findings that resembled grass sickness and a tentative diagnosis of grass sickness was made on the basis of clinical signs and gross pathology.

Mainland Europe

Grass sickness is well recognised in Sweden and Denmark (Bendixen, 1946, Obel 1955 Lannek *et al.,* 1961). The disease has also been reported in Germany (Mayer and Valder, 1968, Schulze *et al.,* 1997), France (Lhomme *et al.,* 1996), Switzerland (Arnold *et al.,* 1981), Holland (Leendertse, 1993) and Belgium (Christmann *et al.,* 1999).

World-wide

A single case of grass sickness has been histologically confirmed in the Falkland Islands (Woods & Gilmour, 1991) and also in Australia (Stewart, 1977). A condition indistinguishable from grass sickness known as mal seco is recognised in Argentina, the Falklands and Chile. Both the clinical signs and post mortem findings of mal seco are similar to those of grass sickness (Uzal *et al.,* 1992). It has been found that the histological lesions in horses with mal seco are also very similar to those described in the ceoliaco-mesenteric ganglia of horses with grass sickness (Uzal *et al.,* 1996). It has been suggested that mal seco and grass sickness are the same disease (Uzal *et al.,* 1992).

PUTATIVE AETIOLOGICAL AGENTS

Plants

In 1918 there was an attempt to identify plants and clovers that may exert a toxic effect on horses. A survey of infected areas in Forfarthshire and Perthshire

was conducted and a search for the following plants all proved negative; tormentil (*Potentilla tormentilla*), hemlock (*Conium maculatum*), wild cheviril (*Chaerophyllum sylvestre*), rough chevril (*Chaerophyllum taenulum*) and stinking mayweed (*matricaria suavolens*) (Guthrie, 1940). Experiments were also conducted to test the toxicity of alsike clover (*Trifolium hybridum).* Two horses were grazed for an entire season on two acres of land sown with alsike clover. A second experiment used alcohol extracts that were fed to a four-year old horse. No ill effects were noticed from these experiments (Tocher *et al.,* 1923).

Bacteria

Clostridium botulinum (Bacillus botulinus)

In 1919, a series of post mortem examinations on horses that had died from grass sickness suggested they succumbed to a very acute toxaemia of bacterial origin (Tocher *et al.,* 1923). A large anaerobic bacillus grown from portions of stomach and intestines had the morphological character and toxigenic properties of *Bacillus botulinus.*

In vivo experiments involved injecting horses with *Bacillus botulinus* toxin obtained from culture (Buxton, 1922, Tocher *et al.,* 1923). The horses died after developing dysphagia, becoming recumbent and showing evidence of cardiac and respiratory failure. Post mortem findings were similar to horses that had died of grass sickness. It was concluded that the bacillus and its toxin produced lesions that were identical to those found in horses suffering from grass sickness (Buxton, 1922, Tocher *et al.,* 1923).

In 1921, a therapeutic antitoxin was produced from known strains of *Bacillus botulinus.* In those cases in which an ultimate recovery did not occur, the acute symptoms of the disease were ameliorated by the intravenous administration of antitoxin (Buxton, 1923).

A vaccination trial was conducted with a toxin/antitoxin mixture (Tocher *et al.,* 1923). The trial consisted of 1,316 horses, 961 horses were immunised and 355 were left as non-treated controls. The number of cases dropped during the trial and reduction in mortality of 6.5% was found to be significant; $P<0.0001$ (after Tocher *et al.,* 1923).

In 1924, the *Bacillus botulinus* theory came under intense criticism. The bacillus isolated from spleens of grass sickness cases had not been shown by scientific methods to be identical to *Bacillus botulinus.* The anti-botulinus serum was not made from the grass sickness organism and some researchers felt that immunisation did not make any appreciable difference to mortality. Finally, an article in The Lancet in 1927 concluded that there was no association between grass disease and botulism (Anon, 1927).

In an attempt to resolve this issue, Walker (1929) stomach tubed ponies with varying doses of cultures of either type A or type B toxin of *B. botulinus.* He reported that the clinical symptoms and post mortem findings produced by botulism in horses are quite different from those produced by grass sickness.

More recently, Poxton *et al.,* (1997) investigated the role of *Clostridium botulinum* (type C) in grass sickness. Serum from grass sickness cases and control horses contained antibodies to the C1 toxin and cell-surface antigens but no clear

relationship was found between disease and antibody levels. Hunter *et al.*, (1999) demonstrated the presence of *Clostridium botulinum* (type C) toxin in the ileum and faeces of horses with grass sickness.

Clostridium perfringens

Ochoa and de Velandia (1978) investigated the role of *Clostridium perfringens* (type A) in recovered cases of grass sickness in Columbia. These horses (diagnosed by clinical signs only) had seroneutralisation titres higher than horses that had no record of having the disease. They concluded that enterotoxic *Clostridium perfringens* (type A) is involved in clinical cases of grass sickness as an etiologic agent or as a complicating organism. In contrast, Gilmour *et al.*, (1981) found a negative serological association between cases of grass sickness in Scotland and *Clostridium perfringens* (type A). The sera from acute and chronic cases of the disease failed to neutralise either crude or partially purified enterotoxin.

Insect vectors

In 1934 it was noticed that some of the areas of maximum incidence of the springtail *(Sminthurus viridis)* sometimes coincided with areas affected by grass sickness. Numerous sweepings of springtail and other insects have failed to produce any definite symptoms of grass sickness when fed to horses (Lloyd, 1934).

An extensive survey of the insect populations of infective and non-infective pastures in South Cardiganshire and Pembrokeshire was also made. No relationship between pastures and insect populations was found (Anon, 1936).

Fungi

A variety of non-endophytic, toxin producing fungi have been isolated from the stomach, colon and rectum of healthy horses and those with grass sickness (Doxey *et al.*, 1990). The survey did not associate any particular fungal species with equine grass sickness.

Robb (1996) investigated three groups of fungi (*Fusarium* spp., endophytic fungi and *Penicillium* spp.) which are toxigenic and grow at the base of plants. Endophytic fungi were identified in less than twenty percent of plants examined on grass sickness premises and *Penicillium* spp were not identified at all. The infrequency of these organisms in fields where grass sickness has occurred makes their involvement in the disease unlikely (Robb, 1996). *Fusarium* spp however, were found on all samples examined but no conclusions can be made of its effect on nerve cells (John, 1996, Robb, 1996). Uzal *et al.*, (1996) failed to reproduce mal seco by feeding horses a plant (infected by an endophytic fungus) native to Argentina known as *Festuca argentina.*

EPIDEMIOLOGICAL INVESTIGATIONS

The failure to create an experimental model of the disease by the use of infectious or poisonous materials has made it necessary to look elsewhere for an explanation for grass sickness (McKay, 1958). Identification of predisposing causes of grass sickness may be assisted by identification of those horses and premises that are at a higher than normal risk of disease.

Since the 1930's a number of epidemiological investigations have been undertaken to identify risk factors for grass sickness (summarised in table 2.2). Risk factors for grass sickness that have been investigated are summarised in table 2.3. In the following description, 'P' values are given if there is a statistically significant relationship between the factor and disease at a 5% level. Odds ratios (OR) and 95% confidence intervals (95% C.I.) are also cited if available.

Other Equids.

Dysautonomias with similar histopathology to grass sickness have been reported in equids other than the horse. In 1977 it was reported that acute grass sickness occurred in a Przewalski's horse, and chronic grass sickness in a common zebra at Whipsnade zoo (Ashton *et al.*, 1977). Prior to this the disease had only been reported in the horse, pony and donkey. Histological examination of the autonomic ganglia from both cases revealed lesions consistent with a diagnosis of grass sickness (Ashton *et al.*, 1977).

Breed

Anecdotal reports suggest that heavy, draught horses are at increased risk of grass sickness. In a univariate analysis, heavy horses were found to be at increased risk (OR = 5.0, P = 0.03) when compared to all other breeds in a case control study (Wood *et al.*, 1998). However, after adjusting for confounding by other variables, the relationship between breed and grass sickness was no longer significant.

Age

This was one of the first risk factors to be identified. Guthrie (1940) found the highest prevalence to be in four-year olds and Greig (1942) noted that the prevalence of disease was very high between the ages of three and six years. In 1939 and 1940, the percentage mortality (and morbidity) of the different ages was calculated using the National Farmers' Union Insurance Society figures (Greig, 1942). A total of 6617 horses were insured, 3.49% of these horses died of grass sickness. The age of horse at which mortality was greatest was 4 years (7.29% mortality).

More recent investigations have also shown that younger animals are more likely to be affected by grass sickness than older animals (Gilmour & Jolly, 1974; Doxey *et al.*, 1991b, Wood *et al.*, 1998). Gilmour & Jolly (1974) reported that equine grass sickness is more frequently encountered in animals between the ages of two and seven years of age than in the younger or older age groups.

In a telephone-based case control study, Barrett *et al.*, (1992) reported that the age of grass sickness cases was significantly younger than that of other cases of fatal colic ($P<0.02$). In this study grass sickness cases were all aged between 2 and 12 years.

Wood *et al.*, (1998) reported that horses between the ages of three and five years were at nearly a six fold greater risk of disease (OR = 5.7) compared to those horses which were ten years or older. Horses which were less than three years old had almost a 5 fold greater risk (OR = 4.8) and those aged between six and nine had a three fold greater risk (OR = 3.1). After adjusting for the confounding effects of sex, time since grass sickness last occurred on the premise, contact with previous grass sickness and time since field change, the relationship between age and disease was still significant. Horses between the ages of three and five years were at greatest risk of grass sickness (OR = 7.1, P = <0.001, 95% C.I. 3.1-16.3).

It has been suggested that older animals are resistant to equine grass sickness. The longer they spend in a field, and the older they get, the less likely they are to become affected (Wood *et al.*, 1998). It is also possible that non-fatal exposure to the causative agent of grass sickness induces resistance to the disease.

Interestingly, grass sickness is rarely reported in foals and horses less than one year of age. The reasons for this are unknown and very rarely referred to in the literature. Out of three publications where precise age data is given on 212 grass sickness cases, 6% of cases were under two years of age (VCOU 1970, Gilmour and Jolly 1974, Barrett *et al.*, 1992).

Pasture

As the name suggests, the occurrence of grass sickness is strongly associated with access to grass. Animals kept solely on pasture experience the disease with greater frequency than those that are stabled for part of the day (Gilmour and Jolly 1974). Rare exceptions to this have been reported where entirely stable-kept horses have suffered the disease. Lannek *et al.*, (1961) reported seven horses dying in two stable yards in Sweden. In 1927, the disease was reported in a number of pit ponies that received no grass at all (Forsyth, 1941).

Guthrie (1940) observed that in Scotland most cases occurred on temporary pastures, especially those of two or three years old. However, in England and Wales, more than 90% have been reported to occur on permanent pastures. More recent studies have reported that there is no significant association between the nature of pasture and grass sickness (Gilmour & Jolly 1974, Doxey *et al.*, 1991b). Grass sickness is however more likely to occur on previously affected pastures than those pastures where grass sickness has never been recorded (Gilmour and Jolly 1974, Wood *et al.*, 1998).

Low levels of pasture selenium have been implicated in grass sickness. Macpherson (1978) noted that the majority of grass sickness cases appeared on farms where muscular dystrophy in cows was a problem. Selenium levels in horses suffering from grass sickness were reported to be low (Macpherson, 1978). However, blood levels from apparently healthy horses on these farms were also

low thus providing little evidence to support the theory that selenium deficiency was associated with grass sickness.

Change of pasture/premise

It has been observed frequently that horses suffer from grass sickness shortly after moving from one pasture to another. Greig (1942) reported on fifteen horses that had succumbed to grass sickness after changing pastures. He observed that ten out of the fifteen grass sickness cases had changed pasture in the preceding two days.

Doxey *et al.*, (1991b) found that 45% of cases in their study had moved from either one premises or one field to another in the two weeks prior to illness. Only 3% of horses in their control population regularly moved to fresh pasture at less than four-week intervals.

In the study by Wood *et al.*, (1998) a seventeen fold increase in risk of disease was found when horses had changed pasture in the two weeks prior to disease compared to those that had changed pasture in the last three months ($P = 0.021$, $OR = 17.4$). There was a five fold increase in risk if the horse had changed pasture within the last two to four weeks ($P = 0.004$, $OR = 5.3$) and nearly a three fold increase if the change was made in the last month ($OR = 2.6$). After adjusting for the confounding effects of sex, time since grass sickness had last occurred on the premise, contact with previous grass sickness and age, the relationship between change of pasture and disease was still significant. Horses that had

changed pastures within the previous two weeks were at greatest risk of disease (OR = 29.7, P = <0.001, 95%CI 6.7-130).

Diet

In a study by Gilmour and Jolly (1974) a high proportion of animals which experienced grass sickness were in groups receiving no supplement or only concentrates compared with those receiving hay and concentrates. This could potentially be confounded by stabling since most animals receiving concentrates only are kept outdoors whereas most receiving hay and concentrates are stabled. Wood *et al.*, (1998) found no evidence that supplementary feeding of hay or forage alone was associated with decreased risk of disease.

A change of diet has been implicated in the onset of grass sickness. Begg (1936) stated that the disease was always associated with a change of diet. McKay (1958) suggested that winter feed might affect susceptibility to disease due to the lower levels of protein in winter feed stuffs compared to young grass.

Body Condition

Doxey *et al.*, (1991b) found that horses in good body condition were more likely to contract grass sickness (P<0.005). However, work by Wood *et al.*, (1998) did not support these findings.

Anthelmintics

The possibility that anthelmintic administration is related to risk of disease requires further investigation. Preliminary work by Wood *et al.*, (1997)

showed that the more frequently animals were given anthelmintics, the more likely they were to be affected by grass sickness (OR = 2.0, P = 0.002, 95%CI 1.1-3.8). However, this finding was not confirmed by multivariate analysis on the full dataset (Wood *et al.*, 1998). It has been suggested that frequent removal of worms might interfere with gut function in some way that increases absorption of the neurotoxin (Milne, 1997b).

Soil

Stewart (1941) studied the soil from 63 farms on which grass sickness had occurred during the summer of 1938 in Aberdeenshire. Soil samples were taken within a few days of case occurrence from case premises. No control soil samples were taken. The results of the soil survey found that i) the occurrence of grass sickness was not confined to soils of any particular geological origin, ii) no relationship between soil texture and the incidence of grass sickness could be made, iii) almost all the soils examined fell into the class of acid soils (pH 5.5 – 6.2), iv) no relationship was found between grass sickness and magnesium, manganese, potassium or potash levels and v) the average values for carbon and nitrogen and for carbon: nitrogen ratios in the soil were higher than for those recorded for cultivated soils in south east England. The sufficiently wide range in values makes the possibility of a relationship between the occurrence of grass sickness and carbon: nitrogen ratio unlikely (Stewart, 1941).

Season

It is consistently reported that there is seasonal variation in the incidence of grass sickness. Begg (1936) stated that: 'the disease makes its first appearance for the season towards the last week in April, just as the grass is beginning to show the first signs of growth and when the weather is getting milder. However, if April is particularly warm following a wet March, then cases appear early and are greater in number. It is noticeable that a spell of warm weather following a wet period brings large number of cases'.

Guthrie (1940) reported that the great majority of cases occur in June, with the peak period in the early part of the month although cases can occur all year round. The Veterinary Clinical Observation Unit (VCOU) report (1970), Doxey *et al.*, (1991c) and Wood *et al.*, (1998) have all reported the month of May as having the greatest number of cases.

Weather

At present there is no good evidence that meteorological factors are important in the occurrence of grass sickness. Pool (1928) observed cases in fine dry, warm weather and suggested that frosty nights were a predisposing factor. Guthrie (1940), Greig (1942) and McKay (1958) have suggested that cases occurred in warm, dry, fine weather and that cold and wet conditions decreased disease incidence. Early morning ground frosts were also implicated in increased disease occurrence. Greig (1942) reported the occasional case during periods of hard frost and even when the ground was covered in snow. Doxey *et al.*, (1991c)

investigated local weather patterns associated with fifteen multiple outbreaks of grass sickness in Eastern Scotland between 1972 and 1988.

The meteorological stations closest to the case premises were located and weather records examined for the two weeks prior to an outbreak. On all fifteen of the case premises mean daily air temperature was below 6°C on only thirty-six occasions (17%) and only on eleven days did the temp exceed 20°C for all the premises studied. Ground frost was often present and 85% of pastures had grass temperatures lower than the previous ten-year average. 73% of premises had lower than average daily rainfall within two weeks of the first case. The conclusion made from this study was that the majority of outbreaks occurred during cooler (temperatures between 7 and 11°C) drier weather associated with irregular ground frosts. No data from control premises was collected for this study.

It has been suggested that weather conditions influence an east/west split in the prevalence of grass sickness in Scotland with eastern Scotland apparently having a higher prevalence of the disease. The weather pattern for eastern Scotland, compared to the west, shows a trend towards slightly cooler, drier weather with frosty mornings (Doxey *et al.*, 1991c).

Association of grass sickness with other animal diseases.

Barrett *et al.*, (1992) investigated the possible association between grass sickness and scrapie. A telephone-based case-control study was performed investigating fatal equine colics in Wales in 1988. Of a total of 84 fatal colics, 17

of these were grass sickness. The remaining 67 cases of fatal colics provided controls for cases of grass sickness. This study concluded that there was no association between grass sickness and scrapie (P=0.06).

Comparison of equine grass sickness (equine dysautonomia) with other species dysautonomias.

Dysautonomias have been reported in cats, dogs and humans. The pathology of these dysautonomias are strikingly similar with the clinical pictures less so. However, the differences in clinical picture can be attributed to interspecies differences (Gaskell, 1987b).

A preliminary epidemiological study in 1987 attempted to identifiy a number of associations with feline dysautonomia. The only convincing finding was the association with age where the age range of affected cats appeared to be skewed towards animals of less than 3 years of age (Gaskell, 1987). Berghaus *et al,* (2001) also found that dogs were at increased risk of canine dysautonomia if they were less than 3 years old. The association with dysautonomia and age is similar for cats, dogs and horses.

Berghaus *et al,* (2001) also presented results to suggest that dogs with dysautonomia were likely to live in rural areas and spend the majority of time outdoors when compared to the control group. Affected dogs were also more likely to have access to pasture land, farm ponds, cattle and, to have consumed wildlife occasionally. Gaskell (1987a) failed to make any association between

feline dysautonomia and cats that were allowed access to the outside and those that were confined to the house.

A seasonal pattern of canine dysautonomia has been identified where most cases occur during February and April (Berghaus *et al,* 2001). This finding can be compared to the seasonal pattern observed in equine grass sickness where most cases occur during April, May and June.

The epidemiology of equine grass sickness has been more fully explored than that of the cat and the dog. Further such investigations in the latter 2 species might identify several common risk factors that may eventually lead to a common aetiological agent.

Table 2.2: Summary of Epidemiological Publications on EGS: 1937 – 1998

Year of Publication	Author(s)	Country of Study	Year(S) of Study	Data Collection Method	Number of GS Cases	Number of Controls
1937	McKay	Scotland	1936	Interview questionnaire	Not given	None
1940	Guthrie	UK	1936 - 1939	Observations	Not given	None
1942	Greig	Scotland	1938 - 1940	Observations	Not given	None
1970	VCOU	Scotland	1968- 1969	Various	105	None
1974*	Gilmour & Jolly.	Scotland	1971	Interview questionnaire	90	2554
1991b*	Doxey, Gilmour & Milne.	Scotland	1972- 1989	Meteorol- ogical office	15 premises	0
1991c*	Doxey, Gilmour & Milne	Scotland	1970 - 1987	Interview & Postal questionnaire	60	1575
1992*	Barrett, Taylor & Morgan	Wales	1998	Telephone questionnaire	17	67
1998*	Wood, Milne & Doxey	UK	1992 - 1995	Postal questionnaire	135	226

VCOU - Veterinary Clinical Observation Unit * - Statistical analyses used

Table 2.3: Epidemiological Observations and Investigations in Grass Sickness Research

	Variable	Risk Factor	Reference(s)
Horse Level	Age	Young horses	VCOU 1970, Greig, 1942, Gilmour & Jolly 1974, Barrett, Taylor & Morgan, 1992. Doxey, Gilmour & Milne, 1991b, Wood, Doxey & Milne, 1998.
	Sex	No consistent findings.	Greig, 1942, Wood, Doxey & Milne, 1998.
	Breed	No consistent findings.	Wood, Doxey & Milne 1998.
	Body Condition	No consistent findings	Doxey, Gilmour & Milne, 1991a, Wood, Doxey & Milne, 1998.
	Grazing	Horses kept solely outdoors.	Guthrie, 1940, Greig, 1942, McKay, 1958, Gilmour & Jolly 1974, Doxey, Gilmour & Milne, 1991b, Wood, Doxey & Milne, 1998.
	Time on premise	Horses that have recently moved premise.	Gilmour & Jolly 1974, Wood, Doxey & Milne,1998.
	Time on pasture	Horses that have recently moved pasture.	Greig, 1942, McKay, 1958, Gilmour & Jolly 1974, Wood, Doxey & Milne,1998
	Supplementary Feeding	No consistent findings	Gilmour & Jolly 1974, Wood, Doxey & Milne,1998
	Change of diet	No consistent findings	Begg 1936, McKay, 1958
	Anthelmintic administration	No consistent findings	Guthrie, 1940, Wood, Doxey & Milne,1997, Wood, Doxey & Milne,, 1998.

	Variable	Risk Factor	Reference(s)
Pasture Level	The pasture	Pasture that has previously given rise to GS	Doxey, Gilmour & Milne, 1991b, Wood, Doxey & Milne,1998.
	Pasture type	No consistent findings	Guthrie, 1940.
	Soil conditions	No consistent findings	Stewart 1941.
Environment	Season	Spring	VCOU 1970, Begg (1936), Guthrie, 1940, Greig, 1942, Doxey, Gilmour & Milne, 1991c, Wood, Doxey & Milne, 1998,
	Weather conditions	No consistent findings	VCOU 1970, Pool 1928, Guthrie, 1940, Greig, 1942, McKay, 1958, Doxey, Gilmour & Milne, 1991c, Wood, Doxey & Milne,1998

Note: Gilmour & Jolly 1974, Gilmour & Milne 1991 and Wood, Milne & Doxey 1998 are the only authors to cite statistical analyses of their results.

CONCLUSION

A number of experimental studies have so far failed to identify the causal agent(s) of grass sickness. However, epidemiology has provided information that has helped understand the disease more fully.

A number of risk factors for grass sickness have been identified. Young horses with access to grass that have recently moved pasture or premise are at greatest risk of disease, especially during the months of April, May and June. Premises are most likely to give rise to a new case of grass sickness if it had previously given rise to the disease. Further understanding of risk factors associated with grass sickness is needed to provide horse owners and veterinary surgeons with greater power to prevent disease.

Case-control studies have been successful in identifying factors that may prevent or cause disease. This method involves the comparison of cases with controls (horses who are free of disease). The comparison reveals exposures that may differ in the two groups and may explain the occurrence of disease. Risk factors may either increase or decrease the risk of disease. A case-control study can quantify the alteration in risk associated with each factor individually and in combination (Schlesselman 1982).

More focused epidemiological studies are needed to better define those horses and premises that are at highest risk of disease, so that disease avoidance advice may be given. Studies investigating interactions between recognised risk factors may shed further light on the causal pathway of the disease. This review has identified gaps in our knowledge that need further investigation. For example,

information about localised weather patterns that precede an outbreak of grass sickness, and what factors in pasture or soil put a premise at higher risk would be particularly useful.

Perhaps the most important feature of epidemiology in unravelling this disease is its ability to accommodate multiple risk factors. The complex nature of the disease makes it unlikely that a single causal organism is responsible for disease. A more likely situation, suggested by the studies described above, is that of a complex causal pathway, where multiple factors must unite to cause disease. Epidemiology can describe and quantify these factors and, in the future, it may be possible to develop mathematical models of the disease.

This review has highlighted that grass sickness is not just a Scottish disease but one that occurs in England and Wales. There is scope to examine spatial and temporal patterns of disease using novel mathematical modelling techniques. Clustering of disease outbreaks in space (location) *or* time may provide clues to its aetiology. Clustering of disease outbreaks in space *and* time can provide evidence of contagion or an underlying spatially and temporally localised process (Diggle *et al.*, 1995). Such analysis may further enhance our ability to predict and prevent episodes of this disease.

Future epidemiological studies should also consider the investigation of immunological and genetic risk factors. There is scope to examine the importance of the major histocompatibility complex in susceptibility for grass sickness. The contribution of population-based research to the development of evidence for the

involvement of major genes is required to determine if certain breeding lines predispose to an increased risk of disease.

Epidemiology has greatly assisted our current understanding of equine grass sickness. Further studies are likely to make significant contributions towards the elucidation of a causal pathway. However, until this pathway is fully understood, epidemiology can inform the efforts of veterinary surgeons and horse owners in their attempt to prevent disease.

CHAPTER 3

SYSTEMIC IMMUNE RESPONSE TO *CLOSTRIDIUM BOTULINUM* TYPE C

INTRODUCTION

PART 1: *Clostridium botulinum.*

C. botulinum is a spore forming, gram positive, anaerobic, rod-shaped bacteria. The spores of this bacteria elaborate a stable and highly lethal toxin that is one of the most potent neurotoxins known, causing botulism through the inhibition of neurotransmitter release from cholinergic synapses. The spores of *C. botulinum* are extremely resistant and can survive for long periods in most environmental circumstances. In its vegetative form, the organism is a common inhabitant of the alimentary tract of herbivores (Smith, 1977).

Toxin types

There are 7 antigenically distinct types of *C. botulinum* classified as A, B, C, D, E, F and G. The organisms are classified by the different toxins they produce. Types A and B are commonly found in soils, while types C, D, E and F are most common in wet environments (Wobeser, 1987).

Types A, B, E and F are common causes of human botulism whilst types C and D are common causes of botulism in animals (Labbe and Shih, 1997). Types A and B toxin were designated in 1919 after different strains that caused outbreaks of human botulism in the USA (Hathaway, 1989). Types C and D appear to cause only animal botulism. Type C was designated in 1922 after causing outbreaks in cattle in Australia (Seddon, 1922) and chickens in the USA (Bengston, 1922). Type D is an organism similar to type C but produces a different type of toxin. Type D was responsible for outbreaks in cattle in South Africa in 1928. Type G is a soil dwelling organism with an apparently low toxigenicity (Simpson, 1989).

Phenotypic and genotypic groups of C. botulinum

There are four recognised physiological groups of *C. botulinum* based on phenotypic characteristics. Each is designated with a Roman numeral (I, II, III, IV). Each group is clearly distinguished from others genetically (Simpson, 1989). Group I contains organisms that produce type A, B and F toxin; Group II consists of type E and F toxin, whilst group III contains types C and D, and finally, group IV produces type G toxin.

Group III botulinum

C. botulinum type C and D are grouped together by their phenotypic properties into Group III.

The nontoxigenic *Clostridium novyi* is a clostridial species that is related to group III *C. botulinum.* Poxton (1984) showed a significant cross-reaction between *C. novyi* and *C. botulinum* type C and D and demonstrated that *C. novyi* and *C. botulinum* types C and D share common antigens.

Group III organisms can only be identified to the species/toxin type level by detection of the major toxin produced: type C and D neurotoxins for *C. botulinum* types C and D respectively and the novyi alpha toxin for *C. novyi* type A. These major toxins are each encoded on separate bacteriophages. The phage-host relationship is consequently unstable and the phage is readily lost. A cycle of phage loss and re-infection is thought to occur *in vivo* (Eklund and Poysky, 1974).

Toxin production by Group III C. botulinum

The toxins produced by group III *C. botulinum* consist of C1, C2, C3 and D toxin (Simpson, 1989). The Cα organism produces C1, C2 and small amounts of D toxin. Type D produces a major amount of type D toxin and a minor amount of C1. Type Cβ produces only C2 toxin (Jansen, 1987). The C3 exoenzyme is encoded on the bacteriophage together with C1 and D toxin.

C. botulinum type C was first isolated in 1922 from the fly larvae of *Lucilia caesar* (green bottle fly) obtained from a carcass of a chicken that had died from botulism in the US (Bengston, 1922). It was also isolated from cattle in Australia in the same year (Seddon, 1922). The organisms isolated from the two separate sources were similar enough to be given the same toxin type. However, differences in the ability of antitoxins to cross-neutralise the toxins from the two organisms led to the designations of subtypes Cα and Cβ (Gunnison and Meyer, 1929). Anti-sera raised against the Bengston strain cross-neutralised the Seddon strain, whereas antisera raised against the Seddon strain only neutralised the homologous toxin. The Bengston strain was designated as Cα and the Seddon strain as Cβ.

Bacterial Phages and group III

Bacteriophages are a group of viruses that infect specific bacterial species (Ekland *et al.*, 1989) and are associated with groups I, II and III. Only with group III strains is there an association with phages and toxigenicity of the bacteria. Eklund *et al.*, (1971) showed that bacteriophages converted non toxigenic cultures of *C. botulinum* type C to a toxigenic state and loss of toxigenicity is permanent once the phage is lost. The prophage-bacterium relationship is relatively unstable

(Eklund *et al.*, 1971, 1974.). Both types C and D no longer produce toxins when cured of their phage. They can become toxigenic once again by re-infecting the organism with the phage isolated from the corresponding toxigenic strain (Eklund and Poysky, 1974).

It has also been demonstrated that a type C strain cured of its phage could be converted into an organism that was indistinguishable from *C. novyi* type A by infecting it with a phage derived from a strain of *C. novyi* (Ekland *et al.*, 1974). It has been suggested that *C. botulinum* type Cβ is derived from Cα after Cα loses the phage responsible for producing type C1 toxin (Jansen, 1987). It has also been discovered that *C. botulinum* types C and D can be interconverted by phage (Eklund and Poysky, 1981). There is an inability to differentiate strains of *C. botulinum* type C from strains of type D after they have ceased to produce neurotoxin and the interconversion of these two types by different phages emphasises the close relationship of members within group III (Eklund *et al.*, 1989).

PART 2: TOXIN ACTION AND ROLE IN PATHOGENESIS

The 7 serotypes of botulinum neurotoxins induce a flaccid paralysis because they block the release of acetylcholine at the neuromuscular junction (Montecucco and Schiavo, 1994). Neurones release acetylcholine by exocytosis of synaptic vesicles from nerve endings. When an action potential arrives, the presynaptic plasma membrane depolarises and voltage gated calcium channels become activated. The rise in intracellular calcium triggers fusion of synaptic vesicles containing acetylcholine to the presynaptic membrane. Botulinum neurotoxin produces a blockade of exocytosis at various sites, including the

cholinergic neuromuscular junction, autonomic ganglia, postganglionic parasympathetic sites, postganglionic sympathetic nerves that release acetylcholine, and the adrenal glands (Wright, 1955; Gunderson 1980; Simpson, 1981).

Botulinum neurotoxins (BoNTs) are synthesised as a single inactive polypeptide chain of 150kDa and are released by bacterial lysis. The neurotoxins are composed of a heavy chain and a light chain bridged by a disulphide bond and are folded into 3 functionally distinct domains, which play different roles in cell intoxication (Montecucco and Schiavo, 1994). The mechanism of neurotoxin cell intoxication consists of four distinct steps involving (1) cell binding, (2) internalisation, (3) membrane translocation and (4) target modification in the cytosol. Whilst the intracellular action of the toxins has been well characterised, steps one to three are much less well experimentally defined.

Progenitor toxins

Botulinum neurotoxins are released in the form of complexes known as progenitor toxins. Progenitor toxins are a set of non-toxic proteins coded for by genes adjacent to the neurotoxin gene (Inoue *et al.*, 1996). It is believed that because the toxin is sensitive to the proteolytic and denaturing conditions found in the stomach, these non-toxic proteins enable the botulinum toxin to reach the intestine undamaged. Once within the intestine, the slightly alkaline pH causes dissociation of the toxin complexes (Schiavo *et al.*, 2000). It is hypothesised that the non-toxic proteins are made to preserve the botulinum toxin from proteolytic attack.

The toxin: non-toxic protein complex is found in three forms: 12S toxin, 16S toxin and 19S toxin. Type C and D toxins are composed of two forms; the 12S and 16S. The 16S toxin consists of a neurotoxin and a haemagglutinin. It is the haemagglutinin that has been found to be important in binding of type C *C. botulinum* toxin to the microvilli of epithelial cells of the small intestine in both in vivo and in vitro tests. The haemagglutinin has not been found to bind to the cells of the stomach or the colon (Fujinaga *et al.*, 1997). It is therefore concluded that the haemagglutinin of type C 16S is very important in the binding and absorption of *C. botulinum* type C toxin in the small intestine (Fujinaga *et al.*, 1997).

1) Cell binding (to neuronal membrane)

After diffusion into the body fluids from the site of production or absorption, BoNT's bind to the presynaptic membrane at the neuromuscular junction (Schiavo and Montecucco, 1997). BoNT protein receptors then drive these neurotoxins into small synaptic vesicles (Matteoli *et al.*, 1996). BoNT receptors are located on the motor neurone plasmalemma at the neuromuscular junction. These receptors must display high affinity if they are to bind minute concentrations of neurotoxins sufficient to cause death (Schiavo and Montecucco, 1997). The receptor for botulinum toxin has not been isolated.

2) *Internalisation.*

The toxin has to enter the cell cytosol to exert its toxic effect. After binding, toxins are internalised inside vesicles of unknown nature in a temperature and energy dependant process (Simpson, 1989; Schiavo and Montecucco, 1997). It is at this stage that the toxin can no longer be neutralised by specific antisera.

3) *Membrane Translocation (into the neuronal cytosol)*

The toxin must undergo conformation change and pass through a low pH step for neurone intoxication to occur (Matteoli *et al.*, 1996; Schiavo and Montecucco, 1997). This process leads to formation of relatively large ion channels and/or perturbations in membranes.

4) *Zinc endopeptidase activity and blocking of neurotransmitter release.*

Clostridial neurotoxins enter nerve cells and block neurotransmitter release via zinc-dependant cleavage of protein components of the neuroexocytosis apparatus (Schiavo and Montecucco, 1997).

The different toxin types have different targets of neurotoxin proteolytic activity. *C. botulinum* type C toxin acts on proteins associated with the presynaptic membrane. BoNT type C cleaves both SNAP-25 (Synaptosomal Associated Protein, 25 kDa) and syntaxin. These targets must play a central role in neuroexocytosis, as they are the only known substrates of this neurotoxin. SNAP–25 is required for axonal growth during development and nerve terminal plasticity in the mature nervous system (Osen-sand *et al.*, 1993). Syntaxin is associated with calcium channels at the active zone of the presynaptic membrane where neurotransmitter release takes place (Montecucco and Schiavo, 1994). The neurotoxicity of *C. botulinum* type C is unique. Whilst all other botulinum neurotoxins can also block transmission, type C is the only clostridial toxin to cause overt neuronal degeneration (Williamson *et al.*, 1995)

PART 3: BOTULINUM TOXINS IN DISEASE

Botulinum toxin derives its potency from its ability to block neuromuscular transmission and cause death through paralysis of airway and respiratory musculature (Labbe and Shih, 1997).

The toxin that causes botulism can be acquired by one of three ways. The classically known botulism, food botulism, is due to ingestion of a preformed toxin within food. In infant botulism, which may prove to be the most common form of botulism (Sugiyama, 1980), toxin is produced in vivo during intestinal growth of the organism. Wound botulism is where the toxin is formed within the wound.

Foodborne botulism

Foodborne botulism is an intoxication that results when preformed botulinum toxin contained in an improperly preserved food is swallowed. Human outbreaks have been caused by types A, B, E and F and also a mixture of A and B (Sugiyama, 1980). In animals, the most common types are C and D whilst A, B and E contribute sporadically. The predominant types vary between species and geographical location. Horses, cattle, mink and fowl are the most commonly affected. *C. botulinum* spores resident in the carcass of a dead animal or in rotting vegetation also yields enough toxin to cause outbreaks (Schiavo and Montecucco, 1997).

The clinical signs of botulism in animals include; anorexia, incoordination, ataxia, flaccid paralysis and laboured breathing. Swallowing difficulty occurs due to paralysis of the tongue and pharynx. Excessive salivation also occurs. Respiratory paralysis causes death of the animal (Schiavo and Montecucco, 1997).

Diagnosis of botulism is often based on historical evidence and clinical signs (Hathaway, 1989).

C. botulinum type C intoxication has been reported in horses in the USA. One outbreak has been associated with consumption of processed alfalfa hay cubes (Kinde *et al.*, 1991) and another reports an outbreak in both horses and mules resulting from exposure to a burial site (Schoenbaum *et al.*, 2000).

Toxicoinfectious (infant) botulism

The spontaneous production of botulinum toxin in the infant gut by ingested *C. botulinum* organisms has been shown to be the underlying cause of infant botulism (Arnon *et al.*, 1978). Infant botulism manifests itself clinically as a symmetrical flaccid paralysis that first affects the muscles of the head, face, mouth and throat (Arnon *et al.*, 1978).

Multiple cases have been reported in the USA (Sugiyama, 1980). Cases have also been reported in England, Australia and Canada (Sugiyama, 1980). Only types A and B *C. botulinum* have been implicated (Sugiyama, 1980). The most striking feature of infant botulism is its age distribution, in which 95% of cases are aged between 3 weeks and 6 months. Under exceptional circumstances of altered intestinal anatomy, physiology and microflora, older children and adults may contract infant botulism (Arnon *et al.*, 1978). In an epidemiological study, the ingestion of honey has been significantly associated with type B infant botulism (Arnon *et al.*, 1979).

Toxicoinfectious botulism has been proved to be the cause of a highly fatal, neuromuscular paralytic syndrome in foals commonly called 'shaker foal syndrome' (Swerczek, 1980). Type B *C. botulinum* was isolated from cases of this

disease and it was suggested that spores of this type are ingested by foals and adult horses from contaminated soil and faecal material with subsequent spore germination, multiplication and production of toxin within the intestine (Swerczek, 1980).

Wound botulism

Wound botulism results from spore germination and colonisation in traumatised tissue. It is the rarest form of botulism. Only types A and B cases have been reported (Sugiyama, 1980).

PART 4: TOXINS THAT AFFECT THE CYTOSKELETON

The C2 toxin and, C3 exoenzyme affect the actin cytoskeleton, causing a characteristic "rounding-up" of cells *in vitro*. Whilst this cytopathic effect is essentially indistinguishable, the toxins are structurally unrelated and functionally have very different mechanisms of action.

C. botulinum C2 toxin

The C2 toxin is located on the bacterial chromosome and is produced by the majority of type C strains and some type D strains, but not by other botulinum toxin-producing organisms. The C2 toxin is produced only during sporulation and not during vegetative growth (Nakamura *et al.*, 1978). C2 toxin production is not governed by the presence of bacteriophages (Eklund and Poysky, 1974). However, it has been proposed that a plasmid may be involved in mediating C2 toxin production as a derivative of a C2 producing type C strain was isolated that no longer produced the C2 toxin (Eklund et al, 1987).

C. botulinum C2 toxin is exceptional in that it has no neurotoxic activity. The C2 toxin belongs to a new class of bacterial ADP-ribosylating toxins which modifies actin (Aktories *et al.*, 1986; Aktories *et al.*, 1987). Cytoskeletal architecture depends on actin filaments (Mauss *et al.*, 1989).

The C2 toxin consists of the enzyme component C21, an ADP-ribosyltransferase that modifies G-actin and the binding component C211 that mediates cell entry of the toxin. The modification of G-actin causes inhibition of actin polymerisation and inhibits ATPase activity (Barth *et al.*, 2000). C2 toxin has also been reported to induce rounding up and subsequent lysis of cultured cells (Ohishi and Odagiri, 1984). The toxin can clearly discriminate between non-muscle and skeletal actin (Aktories *et al.*, 1986).

The *in vivo* effects of C2 toxin appear to be changes in membrane permeability (Simpson, 1982). It induces hypotension (Simpson, 1982), an increase in intestinal secretions (Ohishi, 1983b), vascular permeability (Ohishi, 1983a) and haemorrhage in the lungs (Simpson, 1982).

Mauss *et al.*, (1989) investigated whether botulinum C2 toxin, which modifies G-actin, can affect smooth muscle contractility. They found that C2 toxin inhibited the contractions of guinea-pig ileum myenteric plexus longitudinal muscle. In contrast, contractions of rabbit aortic smooth muscle were not affected by toxin.

Botulinum C2 toxin has also been found to induce fluid accumulation in mouse intestinal loops (Ohishi, 1983b). The secretory response to C2 toxin was initiated after a lag period of 1 to 2 hours. None of the botulinum neurotoxins A to F induced fluid accumulation in mouse intestinal loops.

C. botulinum C3 toxin

Botulinum C3 toxin is another non-neurotoxic toxin. C3 is an exoenzyme that ADP-ribosylates the Rho-family of low molecular weight GTP binding proteins required for the organisation of the microfilament network (Hara-Yokoyama *et al.*, 1995). It is debatable whether the C3 exoenzyme has a role in the pathogenesis of disease as it is not known if the enzyme can enter cells *in vivo*. *In vitro,* C3 toxin can also cause neuronal degeneration (Williamson and Neale, 1998). The neuronal degeneration caused by the C3 toxin is less severe than that caused by C1 toxin.

So why investigate the role of *C. botulinum* type C in equine grass sickness?

The *C. botulinum* hypothesis, first proposed by Tocher *et al.*, (1923) is worthy of re-evaluation for the following reasons:

- The clinical signs of toxicoinfectious botulism (Seddon, 1922) typified by foal botulism, and human botulism (Arnon *et al.*, 1978) are very similar to those of chronic grass sickness.

- Both Tocher *et al.*, (1923) and Walker (1929) investigated the role of types A and B (group 1) *C. botulinum* and not types C and D (group III). Therefore, the dismissal of the botulinum hypothesis in the 1920's might have been premature.

- In addition to type specific C1 neurotoxin, group III strains of *Cl. botulinum* produce C2 and C3 toxins. C2 toxin, at sublethal concentrations, produces

neuronal lesions similar to those found in grass sickness (Morris *et al.*, 1989, Williamson *et al.*, 1995).

- Hunter *et al.*, (1999) have demonstrated the presence of *C. botulinum* type C toxin in the ileum and faeces of horses with grass sickness.

- Hunter and Poxton (2001) have demonstrated lower antibody levels to *C. botulinum* type C cell wall antigen and its C1 toxin in horses with EGS compared to non-affected control horses.

MATERIALS AND METHODS

Study Design

A matched case-control study was employed to compare exposure of EGS cases and healthy control horses to *C. botulinum* type C. The study was carried out between 1999 and 2001. Control horses were matched to case horses by premises at a ratio of two controls per case.

Case Recruitment and Definition

EGS cases

EGS cases that were referred to the Phillip Leverhulme Large Animal Hospital (PLLAH) were automatically recruited onto the study. Further case recruitment involved a phone call from a veterinary practitioner, following our advertisement, to inform us of a suspected case. In the latter method, the horse was either referred to the PLLAH or samples were obtained in the field and submitted by post.

All EGS cases were confirmed histologically either by ante-mortem diagnosis from an ileal biopsy or from autonomic ganglia and/ or an ileal sample obtained at post mortem examination. Any horse that gave a negative result on either of the above tests was subsequently excluded from the study.

Control horses

Horse controls were preferably co-grazers of the EGS case. If it was not possible to obtain co-grazers, horses were obtained either from the pasture nearest

to where the case occurred (n = 30) or, on a minority of occasions (n = 9), the neighbouring premises. Control horses were chosen using pseudo-random method. If there were more than two co-grazers, the possible population of control horses were listed in alphabetical order and the two horses whose names came first in the placed order were selected as the control horses.

Collection of Blood Samples

Blood was collected from 65 cases of EGS. Twelve were chronic cases and 53 were acute cases of the disease. Blood was collected on average 3 days (range = 0 – 29 days) from the onset of clinical signs. Blood was collected from the 130 matched control horses on average 10 days from the onset of the EGS in the case horse (range = 3 – 41 days). Blood samples were collected in sterile vacutainers with no additive and allowed to clot. Serum was collected after centrifugation at 2000g for 10 minutes and stored at –20 °C until assayed.

ELISA Assays for Quantification of Specific Antibodies

1. Antigen Preparation

Three different antigens were used to investigate the role of *C. botulinum* type C in EGS. *C. novyi* type A was used as a phenotypic marker for *C. botulinum* type C (Poxton and Byrne, 1984). EDTA–extraction of surface antigens of *C. novyi* type A (MPRL 2530) and a non-toxin producing *C. botulinum* type C (MPRL 4240) was done following the method of Poxton (1984) (R. Brown, personal communication).

The protein concentration of our *C. novyi* type A EDTA extract was 4.6mg/ml. This was determined by the Coomassie Plus protein assay (Pierce). The protein concentration of *C. botulinum* type C EDTA extract was 439.1µg/ml.

C. botulinum type C (BoNT/C) toxin complex toxoid (1mg/ml) was obtained from a commercial source (Metabiologics Inc, Madison, USA). The final preparation of toxoid contains the neurotoxin and associated proteins that provide stability to the neurotoxin. Residual toxicity was tested for by the manufacturers using the mouse bioassay.

2. Coating of ELISA plates

a) *C. novyi* type A and *C. botulinum* type C surface antigens

Nunc Immuno™ Polysorb™ plates were coated with EDTA-extracted surface antigens of *C. novyi* type A at a concentration of 30µg/ml in coating diluent (0.5M sodium bicarbonate buffer, pH9.6 containing 0.02% sodium azide), as described by Poxton (1984). EDTA-extracted surface antigens of *C. botulinum* type C were diluted to a concentration of 10µg/ml in coating diluent (0.5M sodium bicarbonate buffer, pH9.6 containing 0.02% sodium azide). 100µl of diluted antigen was added to each well and plates were incubated overnight at 4°C. After overnight incubation, plates were washed four times with PBS-T (PBS containing 0.05% Tween 20 and 0.05% sodium azide, pH 7.2).

b) BoNT/C toxoid

Nunc Immuno™ Polysorb™ plates were coated with a type C toxin complex toxoid at a concentration of 5µg/ml in coating buffer and incubated overnight at 4°C followed by four washes with PBS-T. The coating concentration

of this assay was determined by serial dilutions of the antigen in a checkerboard titration. Results were compared with those of a *C. botulinum* type C toxin ELISA (Hunter and Poxton, 2001) run in parallel, using serum of known OD's.

3. Blocking of ELISA Plates

a) *C. novyi* type A and *C. botulinum* type C surface antigens

Plates coated with these antigens were not blocked.

b) BoNT/C toxoid

Plates were blocked with PBS-T containing 5% foetal bovine serum (PBS-TF). 200µl was added to each well and plates were incubated, with shaking, for 1 hour at 37° C and then washed four times with PBS-T.

4. Addition of Samples

a) *C. novyi* type A and *C. botulinum* type C surface antigens

Previous work had determined the optimum dilution for this assay as 1 in 50 (L.C Hunter, personal communication). Samples were diluted in antiserum/conjugate diluent (0.5M phosphate buffer, pH7.4, 0.85% sodium chloride, 0.05% Tween 20 and 0.02% sodium azide). 100µl was added to each well and plates were incubated for 90 minutes at 37° C and then washed four times with PBS-T.

b) BoNT/C toxoid

The optimum dilution for the screening of serum samples was determined as 1 in 50 for the screening of serum samples for IgG to BoNT/C toxoid. Samples

were diluted in PBS-TF. 100 µl was added to each well and plates were incubated for 90 minutes at 37° C and then washed four times with PBS-T.

5. Conjugate

a) *C. novyi* type A and *C. botulinum* type C surface antigens

Rabbit anti-horse IgG (whole molecule) alkaline phosphatase conjugate (Sigma Chemical Co., Poole, Dorset) was diluted 1 in 10000 in antiserum/conjugate diluent and 100µl added to each well. The plates were incubated for 90 minutes at 37°C and then washed four times with wash buffer.

b) BoNT/C toxoid

Rabbit anti-horse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) was diluted 1 in 2000 in PBS-TF and 100µl was added to each well. The plates were incubated for 90 minutes at 37°C and then washed four times with wash buffer.

6. Substrate

For all three assays, alkaline phosphatase substrate tablets; p-nitrophenyl phosphate (Sigma 104) were dissolved in substrate solvent (0.05M sodium carbonate solution, pH 9.8, with 1nM magnesium chloride) to give a concentration of 1mg/ml. 100µl of the substrate solution was added to each well and incubated at room temperature until a positive control standard OD of 1.1 was reached. This took approximately 60 minutes. The absorbance was measured at 405nm, reference 620nm with a Revelation Quick Link plate reader (DYNEX technologies).

7. Controls

A serum sample had been previously identified that gave an OD reading of approximately 1 in the assay to detect IgG to *C. novyi* type A surface antigens. This serum sample was used as a positive control for the ELISA to detect IgG to *C. novyi* type A and *C. botulinum* type C surface antigens. Another serum sample was identified that gave an OD reading of 1 in the assay to detect IgG to the BoNT/C toxoid. This serum sample was used as a positive control for the ELISA to detect IgG to BoNT/ C toxoid. The positive control was used to standardise OD readings between plates.

Every third column, in each plate, was left uncoated and received coating diluent only during the coating stage. Therefore, each serum sample was added to two coated wells and one uncoated well. It is thought that the uncoated well would control for non-specific binding of the serum samples to the plates (L.C. Hunter, personal communication).

The first row in each plate was allocated as a blank row and received only antiserum/conjugate diluent (to *C. novyi* type A and *C. botulinum* type C surface antigens) or PBS-TF (BoNT/ C toxoid).

8. Calculation of ELISA Results

The mean OD for the blank row was automatically subtracted by the plate reader from both the OD readings for the antigen coated and uncoated wells. The OD for the uncoated well for each sample was then subtracted from the mean OD for that sample, in the coated well, to give the final OD value. The OD readings between plates were standardised by correcting the OD by reference to the

positive control. ELISA results were expressed as a percentage of the positive control.

Statistical Analysis

a) The comparison of ELISA OD's in EGS cases and controls.

OD values of IgG in serum of cases and controls were compared using a students T-test for normally distributed data (*C. botulinum* type C and BoNT/C toxoid) and a Mann Whitney U test for data that were not normally distributed (*C. novyi* type A). Data were tested for normality using a normality test and the Anderson-Darling statistic. Both tests used the MINITAB package (version 11). Correlation between IgG responses to different antigens was investigated using linear regression and Pearson's correlation coefficient in MINITAB.

Matched case-control data were analysed using conditional logistic regression analysis in EGRET (version 2.0.3, Cytel Software Corporation). Conditional logistic regression analysis is appropriate for matched case-control studies with a binary outcome. In this study, the binary outcome is EGS (1) or not EGS (0). A matching variable was used to partition the data into matched sets. Observations with the same value for the matching variable were in the same matched set. Age adjusted ELISA OD data were explored as both a continuous and categorical variable. Categorical data were separated into quintiles in S-PLUS.

b) The Relationship between ELISA OD and number of days between onset of disease and serum collection.

Linear regression analysis in MINITAB was used to explore the relationship between ELISA OD and number of days between onset of disease and serum collection in the EGS cases.

c) The relationship between ELISA OD and age.

Linear regression analysis in MINITAB was used to explore the relationship between ELISA OD and age in the EGS cases and controls. Using the data from control horses only, the empirical (statistical) relationship was investigated by fitting simple positive power curves (PPC's) to the data using S-PLUS version 6. Differential equations were formulated to investigate possible mechanistic relationships between ELISA OD and age in control horses. These equations aimed to provide a quantitative assessment of the rate of acquisition of immunity and the age at which the rate of exposure was equal to rate of loss.

d) *Monthly variation in ELISA OD*

Scatterplots were produced in S-PLUS (version 6) to investigate the relationship between ELISA OD and the month of blood sampling. Spline smoothers were used to fit a curve to the data with six degrees of freedom. Smoothing splines are locally cubic splines that minimise a penalised residual sum of squares, drawing a smoothed curve through the data points.

Polyacrylamide Gel Electrophoresis and Western Blotting.

SDS-PAGE

All three antigens (*C. novyi* type A and *C. botulinum* type C surface antigens and *C. botulinum* type C toxin complex toxoid) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 20µl of each antigen was combined with 20µl of reducing sample buffer and reduced by boiling for two minutes. 10µl of each sample was loaded, in duplicate, and run (250v, 40MA, 2 hours) on a 10% resolving gel with a 4% stacking gel. Pre-stained SDS-PAGE standard molecular weight markers (Biorad) were run adjacent to the antigens and also loaded in duplicate. Following electrophoresis the gel was cut in half, each half containing a marker and the three separated antigens. The proteins were visualised in one half of the gel by Coomassie blue staining and the other half by silver staining.

Western blotting

SDS-PAGE was carried out as above except all antigens were diluted to a uniform concentration of 0.5g/ml. Following SDS-PAGE, the proteins were transferred electophoretically onto a nitrocellulose membrane (Hybond-C extra, Amersham Life Sciences) using a TE 22 Mighty Small transfer apparatus (Hoefer).

The gel was measured and the nitrocellulose membrane (NCM) was cut to size. The SDS-PAGE gel and NCM were then placed in transfer buffer (0.025M Tris, 0.10M glycine, 20% Methylene, chilled before use) for approximately 10 minutes whilst apparatus was set up. A 'sandwich' of gel and NCM was assembled, placed in the apparatus and transfer took place at 200MA, for 2 hours

at 4°C. After transfer, the NCM was blocked (5% FBS + TBS) overnight at 4°C. In addition, the post transfer gel was placed in Coomassie blue and stained for 1 hour followed by de-stain overnight to check that all proteins had been transferred.

The NCM was cut into two and placed in pooled serum diluted 1:50 in FBS-TBS. One half was placed in diluted test serum that was considered to have high OD's in all three antigens (OD greater than 0.9, mean 1.29). The other half was placed in diluted test sera that was considered to have low OD's in all three antigens (less than 0.3, mean 0.21). Incubation with test sera was for two hours, with rocking, at room temperature followed by three five-minute washes in TTBS. The detector antibody used was rabbit anti-equine IgG conjugated to horseradish peroxidase (Sigma), diluted 1:1000 in FBS + TBS. Incubation of the NCM in conjugate was for one hour, with rocking, at room temperature followed by another three five-minute washes in TTBS.

After washing, both halves of the NCM was incubated in substrate (Sigma Fast™; 3,3'-diaminobenzidine tablet sets) which developed after two minutes. This procedure was followed by a rinse in distilled water and air-drying.

RESULTS

The detection of antibodies to *C. novyi* type A, *C. botulinum* type C and BoNT/C toxoid in EGS cases and controls.

A wide range of IgG levels to *C. novyi* type A, *C. botulinum* type C and BoNT/C toxoid were detected in histologically confirmed cases of EGS and healthy control horses (table 3.1). Antibody levels were compared between 65 EGS cases and 130 control horses. Horses with EGS were found to have a significantly lower level of serum IgG to *C. novyi* type A surface antigens ($P<0.001$), *C. botulinum* type C surface antigens ($P<0.001$) and BoNT/C toxoid ($P<0.001$) when compared to control horses (figure 3.1a, 3.1b and 3.1c). Due to the small number of chronic cases in the study, the difference in ELISA OD's between chronic cases and acute cases were not examined.

After allowing for matching and age, horses with EGS were found to have a significantly lower level of serum IgG to *C. novyi* type A and *C. botulinum* type C surface antigens and BoNT/C toxoid when compared to their matched controls. Table 3.2 (continuous variables) and 3.3 (categorical variables) gives an estimate of the coefficients, standard errors and P-values for all three antigens tested. The probability of being an EGS case was found to decrease with increasing antibody levels.

Table 3.1: IgG to surface antigens and BoNT/C toxoid in equine serum

Specific Antibody	Statistics	Antibody Level (OD)	
		Cases	Controls
		(n = 65)	(n = 130)
IgG to *C. novyi* type A	Mean	0.338	0.539
	Median	0.296	0.506
	Standard Deviation	0.198	0.265
	Range	0.01 – 0.847	0.070 – 1.662
IgG to *C. botulinum* type C	Mean	0.511	0.662
	Median	0.509	0.657
	Standard Deviation	0.188	0.212
	Range	0.162 – 1.01	0.107 – 1.279
IgG to BoNT/C toxoid	Mean	0.614	0.832
	Median	0.571	0.822
	Standard Deviation	0.305	0.342
	Range	0.050 – 1.430	0.212 – 2.363

Table 3.2: Conditional Logistic Regression Models: Bivariate analysis (age adjusted) 65 Matched Sets, 195 Total Observations. ELISA results presented as percent positivity (continuous variable)

Variable	P-value	Coefficient	Standard error
C. novyi type A ELISA OD	<0.001	-0.043	0.011
C. botulinum type C ELISA OD	<0.001	-0.038	0.011
BoNT/C toxoid ELISA OD	<0.001	-0.020	0.007

Table 3.3: Conditional Logistic Regression Models: Bivariate analysis (age adjusted) 65 Matched Sets, 195 Total Observations. ELISA results presented as percent positivity (categorical variable)

Variable	Coefficient	Standard error	Wald test P-value
C. novyi type A ELISA OD			
0.09 – 23.3%	Ref	Ref	
23.4 - 35.5%	-0.29	0.49	
35.8 – 40.0%	-1.37	0.57	0.01
48.9 – 64.6%	-1.93	0.65	
65.9 – 157.3%	-1.99	0.66	
C. botulinum type C ELISA OD			
10.7 – 41.2%	Ref	Ref	
42.0 – 54.3%	-1.25	0.56	
54.5 – 66.0%	-1.24	0.63	0.01
66.6 – 76.9%	-1.71	0.65	
77.6 – 127.9%	-2.85	0.81	
BoNT/C toxoid ELISA OD			
13.9 – 45.8%	Ref	Ref	
45.9 – 65.5%	0.11	0.52	
65.6 – 82.7%	-1.15	0.65	0.05
82.8 – 107.2%	-1.15	0.65	
107.2 – 237.%	-1.36	0.71	

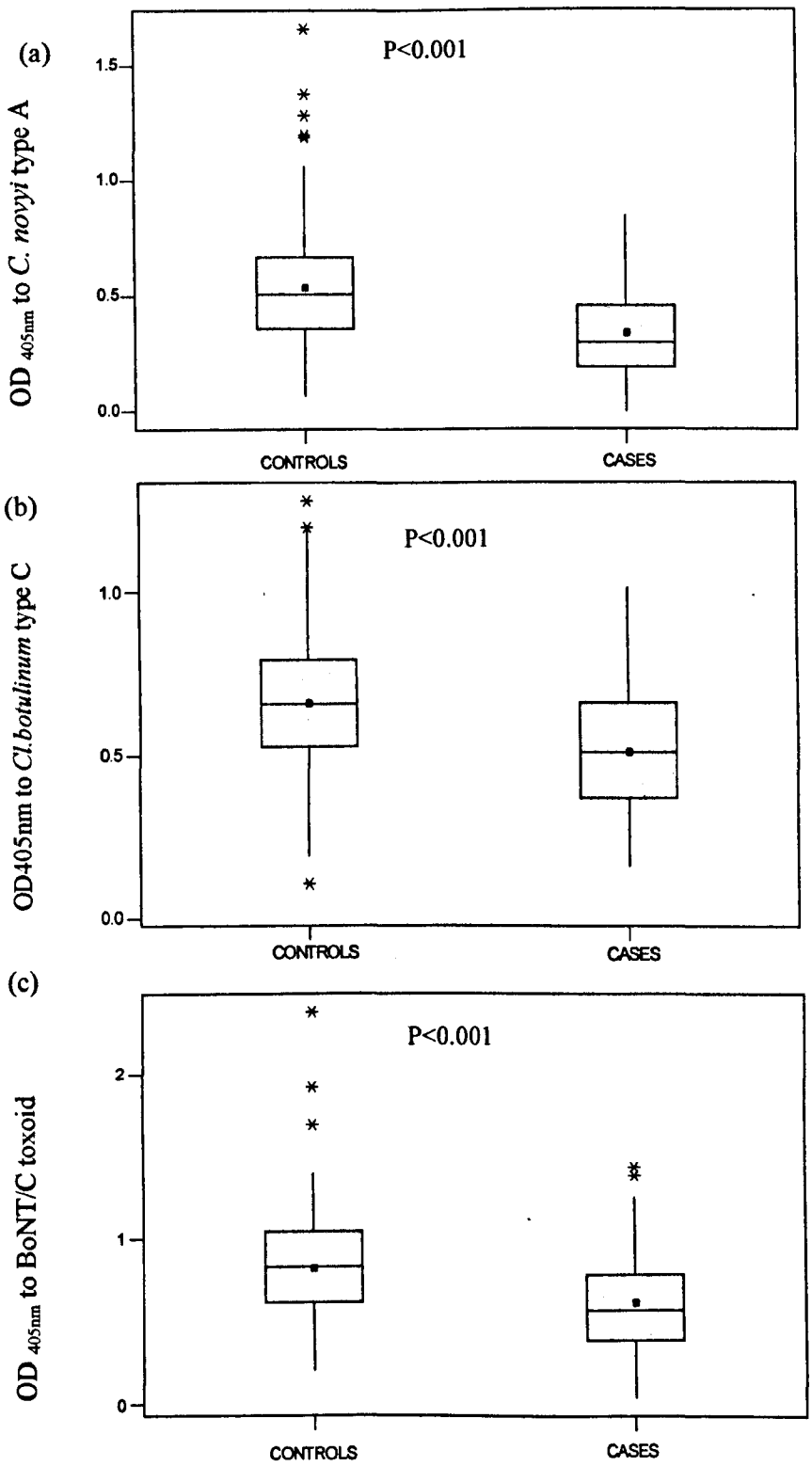


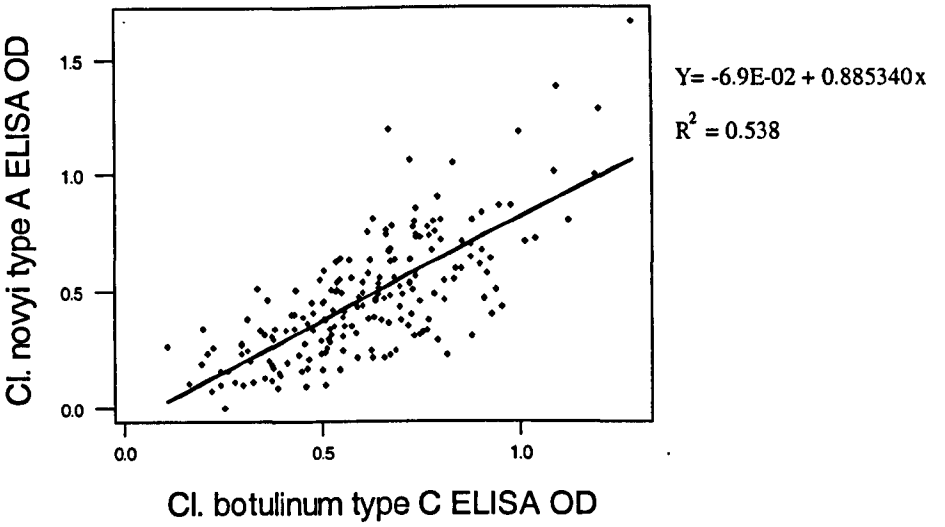
Figure 3.1 (a) IgG to *C. novyi* type A, (b) IgG to *C. botulinum* type C and (c) IgG to BoNT/C toxoid in cases of EGS and control horses. The bottom of the box is at the first quartile (Q1) and the top is at the third quartile (Q3). The mean value is represented by the red dot within the box. Outliers are points outside the lower and upper limits, plotted with asterisks (*). MINITAB considers any value lying between 1.5 and 3 times way from the middle 50% of the data as an outlier.

Table 3.4 shows the correlation coefficients between the three antigens. A positive correlation was found with *C. novyi* type A and *C. botulinum* type C, *C. novyi* type A and BoNT/C toxoid and *C. botulinum* type C and BoNT/C toxoid. The highest level of correlation was found between *C. novyi* type A and *C. botulinum* type C surface antigens. Fitted line plots illustrate the significant ($p<0.001$) associations between the three ELISA antigens (Figures 3.2 a, 3.2 b and 3.2 c).

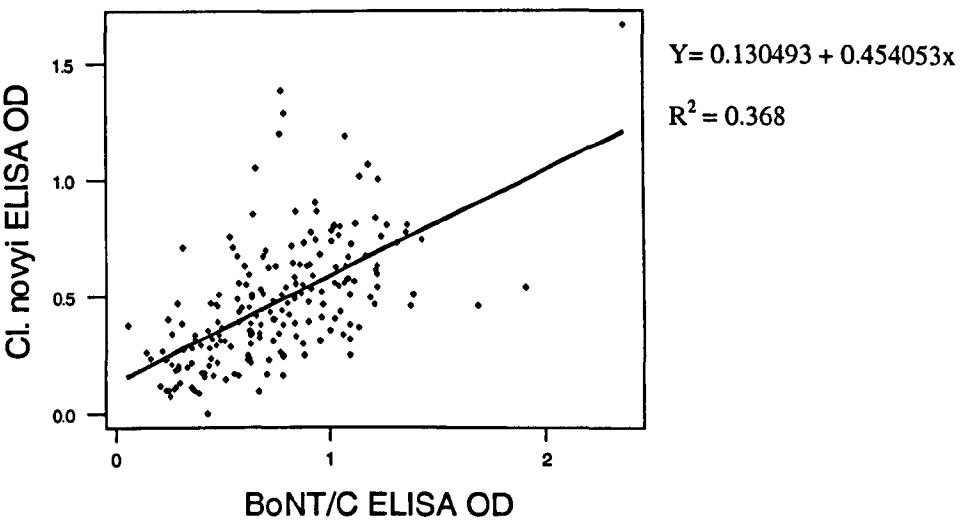
Table 3.4: Pearson's corelation coefficients between between *C. novyi* type A and *C. botulinum* type C, *C. novyi* type A and BoNT/C toxoid and *C. botulinum* type C and BoNT/C toxoid.

ANTIGENS	*C. novyi* type A	*C. botulinum* type C	BoNT/C toxoid
C. botulinum type C	0.733	-	-
BoNT/C toxoid	-	0.573	-
C. novyi type A	-	-	0.598

(a)



(b)



(c)

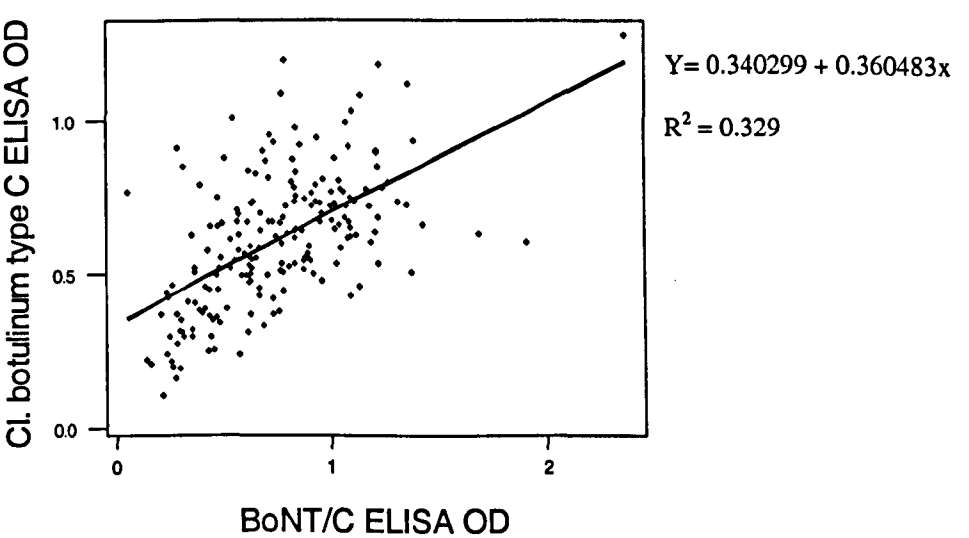


Figure 3.2a, b and c: Linear regression analysis to examine the relationships between ELISA OD's and the three different antigens tested

Antigen Characterisation

SDS-PAGE analysis of *C. novyi* type A, *C. botulinum* type C and BoNT/C toxoid is shown in figure 3.3 and figure 3.4. In the Coomassie stained SDS-PAGE gel of reduced *C. novyi* type A, major protein bands were revealed at approximately 85, 40, 33 25 and 26kDa. Bands of lower protein content are also evident at 77, 37, 36, 30 and 23kDa. In the silver stained gel there were many more bands present due to the increased sensitivity of silver staining. However, the bands were not so discrete. In the Coomassie stained SDS-PAGE gel of reduced *C. botulinum* type C a major protein band was revealed at 40kDA with bands of lower protein content present at 28 and 23kDa. Once again, the silver stained gel revealed multiple bands of a lower protein content especially between 36 and 21 kDa. In the Chemise stained and silver stained SDS-PAGE gel of reduced BoNT/C toxoid there were no clear bands evident.

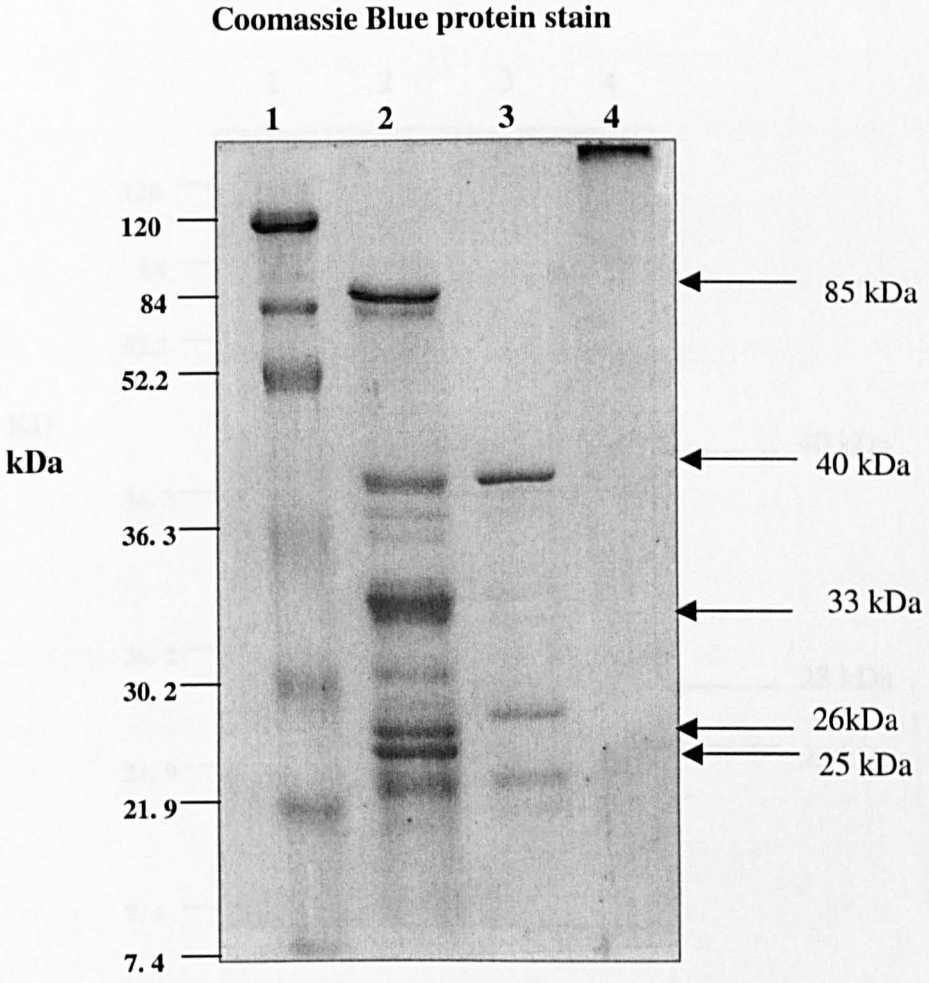


Figure 3.3: 10% polyacrylamide gel stained with Coomassie blue, showing the protein composition of *C. novyi* type A (lane 2), *C. botulinum* type C (lane 3) and BoNT/C toxoid (lane 4). Molecular weight markers are in lane 1.

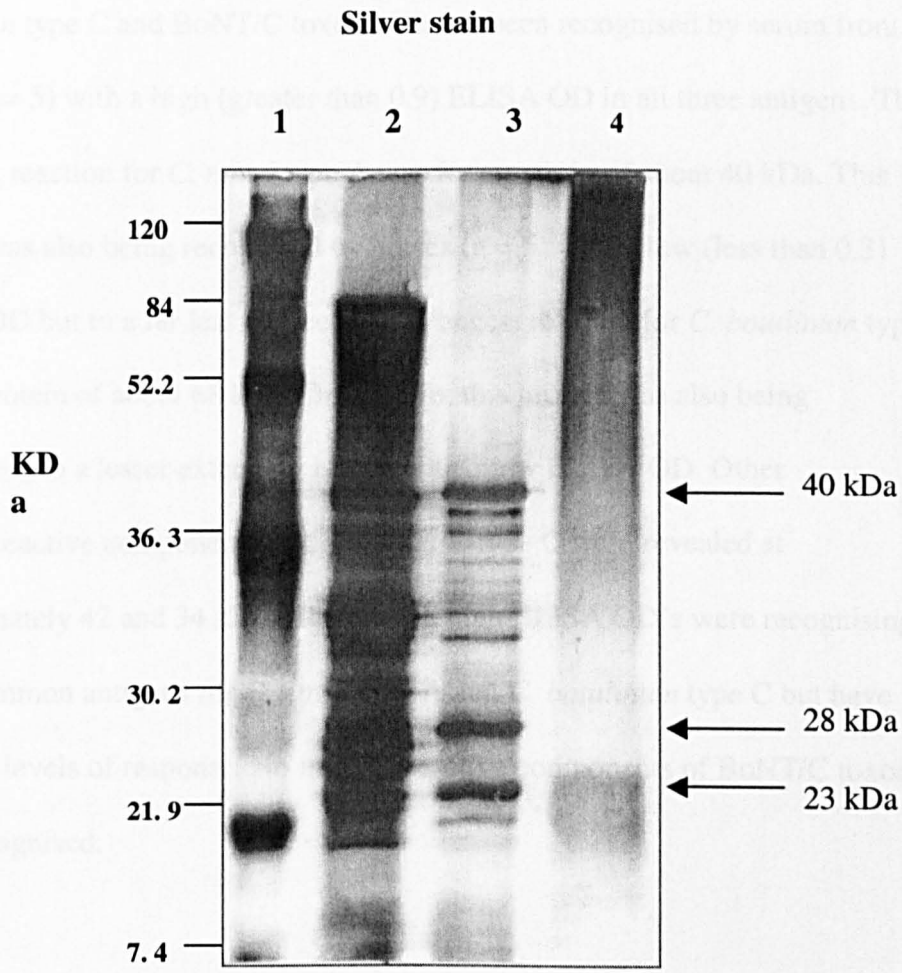


Figure 3.4: 10% polyacrylamide gel; silver stained, showing the protein composition of *C. novyi* type A (lane 1), *C. botulinum* type C (lane 2) and BoNT/C toxoid (lane 3).

Western blot analysis.

Figure 3.5 reveals the immunoreactive components of *C. novyi* type A, *C. botulinum* type C and BoNT/C toxoid that has been recognised by serum from horses (n= 5) with a high (greater than 0.9) ELISA OD in all three antigens. The strongest reaction for *C. novyi* type A was for a protein of about 40 kDa. This protein was also being recognised by horses (n = 5) with a low (less than 0.3) ELISA OD but to a far less degree. The strongest reaction for *C. botulinum* type C was to protein of about 68 kDa. Once again, this protein was also being recognised, to a lesser extent, by horses with a low ELISA OD. Other immunoreactive components of *C. botulinum* type C were revealed at approximately 42 and 34 kDa. Horses with high ELISA OD's were recognising some common antigens for *C. novyi* type A and *C. botulinum* type C but have different levels of response. No immunoreactive components of BoNT/C toxoid were recognised.

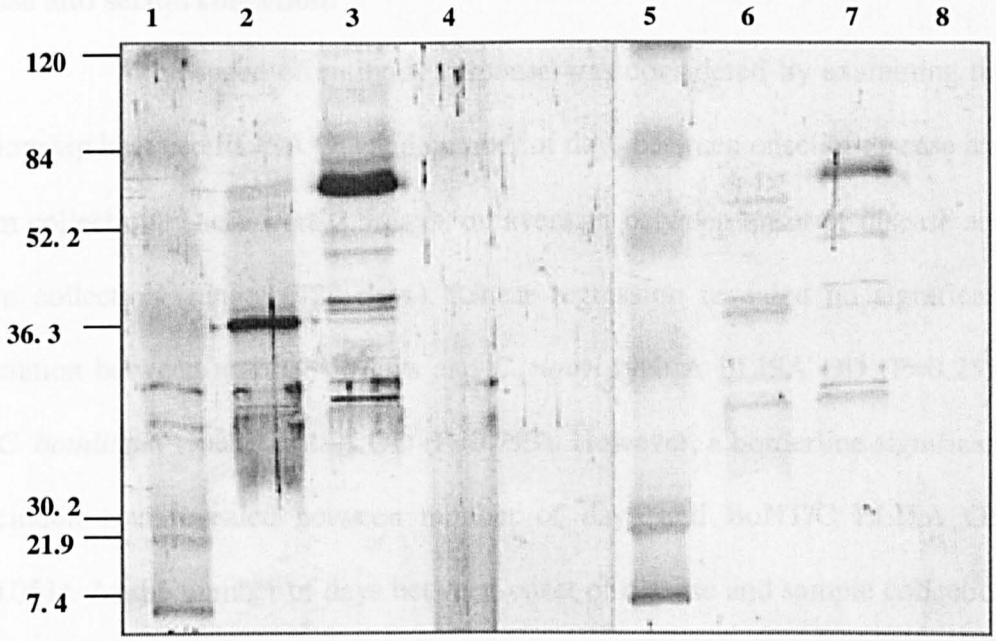
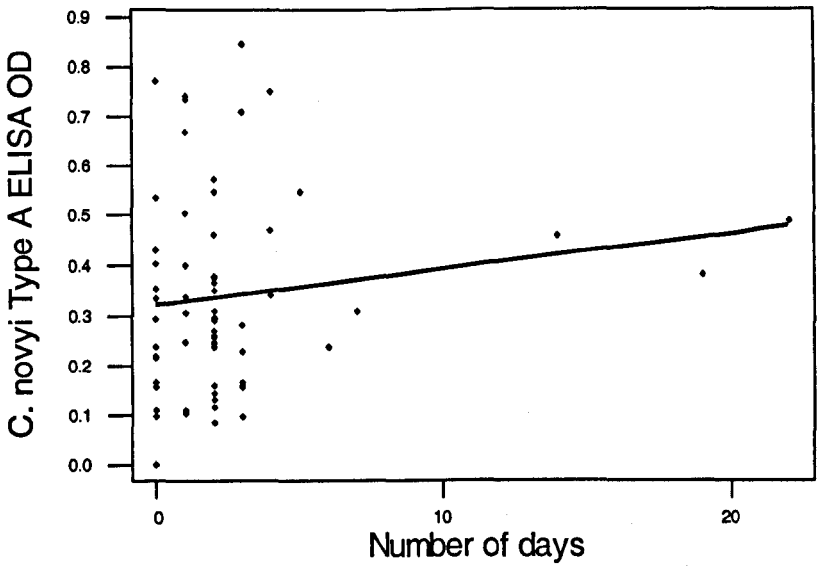


Figure 3.5: Western blot analysis of immunoreactive proteins. Blots have been probed with pooled sera from horses with high ELISA OD's to *C. novyi* type A (lane 2), *C. botulinum* type C (lane 3) and BoNT/C (lane 4) and pooled sera from horses with low ELISA OD's to *C. novyi* type A (lane 6) Cl. *botulinum* type C (lane 7) and BoNT/C (lane 8). Molecular weight markers are in lanes 1 and 5.

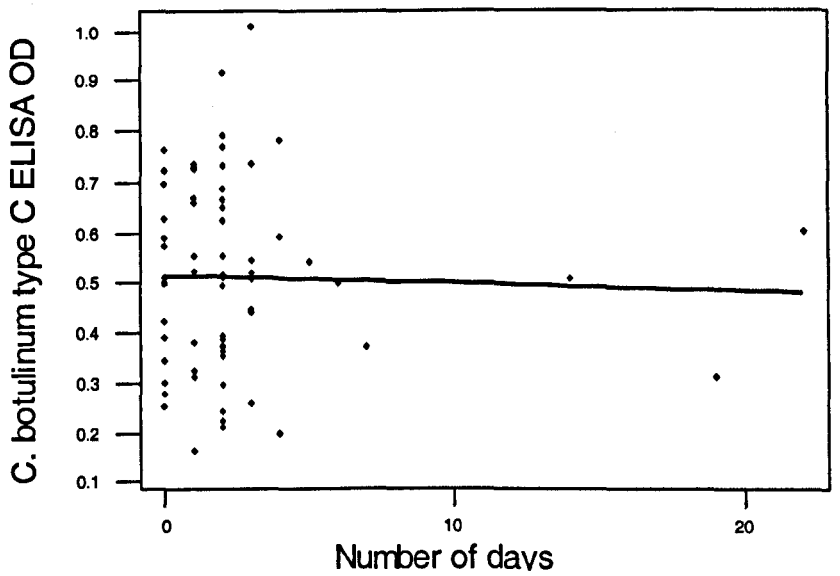
The Relationship between ELISA OD and number of days between onset of disease and serum collection.

The speed of antibody response was considered by examining the relationship between ELISA OD and number of days between onset of disease and serum collection. There were 2.5 days, on average, between onset of disease and serum collection (range; 0-22 days). Linear regression revealed no significant association between number of days and *C. novyi* type A ELISA OD (P=0.255) and *C. botulinum* type C ELISA OD (P=0.793). However, a borderline significant association was revealed between number of days and BoNT/C ELISA OD (P=0.051). As the number of days between onset of disease and sample collection increased, BoNT/C ELISA OD increased. Fitted line plots illustrate these relationships (Figures 3.6 a, 3.6 b and 3.6 c).

(a)



(b)



(c)

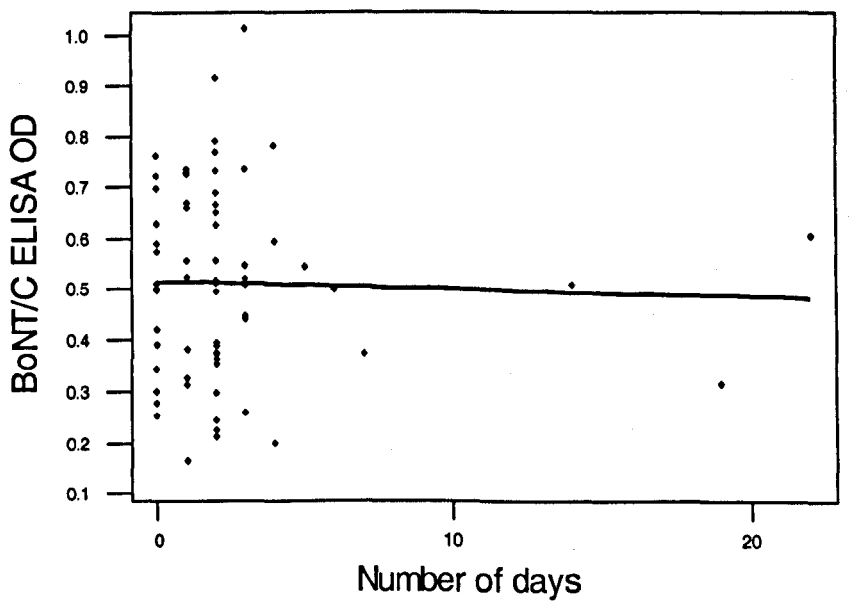


Figure 3.6a, b and c: The relationships between ELISA OD and number of days between onset of disease and sample collection. There appears to be no relationship between the two variables.

The Relationship between ELISA OD and Age

The relationship between ELISA OD and age was initially investigated using linear regression. Linear regression revealed a significant ($p<0.001$) association between age of EGS cases and controls and each of the three antigens. ELISA OD appeared to increase with increasing age of the horse.

Further investigations examined the relationship between ELISA OD and age in control horses only. Both empirical and mechanistic models were fitted to the data. The empirical (statistical) relationship was investigated by fitting a family of regression models to the data. Models were fitted using either polynomial or log-log relationships (appendix 2). For all three antigens, log-log relationships provided the best fit to the data suggesting a positive power curve relationship of the form; $OD = a\ (age)^{b}$, where a and b are parameters. The increase in ELISA OD with increasing age and the positive power curve fitted relationships are demonstrated in figure 3.6, 3.7 and 3.8. Older horses had higher levels of antibodies to *C. novyi* type A, *C. botulinum* type C and BoNT/C and beyond a certain age it was possible that horses have reached and maintained a protective level of immunity. Positive power curves (PPC's) have been fitted to the data using the following equations:

- Log *C. novyi* OD = -0.5100 + 0.2261 (log age)

- Log *C. botulinum* OD = -0.3621 + 0.1877 (log age)

- Log BoNT/C toxoid OD = -0.3363 + 0.2527 (log age)

From the fitting of PPC's to the control horse data there appears to be a positive relationship between ELISA OD and age for *C. novyi* type A, *C. botulinum* type C and BoNT/C toxoid.

To compare those empirical models with a basic mechanistic model we propose the following relationship between ELISA OD (θ) and age (a):

$$d\theta/da = \Delta - \mu\theta$$

We assume Δ is a constant rate of antibody production resulting from a constant antigenic challenge and μ is the per capita rate of decline of antibody in the absence of an antigenic challenge.

The mathematical solution to the above equation is:

$$\theta_{(a)} = \frac{\Delta}{\mu}(1 - e^{-\mu a})$$

A non-linear regression procedure was used to fit this equation to the data and the results have been reported graphically. Coefficients, standard errors and P-values for all three regression models are reported in table 3.5.

For *C. novyi* type A, the model predicted that antibody levels increased rapidly until 9 years of age. At this age the rate of exposure is equal to the rate of loss of antibody production and a point of equilibrium was reached (Figure 3.6). The model gives the point of equilibrium at an ELISA OD of 0.584. The rate of *C. novyi* type A antibody production is given in equation 1:

Cl. novyi OD = 0.367 / 0.628 * (1-exp [-0.628 * age]) (1)

Antibody production to *C. botulinum* type C reached a point of equilibrium at 7 years of age (Figure 3.7). This point of equilibrium occurred at an ELISA OD of 0.584. The rate of *C. botulinum* type C antibody production is given in equation 2:

Cl. *botulinum* OD = 0.576 / 0.823 * (1-exp [-0.823 * age]) (2)

Antibody production to BoNT/C toxoid started to level off at 8 years of age and reached a point of equilibrium at 10 years of age (Figure 3.8). This point of equilibrium occurred at an ELISA OD of 0.903. The rate of BoNT/C toxoid antibody production is given in equation 3:

BoNT/C toxoid OD = 0.496 / 0.549 * (1-exp [-0.549 * age]) (3)

To compare the empirical and mechanistic relationships two-dimensional residual plots have been produced (Kleinbaum *et al.*, 1988). Working residuals for the response values (ELISA OD) have been plotted against age. Loess smoothers from generalised additive models have been used to fit a curve to the data (Figures 3.9–3.14). Loess smoothers use a "locally weighted" linear regression to obtain smoothed values for each value of y, given the values for x. 95% confidence intervals have also been added to the plots

For *C. botulinum* it appeared that the empirical relationship was over predicting the ELISA OD of the older age group whereas the mechanistic relationship was more consistent throughout the age ranges. It was possible that the mechanistic relationship, when compared to the empirical relationship, over

predicted the rate of antibody production. A similar relationship was also seen for BoNT/C toxoid ELISA OD.

Table 3.5: Coefficients, standard errors and P-values for basic mechanistic relationship between ELISA OD and age.

Regression model		**Parameter estimates**		
Response variable	Explanatory variable	Coefficient	Standard error	P-value
Cl.novyi type A ELISA OD	Age	Δ= 0.367 μ = 0.628	Δ = 0.100 μ = 0.178	Δ = <0.001 μ = <0.001
C. botulinum type C ELISA OD	Age	Δ= 0.576 μ = 0.824	Δ = 0.109 μ = 0.165	Δ = <0.001 μ = <0.001
BoNT/C ELISA OD	Age	Δ = 0.496 μ = 0.549	Δ = 0.100 μ = 0.122	Δ = <0.001 μ = <0.001

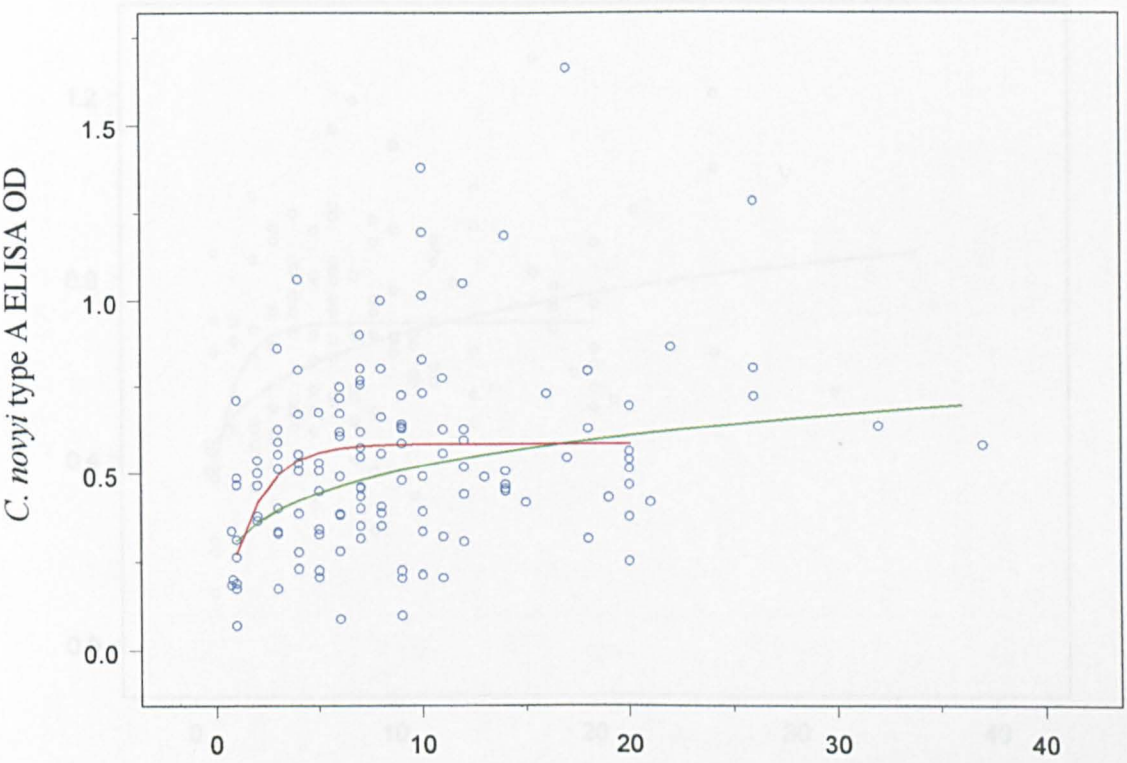


Figure 3.6: The empirical (green line) and mechanistic (red line) relationship between age and *C. novyi* type A ELISA OD. For the empirical relationship, a positive power curve has been fitted to the data using the regression model log age and log *C. novyi* OD (R^2 = 13.8%)

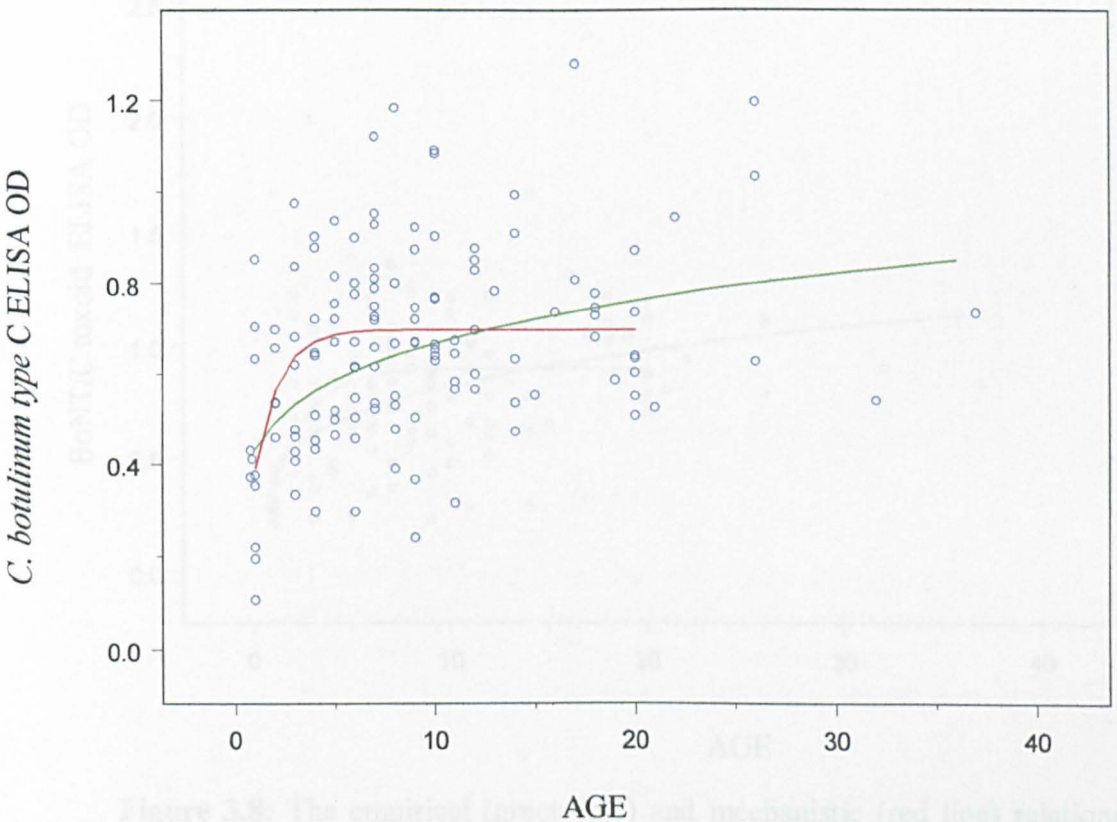


Figure 3.7: The empirical (green line) and mechanistic (red line) relationship between age and *C. botulinum* type C ELISA OD. For the empirical relationship, a positive power curve has been fitted to the data using the regression model log age and log *C. botulinum* OD (R^2 = 22.3 %)

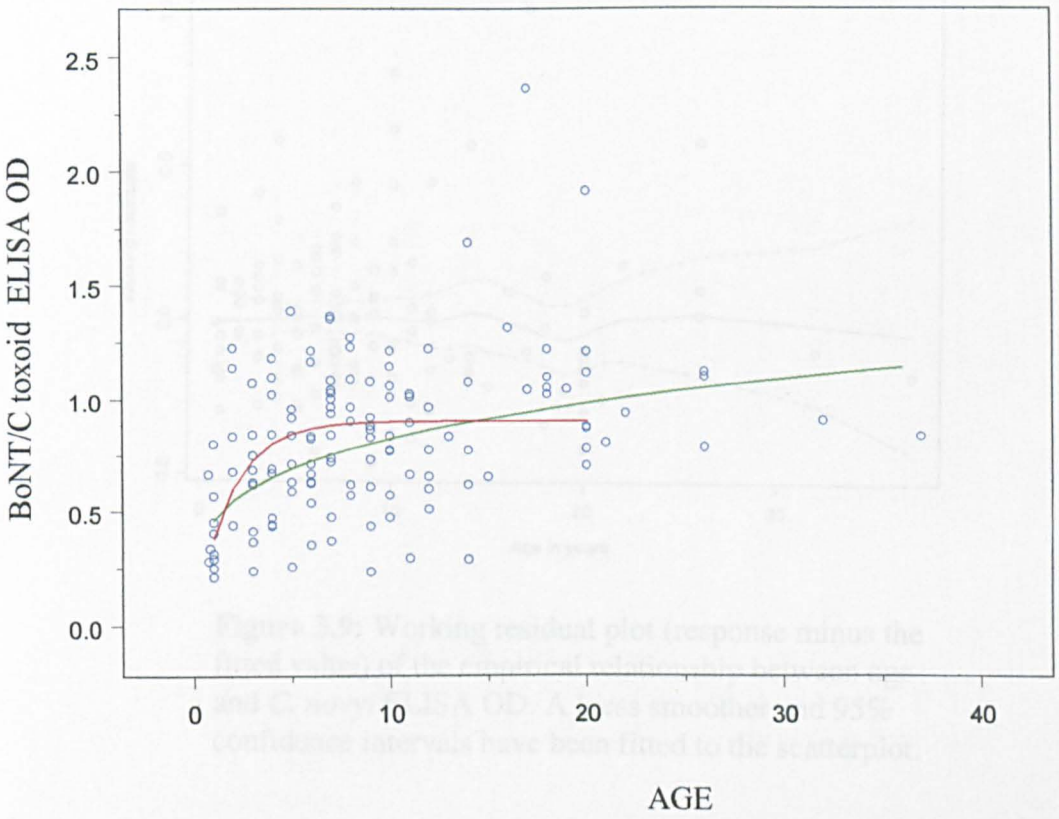


Figure 3.8: The empirical (green line) and mechanistic (red line) relationship between age and BoNT/C toxoid ELISA OD. For the empirical relationship, a positive power curve has been fitted to the data using the regression model log age and log BoNT/C toxoid OD (R^2 = 22.9 %)

Figure 3.9: Working residual plot (response minus the fitted value) of the empirical relationship between age and *C. novyi* ELISA OD. A loess smoother and 95% confidence intervals have been fitted to the scatterplot.

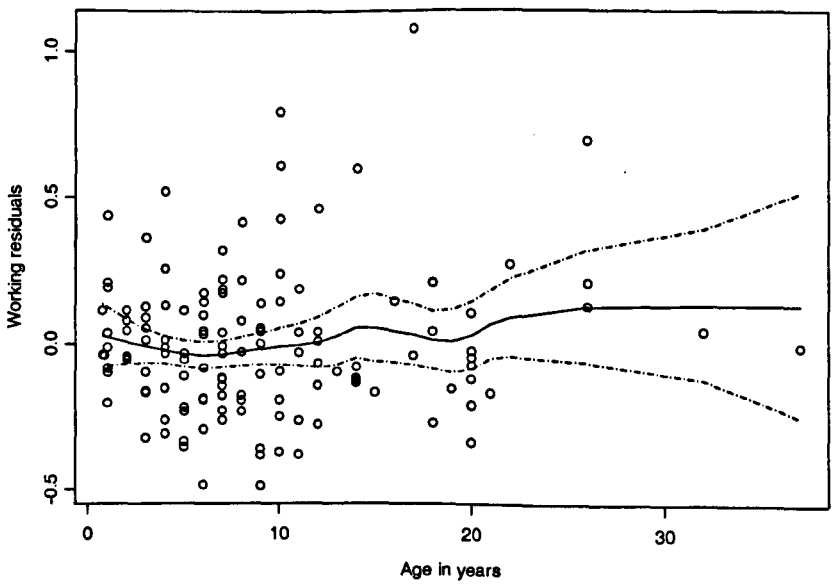


Figure 3.10: Working residual plot of the mechanistic relationship between age and *C. novyi* ELISA OD. A loess smoother and 95% confidence intervals have been fitted to the scatterplot.

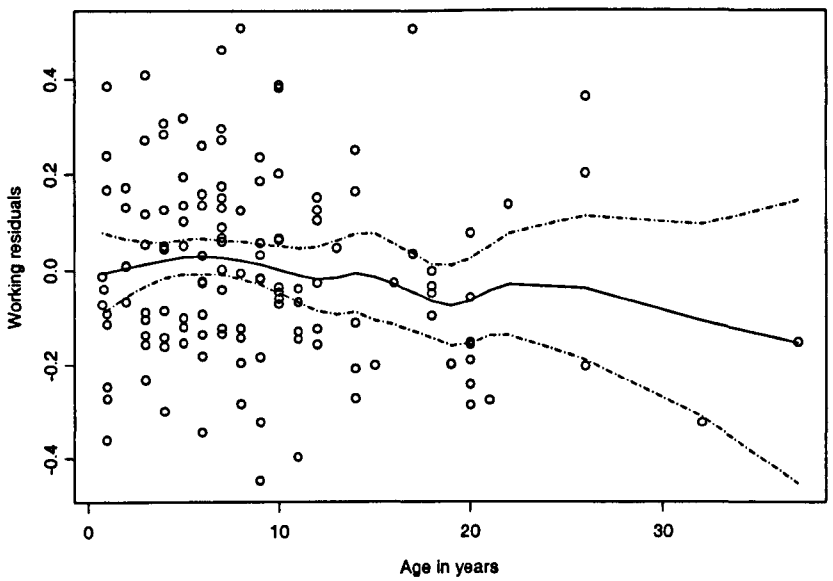


Figure 3.11 : Working residual plot of the empirical relationship between age and *C. botulinum* ELISA OD. A loess smoother and smoother and 95% confidence intervals have been fitted to the scatterplot.

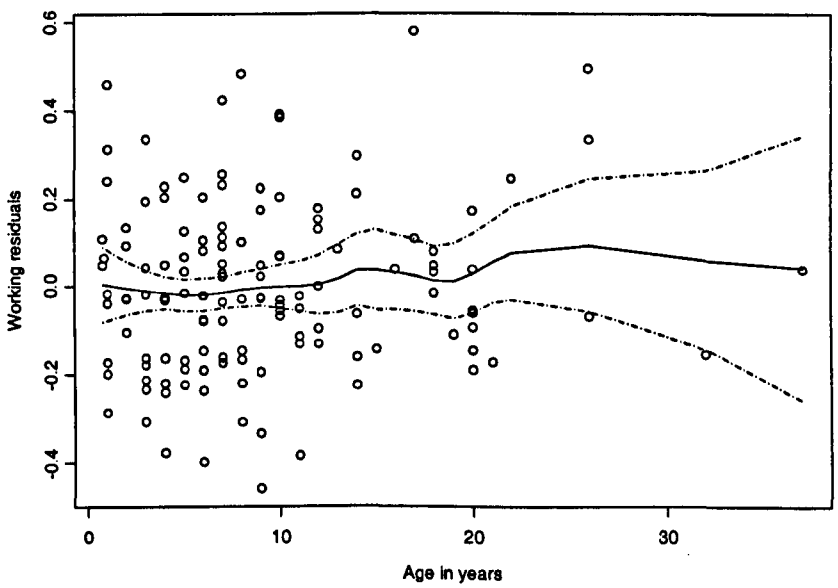


Figure 3.12 : Working residual plot of the mechanistic relationship between age and *C. botulinum* ELISA OD. A loess smoother and 95% confidence intervals have been fitted to the scatterplot.

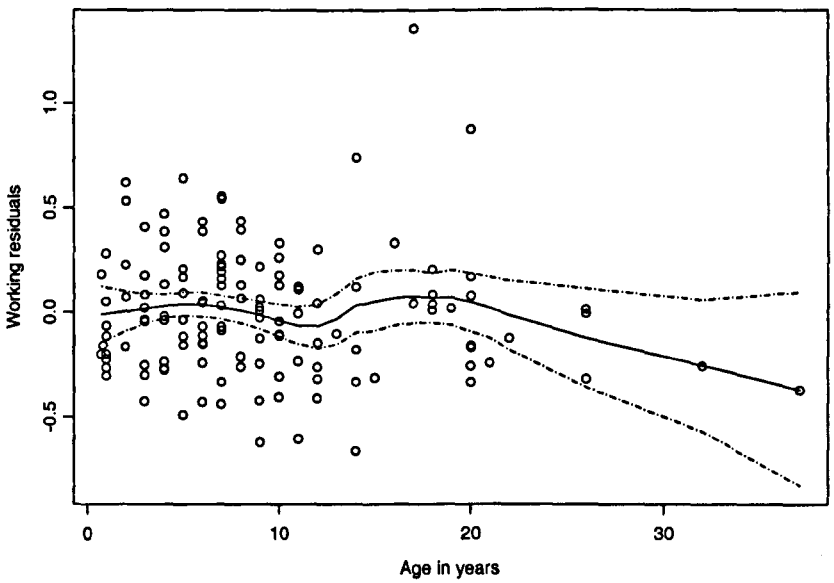


Figure 3.13: Working residual plot of the empirical relationship between age and BoNT/C toxoid ELISA OD. A loess smoother and 95% confidence intervals have been fitted to the scatterplot.

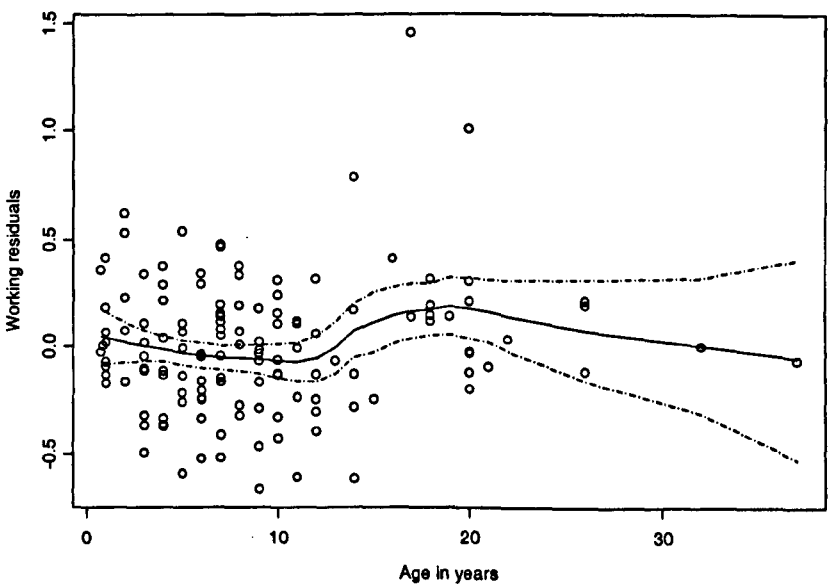


Figure 3.14: Working residual plot of the mechanistic relationship between age and BoNT/C
ELISA OD. A loess smoother and 95% confidence intervals been fitted to the scatterplot.

Monthly Variation in ELISA OD.

The monthly or seasonal variation in antibody level was investigated using the ELISA OD from control horses only. The month in this data set represents the date the blood sample was taken rather than the date EGS occurred. The results are presented in figures 3.15, 3.16 and 3.17.

For *C. novyi* type A, the spline smoothing plot suggests there is little variation in antibody level throughout the year. Horses that were sampled at the beginning of the year had a similar antibody level to those sampled at the end of the year. There does however appear to be a slight increase in ELISA OD from March through to May. The picture is very similar for *C. botulinum* type C and BoNT/C toxoid. There is however, a smaller increase in ELISA OD for *C. botulinum* type C from March to May whereas BoNT/C toxoid has a larger increase in ELISA OD that extends from April to June.

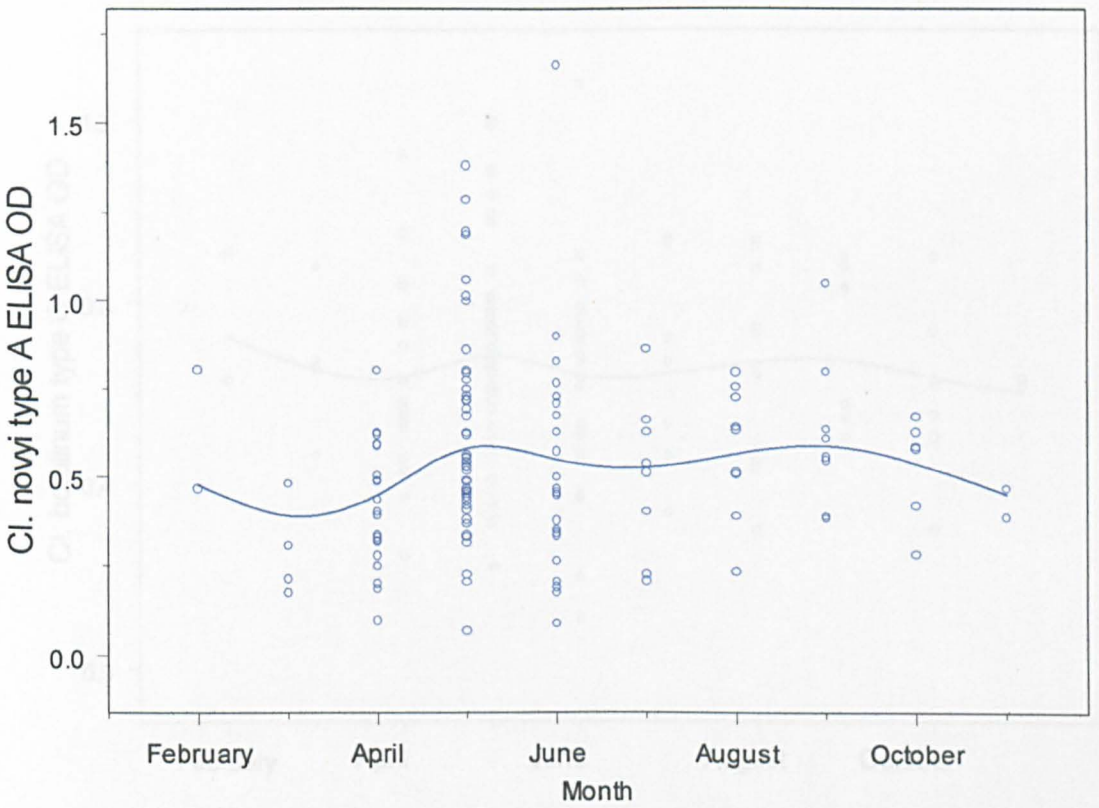


Figure 3.15: Monthly variation in *C. novyi* type A ELISA OD (Control horses only). The ELISA OD corresponds to the month of sampling. A spline smoother has been fitted to the scatterplot.

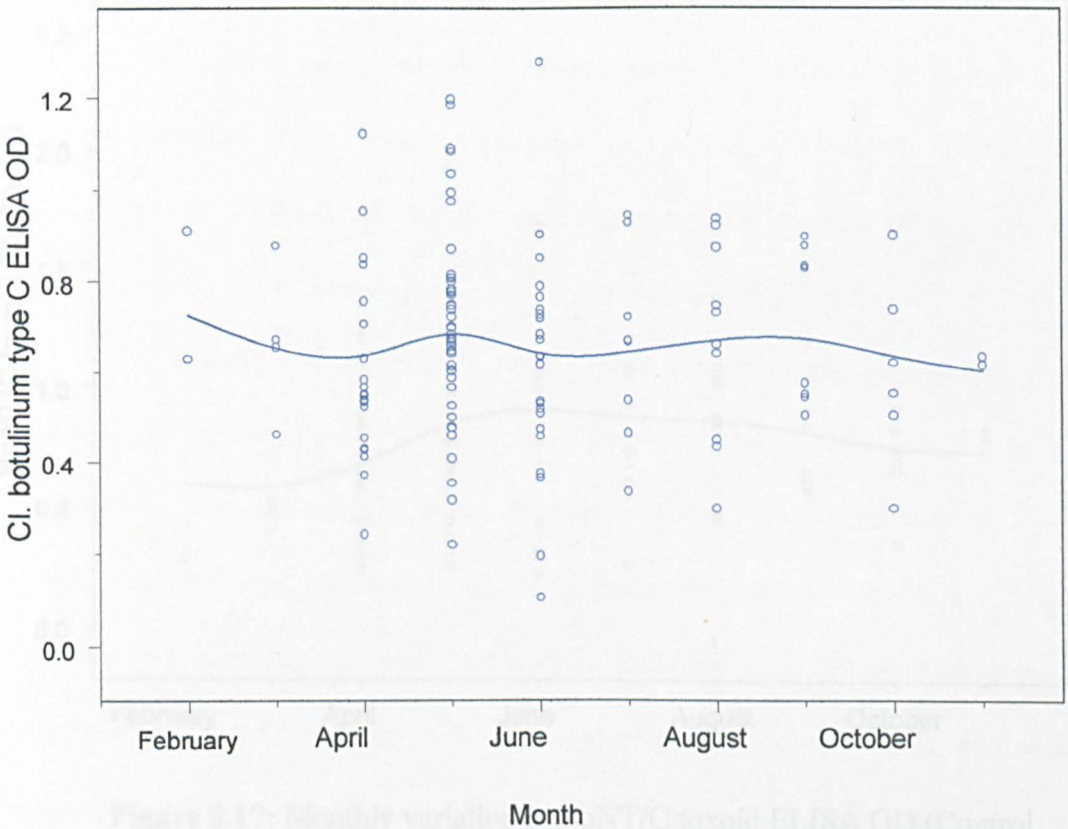


Figure 3.16: Monthly variation in *C. botulinum* type C ELISA OD (Control horses only). The ELISA OD corresponds to the month of sampling. A spline smoother has been fitted to the scatterplot.

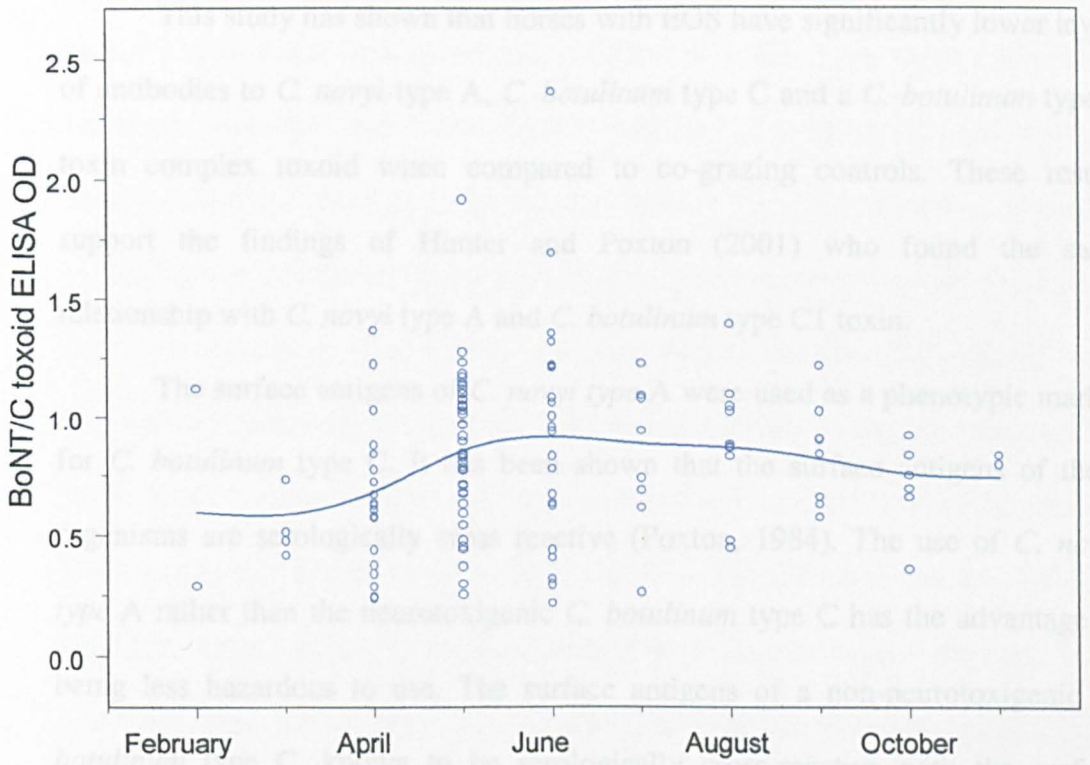


Figure 3.17: Monthly variation in BoNT/C toxoid ELISA OD (Control horses only). The ELISA OD corresponds to the month of sampling. A spline smoother has been fitted to the scatterplot.

DISCUSSION

This study has shown that horses with EGS have significantly lower levels of antibodies to *C. novyi* type A, *C. botulinum* type C and a *C. botulinum* type C toxin complex toxoid when compared to co-grazing controls. These results support the findings of Hunter and Poxton (2001) who found the same relationship with *C. novyi* type A and *C. botulinum* type C1 toxin.

The surface antigens of *C. novyi type* A were used as a phenotypic marker for *C. botulinum* type C. It has been shown that the surface antigens of these organisms are serologically cross reactive (Poxton, 1984). The use of *C. novyi type* A rather than the neurotoxigenic *C. botulinum* type C has the advantage of being less hazardous to use. The surface antigens of a non-neurotoxigenic *C. botulinum* type C, known to be serologically cross-reactive with the surface antigens of *C. novyi* type A (Hunter, 2001) was also used. The use of a *C. botulinum* type C toxoid was also considered safer to use than the toxin. A validation experiment comparing the antibody response to toxin and toxoid into was not undertaken due to the lack of availability of toxin.

A measurable antibody response to all three ELISA antigens was found to be present in all horses with a significantly higher level in matched controls. The highest level of correlation was seen between the surface antigens *C. novyi type* A and *C. botulinum* type C. Interestingly, western blot analysis suggested that although horses were recognising common proteins in the two antigens, the strongest response to each antigen was to different bacterial epitopes. Western blot analysis failed to recognise any immunoreactive proteins for the toxoid under both reducing and non-reducing conditions. It is suggested that the antigen underwent protein degradation at the recommended storage conditions of 4°C and

should have perhaps been stored at - 20 °C. Further evidence for breakdown of the BoNT/C toxoid comes from the fact that initial ELISA optimisation produced a satisfactory response at a serum concentration of 1:200. Three weeks later when the batch sera was tested, the ELISA had to be re-optimised and a serum concentration of 1:50 had to be used to produce a good response. A further possibility was that the antigen was significantly non-protein (e.g. carbohydrate).

Antibodies to the surface antigens and to the BoNT/C toxoid were detected in horses with and without EGS which suggests widespread exposure of horses to *C. botulinum* type C (or a cross reactive organism) and its neurotoxin. The significantly higher levels of IgG to both the surface antigens and BoNT/C toxoid in control animals suggested that these horses have been exposed to *C. botulinum* type C and may have mounted a systemic immune response that provided protection against EGS. Epidemiological evidence suggests that resistance can occur to EGS. There is a decreased prevalence of the disease in older horses, in horses that have been in contact with cases of grass sickness, and horses that have been grazing a particular pasture for a longer length of time (Gimour and Jolly, 1974; Doxey *et al.*, 1991a and Wood *et al.*, 1998). This resistance may be in the form of an immune response to the aetiological agent. Some experimental ponies were found to be resistant to the toxic factor present in plasma from acute EGS cases that had been shown to cause neuronal chromatolysis when injected into other ponies (Doxey *et al.*, 1997). It is hypothesised that the putative neurotoxin is produced enterically and that toxin present in the serum results from gastrointestinal absorption (Griffiths *et al.*, 1994). This effect explains why systemic antibodies are produced in response to

an enteric organism, but also suggests that the study of the mucosal immune system may prove valuable.

Endogenous antibody production to botulinum toxin has been demonstrated in a case of adult intestinal colonisation botulism (Griffin *et al.*, 1997). Cattle (Jean *et al.*, 1995) and horses (Whitock and Buckley, 1997) recovering from type C botulism have been shown to develop protective immunity against the organism. Hunter and Poxton (2001) suggest that horses in contact with EGS cases may be exposed to low levels of toxin resulting in a subclinical condition with the generation of a protective immune response.

The project hypothesis was that EGS was associated with toxicoinfection with *C. botulinum* type C. The diagnosis of toxicoinfectious botulism requires the demonstration of the presence of both the toxin and the toxin-producing organism in the affected animal. Infant botulism is diagnosed in this way (Hathaway, 1979). The ability to detect the toxin and the toxin-producing organism in EGS cases from this study was beyond the scope of this project. Hunter *et al.*, (1999) have however, demonstrated the presence of *C. botulinum* type C toxin in the ileum and faeces of horses with EGS. *C. botulinum* type C is reported to be a fastidious organism with successful culture and isolation (with or without enrichment) being very difficult (L.C. Hunter, personal communication). As a result, it is possible that *C. botulinum* type C may not be detectable in all cases of EGS. Even if the organism is detected in faeces after enrichment it does not necessarily mean that the organism was producing toxin *in vivo*, and conversely an organism that was producing toxin *in vivo* may not be detected in culture due to loss of the prophage.

C. botulinum is commonly found in soil with different types found in different areas. Type C is the predominant type found in Europe (Johnston and

Whitock, 1987). The presence of *C. botulinum* in the soil probably accounts for its presence in mammal, bird and fish intestines (Smith, 1977). The faeces of herbivores commonly contain the spores of *C. botulinum* and have been found in healthy foals and adult horses without any evidence of disease (Swerczek, 1980). If *C. botulinum* type C commonly inhabits the intestines of horses in the UK there is a need to identify the trigger for rapid growth and sporulation that leads to toxin production *in vivo.*

The model-based relationships between age and ELISA OD provided a possible explanation of why younger horses are at greater risk of disease. These are very basic models with the assumptions that the horses have already mounted a primary immune response and that the antigenic challenge is constant. Alternative formulations will be considered in the future with varying age and varying exposure, for example. For all three antigens the point of equilibrium, where the rate of antibody production was equal to the rate of loss, was not reached until the horses were at least 7 years of age. This is compatible with the clinical picture where horses between the ages of 2 and 7 years are at greater risk (Wood *et al.,* 1998). Up to the age of 7, (9 for *C. novyi* type A, 10 for BoNT/C toxoid) it is possible that horses have not received enough exposure to the aetiological agent to produce a protective systemic antibody level. It is possible therefore that horses under the age of 10 years are candidates for vaccination to provide the age related protection highlighted in this study. An approved type C vaccine is currently not available for horses. An ideal vaccine would be one that consists of type C1 and C2 toxins. A commercial type B vaccine exists but cross immunity does not exist between types B and C (Kinde *et al.,* 1991). There is a

commercial type C vaccine available for mink but is not approved for use in horses.

The relationship between ELISA OD and number of days between onset of disease revealed no significant associations. However, an interesting pattern was observed for the BoNT/C antigen. As the number of days between onset of disease and sample collection increased, BoNT/C ELISA OD increased. The longer duration of disease of the few chronic cases in this study could, in theory allow time for an immune response to develop as it is these few chronic cases that add weight to the observation. Hunter and Poxton (2001) observed an increase in ELISA OD in chronic cases that were sampled more than once during the course of the disease. However, Hunter and Poxton (2001) stated that it is doubtful whether these observations could be interpreted as seroconversion, as the majority of increases in ELISA OD were very small changes and decreases in OD were also observed. The fluctuations in OD levels over the course of the disease could be due to the *in vivo* neutralisation of the organism and toxin, resulting in a reduction in the level of IgG available for detection by ELISA. This may also explain why horses with EGS have a significantly lower level of IgG to these antigens compared to healthy control animals (Hunter and Poxton, 2001). On the contrary, it should also be noted that there is a lag period between exposure to toxin and/or bacterial organism and a detectable antibody level. Tizzard (1992) states antibodies in serum become detectable about one week after exposure and climbs for 10 to 14 days before declining and disappearing within a few weeks. After a second exposure the lag period only lasts for 2 to 3 days and antibody level rises rapidly and may remain detected for months or even years after exposure. Therefore, we could go back to the original idea that EGS cases have

had no previous exposure to the bacteria and its toxin whilst control animals have had previous exposure and have mounted a protective level of immunity.

It was thought that horses may have a seasonal variation in exposure to *C. botulinum* type C. The results of this study provide no convincing evidence to support this hypothesis. There is a possible increase in exposure, reflected by antibody level, during the spring when the disease was most prevalent but this could be just an incidental finding. It is possible that the older, protected horses maintain a protective level of antibodies throughout the year due to either a continuous challenge throughout the year or, that horses receive a challenge at particular times of the year and their antibody level remains at a constant level throughout the year.

In conclusion, this study has provided further evidence for the role of *C. botulinum* type C in EGS. It is hypothesised that this organism produces toxins enterically that attack the nerve terminals in the gut wall. Hunter and Poxton (2001) provided evidence for the role of the C1 toxin whereas a type C1 toxin complex toxoid has been used here. Further studies are needed to investigate the role of C2 and C3 toxins, as the reagents were not available for this study. It is possible that the C2 and C3 toxins contribute to the wide range of clinical signs observed in EGS. In addition, it has to be determined whether *C. botulinum* type C is the primary cause of EGS or whether it is part of a causal pathway and plays a secondary role to another factor that causes reduced gut motility and/or alteration of the gastrointestinal environment.

ACKNOWLEDGEMENT

The assistance of Professor Ian Poxton, Mr Robert Brown and Dr. Leonie Hunter for the advice and guidance with the serological assays in this chapter are gratefully acknowledged. They are also acknowledged for providing the *C. novyi* type A and *C. botulinum*

CHAPTER 4

A CASE-CONTROL STUDY TO IDENTIFY HORSE-LEVEL AND PASTURE-LEVEL RISK FACTORS FOR EQUINE GRASS SICKNESS.

INTRODUCTION

Equine grass sickness (EGS) is a largely fatal disease of all equidae with an unknown aetiology. Previously, associations between the disease and a number of infective or toxic agents have been investigated. Until recently none were convincingly associated with disease. The failure to identify the causal agent of EGS has meant that epidemiology has played a key role in helping to understand the disease more fully by identifying risk factors.

One of the most useful epidemiological tools to identify risk factors in a rare disease such as EGS is the case-control study. In case-control studies individuals with a particular condition or disease (cases) are selected for comparison with a group of individuals in whom the condition or disease is absent (controls). The case-control study proceeds from effect to cause and is therefore commonly called a retrospective study. The cases and controls are compared with respect to past or present exposures that may potentially be relevant to the development of the condition or disease. The case-control study selects subjects on the basis of the presence or absence of the condition or disease of interest and can quantify the alteration in risk associated with each factor individually and in combination (Schlesselman 1982). Risk factors may either increase or decrease the risk of disease.

A number of risk factors for EGS have been identified. A case-control study was employed by Wood *et al,* (1998) who identified several horse-level risk factors for EGS. Young horses that had a recent change of pasture, on premises where EGS had recently occurred, especially during the months of April, May and June were found to be at greatest risk of disease. Other epidemiological studies identifying risk factors for EGS have been undertaken but there are still areas of

the disease that need further investigation in order to better define those horses and premises that are at highest risk of disease. For example, we have yet to identify particular soil, pasture properties and weather conditions that put a premises at risk. Wood *et al,* (1998) concludes that risk factors such as breed and anthelmintic administration need further study. The epidemiological review in Chapter 2 examines the previous studies undertaken and highlights the need for investigating interactions between recognised risk factors that may ultimately lead to a recognised causal pathway of disease where multiple factors must unite to cause disease.

The epidemiological investigation undertaken here used a matched case-control study design. This study has several advantages to those previously published. Firstly, all EGS cases recruited to the study have been have been histologically confirmed. Secondly, horse controls have been matched to cases by premises in the assumption that controls would be exposed to the same environmental factors as the cases. In addition, control premises were matched to case premises on time in order to control for the seasonal distribution of the disease.

The aim of this investigation was to identify managemental and environmental risk factors in combination with serological evidence of exposure to *C. botulinum* type C (Chapter 3) in order to identify those horses and premises at greatest risk of disease. Ultimately, this information could be used in disease avoidance strategies.

MATERIALS AND METHODS

Study design

The study was carried out between 1999 and 2001. Sample size estimates using EpiInfo software indicated that an unmatched study of 60 cases with 120 controls will give the study power in excess of 99% to detect odds ratios greater than 6.0 with 95% confidence. Odds ratios of this magnitude and greater were expected for variables relating to the primary hypothesis i.e. ELISA OD for antibody to *C. botulinum* toxoid (assuming 90% exposure in cases, 10% in controls). This sample size was also estimated to give in excess of 80% power to detect odds ratios of 3.0 or greater in variables relating to the subsidiary hypotheses; e.g. high grass nitrogen content (assuming exposure of 40% in cases, 20% in controls). Matching of cases and controls was expected to increase the power of the study. The target of sixty cases of EGS was exceeded. The first case of EGS was recruited in March 1999; the 66th and final case was recruited in May 2001. Following recruitment of cases to the study, field visits were subsequently made to EGS case premises and control premises.

Case Selection

EGS cases were recruited to the study in one of two possible ways. Cases that were referred to the Phillip Leverhulme Large Animal Hospital (PLLAH) were automatically recruited whilst another method involved a phone call from a veterinary practitioner to inform us of a suspected case. In the latter method, the horse was either subsequently referred to the PLLAH or samples were obtained in the field and submitted by post. Any horse that gave a negative result on ileal biopsy or post mortem examination of sympathetic or enteric ganglion was

subsequently excluded from the study. Due to time and financial limitations, cases were not accepted onto the study if more than two and a half-hours away by car from the PLLAH.

Control selection

Two control horses were selected per EGS case. Horse controls were preferably co-grazers of the EGS case. If it was not possible to obtain co-grazers, horses were obtained either from the pasture nearest to where the case occurred or, on a minority of occasions, the neighbouring premises. If there were more than two co-grazers, the possible population of control horses were listed in alphabetical order and the two horse whose names came first in the placed order were selected as the control horses.

Two premises controls were selected per EGS case. Premises controls were selected from a population of horses that were referred to the PLLAH. A random number generator (Epi-info, version 6.04) selected PLLAH case numbers from the previous year's referrals. For example, premises control sampling in 2000 used referrals from 1999. No PLLAH case was selected if it was referred for colic. The selected referrals were listed in alphabetical order of horse name and a telephone call was made to the owner. A request was made for a field visit to premises on which their horse was currently kept. The criteria that had to be satisfied by selected control premises were i) they must not have had a case of EGS in the previous 12 months and ii) they must not be more than two and a half hours away from the PLLAH. Where more than one EGS case occurred on a premises, two premises controls per case were identified to increase the power of the study. Due to the foot and mouth outbreak, control premises that were visited

during March, April and May of 2001 were equine premises only (i.e. no sheep, cattle, pigs etc.).

Questionnaire design

Three separate questionnaires were designed for this study (see appendix 3). The first was for the EGS case owner (horse and premises information), the second was for the control horse owner (horse information only) and the third was for the premises control owner (premises information only). The same questions were asked to the EGS case owner and control horse owner about the horse and the same questions were asked to the EGS case owner and premises control owner about the premises. For information about the premises the questionnaire was administered to the either the owner of the horse or the owner or manager of the premises. This latter depended on the knowledge of the horse owner and the availability of the premises owner.

The questionnaire was piloted to horse owners that worked at the PLLAH. The questionnaires requested information on; i) the horse (age, sex, breed and present use); ii) its recent management (time on premises, recent routine, time on recent routine, time on current pasture and any recent change of routine or pasture); iii) feeding (diet composition and any recent changes in feed type or quantity); iv) worming (frequency, time since last worming, time since penultimate worming and brand of wormer used at the last and penultimate worming); v) previous illnesses; vi) relatives diagnosed with EGS; vii) previous EGS cases on the premises; and finally viii) premises management details (ploughing, fertilisation, harrowing, other species). Unless otherwise specified,

many of the details about the case and control horses in the questionnaire related to the two weeks prior to the onset of clinical signs of EGS in the case horse.

Sample collection 1: Case and control horses

Blood and faeces were collected from both the case and control horses. Serum was collected and stored as described by the methods in chapter 2. Faeces from EGS cases were obtained either from rectal examination (performed as part of the clinical evaluation) or were collected post mortem. Faecal samples that were obtained post mortem were collected within 15 minutes of death. For the control horses, owners were requested to stable each horse for at least one hour prior to the field visit. Faecal samples were then collected from a fresh faecal pat within the stable. A minimum of 300g of faeces was collected from each EGS case and control horse and stored at –20 °C until assayed.

Sample collection 2: Case and control premises

Soil and Pasture

Soil and pasture samples were collected from EGS case and control premises. Samples were obtained by walking a 'W' shape across the field stopping to sample every 10 to 20 paces depending on the size of the field. Soil sampling involved the use of a trowel that was placed into the ground to a depth of approximately 2 inches. The trowel was pulled out of the ground bringing with it a sample approximately 2 inches by 2 inches in size. Up to 1 kg of soil was collected from each field sampled. Samples were collected into a polythene bag that was subsequently labelled and stored at –20 °C until assayed. Pasture samples were collected by taking 5 to 15 plucks of grass at each stop during the W' shaped

walk across the field. The number of plucks depended on the length of the sward. The shorter the sward, the greater the number of plucks. Ungrazed 'roughs' of pasture were not sampled. A minimum of 200g of pasture was collected into a polythene bag that was transported chilled and subsequently stored at –20 °C until assayed.

Meteorological Office Data

Meteorological surface data for each case and control premises were collected from the Meteorological Office database at; http://www.badc.rl.ac.uk. This database is known as MIDAS (Met Office Integrated Data Archive System). A username and password were required to access the data.

The EGS case and control premises location was converted into a 4 figure Ordnance Survey (OS) grid reference using The OS Gazetteer of Great Britain (third edition). Using the OS four figure grid reference for each case and control premises two daily observation weather stations for each premises were identified. Daily rainfall and temperature data was available from an 'Ordinary Climatological Station'. This station type records daily precipitation amount (Met domain: DLY3208, Id type: RAIN) and 24 hour max/min temperatures (Met domain: DLY3208, Id type: DCNN). Daily rainfall data were also collected from another station type known as a 'Daily Rainfall Station' (Met domain: WADRAIN, Id type: RAIN). Data were collected from such a station in addition to the Ordinary Climatological Station due the daily rainfall stations often being nearer to each case and control premises. Data collected from the above weather stations consisted of i) daily rainfall total, ii) daily minimum temperature and iii), daily maximum temperature. Where possible, daily minimum grass temperature

was also collected. The daily temperature range was calculated from the minimum and maximum daily temperature.

Laboratory procedures 1: Case and control horses

Serological assays

Enzyme linked immunosorbent assays (ELISA's) to investigate the exposure of EGS cases and control horses to *C. botulinum* type C were carried out as described in Chapter 2. Three different antigens were used; *C. novyi* type A surface antigen, *C. botulinum* type C surface antigen and a *C. botulinum* type C toxin complex toxoid (BoNT/C toxoid).

As part of an evaluation of parasite status, serum was used in an ELISA to test both case and control horses for tapeworm (*Anoplocephala perfoliata*) status. The method used was that developed and described by Proudman *et al.*, (1998).

Faecal Analysis

In addition to the tapeworm ELISA, a faecal egg count (FEC) was also used to evaluate the parasite status of case and control horses. 5 – 10g of faeces were taken out of the main (>300g) faecal sample prior to it being stored at –20°C. A FEC test was carried out within 3 days of sample collection using the McMasters method.

The bulk of the faecal sample was transported, overnight with ice, to the Analytical Services Department at the Scottish Agricultural College (SAC), Edinburgh. Here the samples were analysed for dry matter, pH, total nitrogen and ammoniacal nitrogen.

Laboratory procedures 2: Case and control premises

Soil samples

As described for faecal samples, soil samples were transported, overnight with ice, to the Analytical Services Department at the (SAC), Edinburgh. Here the samples were analysed for dry matter, pH, extractable nitrogen, ammoniacal nitrogen and total nitrogen.

Pasture Samples

Pasture samples were transported, overnight with ice, to the Analytical Services Department at the (SAC), Edinburgh. Samples were analysed for dry matter and total nitrogen.

Data Storage

Data from the questionnaires were entered into a Microsoft Access database along with the results of the ELISA's (*C. botulinum* antigens and tapeworm ELISA) and the FEC's. Separate databases were set up in Microsoft Access for i) Met. Office data; ii) SAC faecal analysis and iii) SAC soil analysis. All data were subsequently exported to EGRET where all the statistical analyses were undertaken.

Statistical analysis

Descriptive statistics

Data from faecal analysis, soil analysis, pasture analysis and Meteorological Office data were tested for normality using the Anderson-Darling statistic in the MINITAB® statistics package (version 11.21). A students T test

was used to statistically compare data that were normally distributed whilst a Mann Whitney U test was used to statistically compare non-parametric data from case and control premises. Boxplots, bar charts and regression plots were produced using the MINITAB® statistics package (version 11.21). Throughout, the critical probability was 0.05.

Univariable and multivariable analysis

As horse controls were matched to EGS cases by premises and premises controls were matched to EGS premises by date, a matched analysis was performed (Schlesselman, 1982). Horse-level and premises level variables were analysed separately. Matched sets were identified by means of unique number allocated to each EGS case and control triplet.

The dependent variable for the analyses was grass sickness (case) or not grass sickness (control). A table to define the independent variables entered in the analysis is in appendix iv. For both continuous and categorical variables, univariable odds ratios and exact confidence intervals were calculated using conditional logistic regression analysis (Hosmer and Lemeshow, 2000) (EGRET, version 2.0.3, Cytel Software Corporation). The conditional logistic regression model is commonly used to model matched binary data. Multivariable logistic regression models were constructed using backward elimination procedures. Variables were considered for inclusion in a multivariable model if their univariable P-value was < 0.3. Variables with a term wise Wald test $P<0.05$ or variables that improved the fit (likelihood ratio chi squared statistic $P<0.05$) with a reduction in residual deviance were left in the model. Interaction terms were tested between all plausible biological terms.

Prior to multivariable analysis, linear transformation of continuous variables were considered. Variables were centred (variable mean subtracted from each variable value) and squared (centred value squared) in addition to being categorised. In this instance, variables were categorised into quintiles. Categorising variables enables the identification of non-linear relationships whereas centering variables can reduce the effect of multicollinearity. After transforming continuous variables it was determined that all variables remaining in the final models should remain in their original form.

The effect of confounding was also considered when constructing the final multivariable logistic regression models. Any variable that markedly altered the coefficient and odds ratio of other main effect variables when removed from the model was considered as a confounding variable and dealt with appropriately. A confounding variable was suspected when a change in odds ratio of around 25% occurred in any of the remaining model variables when it was added or removed from the model.

The fit of the models was assessed by calculating the sensitivity (proportion of true cases predicted by the models) and specificity (proportion of true controls that are predicted by the models). Varying predicted-value cut-off points were used. Fitted values (estimated expected conditional probabilities associated with each observation) were calculated in EGRET.

RESULTS

PART A: HORSE-LEVEL VARIABLES

Descriptive analysis

Sixty-six cases of grass sickness from 58 different premises were recruited to the study during the period of March 1999 and May 2001. Thirty-five EGS cases were recruited during 1999 whist 16 cases were recruited in 2000 and 15 in 2001. Forty-seven EGS cases were recruited to the study via the PLLAH whilst 19 cases were recruited directly from veterinary surgeons. Fifty-six per cent of EGS cases were mares whilst 44% were either geldings or stallions. Forty seven per cent of cases were not in work. These horses were usually either breeding mares or they were unbroken, young animals. Five percent of EGS cases were actively competing whilst the remaining 48% were general riding horses. EGS occurred in a wide variety of breeds. For statistical analysis the large numbers of different breeds were grouped; Thoroughbred (EGS cases, n = 10), Thoroughbred cross (n= 4), hunter/mixed breed (n=23), Warmblood and other (n=3), Arab and Anglo Arab (n=6), native breeds (n=9) and welsh mountain ponies and cobs (n=21). Native breeds included Shetland ponies, Shires, Connemaras, and Fell ponies. 'Others' included breeds such as an Icelandic pony and an Apaloosa.

During the study, 100% EGS case owner compliance was received. There was also 100% control horse owner compliance and 98% premises control owner compliance.

Univariable analysis

Results of univariable analysis of binary and categorical variables and continuous variables are shown in tables 4.1 and 4.2 respectively.

1.The horse.

Univariable analysis revealed a significant ($p = <0.001$) association between EGS and age. The age group at which horses were most at risk were horses aged 4 to 5 years where they were twice as likely to suffer disease than horses that were aged three or less (referent group). Horses aged 6 to 7 years were more than 1.5 times less likely to suffer disease, horses aged 8 to 11 years were more than two times less likely and horses over the age of 12 were 25 times less likely to suffer disease than the referent group. A significant ($p = 0.002$) association was found between the occurrence of EGS in different breeds. Using Thoroughbred cross as a referent breed, Arabs and Anglo Arabs were seventeen times more likely to suffer EGS whilst Welsh Mountain ponies and cobs were over nine times more likely and native breeds nearly seven times more likely. The significant association between EGS and breed was not confirmed in the multivariable analysis.

2. Horse management

Time spent on the current pasture was significantly ($p = <0.001$) associated with EGS. Analysis of this continuous variable revealed that horses that had grazed the pasture for 14 days or less were most at risk. However, analysis of the binary variables revealed that horses were three times more likely to suffer EGS if they had grazed the pasture for 14 days or less ($p = 0.001$), three times

more likely to suffer EGS if they had grazed the pasture for 21 days or less (p = 0.001), and nearly nine time more likely to suffer EGS if they had grazed the pasture for 56 days or less (p = <0.001). This latter variable 'grazed the pasture for less than 56 days' was also found to be significant in the final multivariable model.

There were several variables found to be significant in the univariable analysis that were not found to be significant in the multivariable model. Univariable analysis revealed a significant (p = 0.006) association between time spent on the current premises and EGS. Horses that had been on the premises less than 63 days were most at risk. It is likely that time on premise was both confounded by and correlated with time on pasture. A significant (p = 0.04) association was also found between the number of hours grazed during the week leading up to disease and EGS. When comparing two groups; horses were more than 3.5 times more likely to suffer disease if they had grazed for more than 77 hours in the previous week when compared to those horse that had grazed for 77 hours or less.

3. Feeding

A significant association (p = 0.05) was found between EGS and the feeding of hay. Horses that had hay in their daily diet were almost two and a half times less likely to suffer disease that those who didn't have hay in their diet. In this context hay includes dry hay, soaked hay, horsehage and haylage. Interestingly, it appears that haylage is exerting the protective effect. A significant (p = <0.001) association was also found between horses that had a recent change in feed type and/or quantity and EGS. Horses were six times more likely to suffer

disease if they had a recent (within 14 days) change in feed type or quantity. Thirty-two percent of EGS cases had a change in the last 14 days whilst only 10% of control horses had a change. Table 4.3 lists the types of feed change recorded in the questionnaires.

4. Worming

A significant association ($p = 0.03$) was found between worming a horse with Ivermectin on the penultimate worming occasion and EGS. If the horse had been wormed with Ivermectin on that occasion they were more than four times more likely to suffer disease. In contrast, horses that were wormed with Ivermectin on the last occasion were almost twice as likely to suffer disease but this association was not found to be significant in the univariable analysis ($p = 0.24$). A significant ($p = <0.001$) association was also found between EGS and faecal egg count. Cases were more likely to have zero counts than controls. This significant association was however, strongly confounded by the use of ivermectin. After allowing for the confounding effect of ivermectin, the relationship between EGS and faecal egg count was no longer significant.

5. Exposure to EGS.

A history of grazing the same pasture as an EGS cases (either during the current outbreak or a previous one) was associated with a twenty-fold reduction in disease risk. We have not been notified of any of the control horses developing EGS at anytime during the study period. In this study, 18% ($n = 12$) of cases had exposure to EGS whilst 83% of controls had exposure to the disease. There was

no significant association if there was a history of grazing the same pasture as an EGS case during a previous outbreak only.

A borderline significant association (p = 0.059) was found between having a relative that has had EGS and the development EGS. If the horse had a relative with EGS, then it was nearly four times more likely to suffer disease than if it had not.

Multivariable analysis

1. Horse-level risk factors

Four variables were included in the final multivariable conditional logistic regression model (Table 4.4). These were age, hay in daily diet, the use of ivermectin as the penultimate wormer and a change of feed type or quantity. EGS was associated with younger horses (OR 0.79 per increase in one year, 95 per cent CI 0.69 to 0.91, P = <0.001), having hay in the daily diet (OR 0.29, 95 per cent CI 0.09 to 0.83, P = 0.02), having ivermectin as the penultimate wormer (OR 5.64, 95 per cent CI 1.23 to 25.84, P = 0.03) and having a change of feed type or quantity in the 14 days prior to disease (OR 7.71, 95 per cent CI2.31 to 26.10, P = <0.001). All of these variables were found to be significant in the univariable analysis. The P values have remained the same for all variables except hay in daily diet (0.05 to 0.02). In the multivariable model, the OR for all variables moved further away from 1 when compared to the univariable model.

2. *Horse-level risk factors and C. novyi type A ELISA OD*

A multivariable conditional logistic regression model was constructed using horse-level variables as described above in addition to serological data from the ELISA to detect antibodies to *C. novyi* type A. The serology results are described more fully in Chapter 3. Five variables were included in this model (table 4.5). These were age, *C. novyi* antibody level, feed change, grazed pasture less than 56 days and ivermectin as the penultimate wormer. EGS was associated with younger horses (OR 0.80 per increase in one year, 95 per cent CI 0.67 to 0.95, P = 0.09), low level of antibodies to *C. novyi* type A (OR 0.96 per 1 per cent increase in per cent positivity, P = <0.001), having a change of feed type or quantity in the 14 days prior to disease (OR 3.81, 95 per cent CI 1.07 to 13.55, P = 0.04), grazing a pasture for less than 56 days (OR 6.09, 95 per cent CI 1.53 to 24.22, P = 0.01) and having used ivermectin as the penultimate wormer (OR 4.94, 95 per cent CI 1.01 to 24.15, P = 0.048). In this model, ORs for all variables except ivermectin moved closer to one and all P values except *C. novyi* antibody level increased when compared to the univariable model.

3. *Horse-level risk factors and C. botulinum type C ELISA OD*

A multivariable conditional logistic regression model was constructed using horse-level variables and serological data from the ELISA to detect antibodies to *C. botulinum* type C. Five variables were included in this model (table 4.6). These were age, *C. botulinum* antibody level, ivermectin as the penultimate wormer, feed change and grazed pasture less than 56 days. EGS was associated with age (OR 0.80 per increase in 1 year, 95 per cent CI 0.68 to 0.94, P =0.007), low level of antibodies to *C. botulinum* type C (OR 0.96 per increase in 1 per cent

positivity, 95 per cent CI 0.93 to 0.98, P = 0.003, ivermectin as the penultimate wormer used (OR 6.08, 95 per cent CI 1.15 to 17.35, P =0.029), having a change of feed type or quantity in the previous 14 days (OR 5.04, 95 per cent CI 1.15 to 17.35, P = 0.029) and having grazed a pasture for less than 56 days (OR 4.79, 95 per cent CI 1.32 to 17.32, P = 0.017). In this model, when comparing to the univariable model the ORs remained the same for age and antibody level, moved further away from 1 for ivermectin but closer to 1 for feed change and grazed pasture less than 56 days. All P values increased except for ivermectin.

4. Horse-level risk factors and BoNT/C toxoid ELISA OD

A multivariable conditional logistic regression model was constructed using horse-level variables and serological data from the ELISA to detect antibodies to BoNT/C toxoid. Five variables were included in the final model (table 4.7). In this model EGS was associated with age (OR 0.80 per increase in 1 year, 95 per cent CI 0.68 to 0.94, P =0.005), low level of antibodies to BoNT/C toxoid (OR 0.98 per increase in 1 per cent positivity, 95 per cent CI 0.96 to 0.99, P = 0.01), ivermectin as the penultimate wormer used (OR 6.73, 95 per cent CI 1.41 to 33.11, P =0.019), having a change of feed type or quantity in the previous 14 days (OR 4.83, 95 per cent CI 1.41 to 16.47, P = 0.012) and having grazed a pasture for less than 56 days (OR 5.48, 95 per cent CI 1.48 to 20.30, P = 0.011). When compared to the univariable model the OR's remained the same for age and antibody level, moved further away from 1 for ivermectin but closer to 1 for feed change and grazed pasture less than 56 days. All P values increase except for ivermectin.

Sensitivity and Specificity of multivariable models

The fit and predictive ability of the 8 horse-level models were further assessed by calculating the sensitivity and specificity of each of the models at various cut-off points (tables 4.8 to 4.11). When a cut-off point of 0.3 was selected (i.e. if the predicted probability of becoming an EGS case is above 0.3 and the horse is predicted to develop EGS), the model that gave the best fit and predictive ability was that which had the variables age, antibody level to *Clostridium botulinum* type C, ivermectin as the penultimate wormer, feed change in the previous 14 days and grazing a pasture for less than 56 days. . The sensitivity of this model was 88% and the specificity was 79%.

Further analysis

Further analyses were carried out investigating the association between the anthelmintic ivermectin and EGS. Univariable analysis revealed no significant association between EGS and the number of days between disease occurrence and the use of ivermectin (OR 1.009, 95% CI 0.99 to 1.03, P = 0.44).

Three multivariable models were produced using the variables age, faecal egg count the use of ivermectin as the final or penultimate wormer (table 4.12). Whist taking into account age and faecal egg count horses were twice as likely to develop EGS if they had ivermectin as the last wormer (P=0.3, 95% CI 0.5 – 9.2) whilst horses that had ivermectin as the penultimate wormer were nearly 7 times more likely (P=0.03, 95% CI 1.2 – 38.5) and horses were nearly 12 times more likely of they had ivermectin on both occasions.

TABLE 4.1: Univariable Analysis of binary and categorical variables with odds ratio, 95% confidence intervals (95% CI)

Variable		Case % (n)	Control % (n)	Odds ratio	95% CI	P-value
The horse						
Sex	Mare	56 (37)	61 (81)	Ref		
	Geldings and stallions	44 (29)	39 (51)	0.79	0.42 - 1.47	0.45
Breed	TB cross	6 (4)	24 (32)	Ref		
	TB	15 (10)	11(15)	4.62	1.19 - 17.89	
	Hunter/Mixed	23 (15)	22 (29)	4.18	1.22 - 14.25	
	WB & Other	5 (3)	10 (13)	0.93	0.09 - 9.22	0.002
	Arab/Anglo	4 (6)	2 (2)	17.21	2.11 -140.2	
	Arab	14 (9)	11 (15)	6.69	1.52 - 29.46	
	Native Welsh Mountain /Cob	32 (21)	20 (26)	9.42	2.44 - 36.35	
Use	Not in work*	47 (31)	45 (60)	Ref		
	General/Hack	48 (32)	47 (61)	1.06	0.53 - 2.11	0.59
	Competing	5 (3)	8 (11)	0.53	0.14 - 2.03	
Horse Management						
Recent Routine	Stabled part of day or night.	22 (14)	29 (38)	Ref		
	Grass24 hours	78 (51)	71(94)	2.54	0.85 - 7.60	0.09
Change of routine in the last 14 days	Yes	21(14)	14 (19)	2.38	0.82 – 6.88	0.11
	No	79 (52)	84 (113)	Ref		
Inconsistent routine	Yes	12 (8)	11 (14)	1.42	0.67 - 3.01	0.36
	No	88 (58)	89 (118)	Ref		
Grazing pasture < 14 days	Yes	33 (20)	18 (24)	3.18		
	No	67 (44)	82 (106)	Ref	1.32- 7.65	<0.001

Grazing pasture	Yes	39 (24)	24 (32)	3.35		
< 21 days	No	61 (40)	82 (98)	Ref	1.34 - 8.41	<0.001
Grazing pasture	Yes	70 (44)	51 (66)	8.41		
< 56 days	No	30 (20)	49 (64)	Ref	2.43 - 29.13	<0.001
Feeding						
Hay	Yes	36 (24)	48 (63)	0.43		
	No	64 (42)	52 (69)	Ref	0.18 - 0.98	0.05
Concentrates	Yes	50 (33)	55 (72)	0.67		
	No	50 (33)	45 (60)	Ref	0.28 - 1.63	0.38
Course Mix	Yes	32 (21)	36 (47)	0.70		
	No	68 (45)	64 (85)	Ref	0.30 – 1.63	0.41
Cubes	Yes	11 (7)	14 (19)	0.36		
	No	89 (59)	86 (113)	Ref	0.07 – 1.90	0.23
Oats	Yes	11 (7)	5 (7)	7.19		
	No	89 (59)	95 (125)	Ref	0.81 – 64.17	0.07
Barley	Yes	8 (5)	10 (13)	0.57		
	No	92 (61)	10 (119)	Ref	0.12 – 2.75	0.49
Bran	Yes	9(6)	8 (11)	1.28		
	No	91 (60)	92 (121)	Ref	0.24 – 6.89	0.77
Sugar Beet	Yes	12 (8)	17 (23)	0.46		
	No	88 (58)	83 (109)	Ref	0.14 – 1.54	0.21
Mollichop	Yes	9 (6)	14 (18)	0.80		
	No	91 (60)	86 (114)	Ref	0.13 – 1.76	0.27
Alfalfa	Yes	8 (5)	8 (11)	0.81		
	No	92 (61)	92 (121)	Ref	0.17 – 3.93	0.79
Hi-Fi	Yes	9 (6)	10 (13)	0.87		
	No	91 (60)	90 (119)	Ref	0.25 – 3.07	0.83
Dry Hay	Yes	20 (13)	20 (26)	1.0		
	No	80 (53)	80 (106)	Ref	0.38 – 2.66	1.0

Wet Hay	Yes	11(7)	10 (13)	1.47		
	No	89 (59)	90 (119)	Ref	0.64 – 3.35	0.36
Haylage	Yes	12 (8)	18 (24)	0.25		
	No	88 (58)	82 (108)	Ref	0.05 – 1.25	0.09
Supplements	Yes	29 (19)	37 (49)	0.54		
	No	71 (47)	63 (83)	Ref	0.23 - 1.24	0.15
Change of feed type/quantity in the last 14 days.	Yes	32 (21)	10 (13)	6.09		
	No	68 (45)	90 (119)	Ref	2.23 - 16.61	<0.001
Intermittent feeding	Yes	6 (4)	8 (10)	0.71		
	No	94 (62)	92 (122)	Ref	0.167 – 3.00	0.64
Worming						
Ivermectin (last worming)	Yes	33 (22)	28 (37)	1.88		
	No	66 (44)	78 (95)	Ref	0.66 - 5.39	0.24
Moxidectin (last worming)	Yes	32 (21)	29 (38)	1.75		
	No	68 (45)	71 (94)	Ref	0.47 - 6.40	0.40
Benzimidazole (last worming)	Yes	15 (10)	22 (29)	0.41		
	No	85 (56)	78(103)	Ref	0.13 - 1.31	0.13
Pyrantel (last worming)	Yes	15 (10)	17 (22)	0.79		
	No	85 (56)	83 (110)	Ref	0.25 - 2.52	0.70
Ivermectin (Penultimate worming)	Yes	39 (25)	29 (38)	4.42		
	No	61 (41)	71 (94)	Ref	1.17 - 16.71	0.03
Moxidectin (Penultimate worming)	Yes	9 (6)	11 (14)	0.76		
	No	91 (60)	89 (118)	Ref	0.22 - 2.7	0.67
Benzimidazole (Penultimate worming)	Yes	9 (12)	18 (24)	1.24		
	No	89 (54)	82 (110)	Ref	0.41 - 3.73	0.71
Pyrantel (Penultimate worming)	Yes	15 (10)	20 (26)	0.53		
	No	85 (56)	80 (106)	Ref	0.17 - 1.66	0.28

Exposure to EGS						
Relative with EGS	Yes No	12 (8) 88 (58)	5 (7) 95 (125)	3.78 Ref	0.95- 15.04	0.059
Grazed an EGS pasture? (previous outbreak)	Yes No	6 (4) 94 (62)	14 (18) 86 (114)	0.29 Ref	0.06 – 1.47	0.135
Grazed an EGS pasture? (current or previous outbreak)	Yes No	27 (18) 73 (48)	83 (110) 17 (22)	0.05 Ref	0.02 - 0.15	<0.001

* = Retired, not in use or unbroken.
TB= Thoroughbred
WB= Warmblood

TABLE 4.2: Univariable analysis of categorised continuous variables with odds ratio, 95% confidence intervals (95% CI) and P – values.

		Case % (n)	Control % (n)	Odds ratio	95% CI	P-value
Age (years)	<1 – 3	28 (18)	20 (26)	Ref		
	4 – 5	35 (24)	13 (17)	2.03	0.70 – 5.81	
	6 – 7	17 (11)	17 (23)	0.62	0.20 – 1.94	<0.001
	8 – 11	18 (12)	23 (30)	0.45	0.13 – 1.59	
	>12	2 (1)	27 (36)	0.04	0.005 – 0.33	
Time on premises (days)	9 – 63	26 (17)	17 (23)	Ref		
	70 – 364	30 (20)	23 (30)	0.63	0.18 – 2.22	
	365 – 730	23 (15)	16 (21)	0.49	0.14 – 1.75	0.01
	731 – 1460	9 (6)	23 (30)	0.11	0.02 – 0.51	
	1461 – 7280	12 (8)	21 (28)	0.23	0.06 – 0.85	
Time on pasture (days)	1 – 14	33 (21)	19 (24)	Ref		
	15 – 28	19 (12)	18 (23)	0.81	0.23 – 2.90	
	29 – 84	23 (15)	19 (24)	0.46	0.14 – 1.54	<0.001
	85 – 196	17 (11)	23 (31)	0.16	0.04 – 0.67	
	>196	8 (5)	21 (27)	0.05	0.007 – 0.28	
Time on recent routine (days)	1 – 28	32 (21)	24 (31)	Ref		
	29 – 56	22 (14)	16 (21)	0.86	0.27 – 2.8	
	57 – 140	14 (9)	17 (23)	0.37	0.09 – 1.39	0.12
	141 – 476	17 (11)	20 (26)	0.30	0.07 – 1.2	
	>476	15 (10)	23 (30)	0.29	0.09 – 0.94	
Hours grazed per day	4 – 7	11 (7)	15 (20)	Ref		
	8 – 12	9 (6)	12 (16)	1.44	0.23 – 8.92	0.09
	>12	80 (53)	73 (96)	3.97	0.94 – 16.80	
Hours grazed in week	8 – 77	17 (11)	33 (42)	Ref		
	>77	83 (54)	67 (85)	3.59	0.94 – 13.77	0.04
Faecal egg count (eggs per gram)	0	79 (49)	60 (76)	Ref	0.32 – 2.2	
	25 – 75	13 (8)	9 (12)	0.84	0.05 – 0.78	<0.001
	100 – 450	5 (3)	15 (19)	0.20	0.006 – 0.48	
	>450	3 (2)	16 (20)	0.05		
Tapeworm ELISA OD	0 – 0.02	21 (13)	18 (24)	Ref		
	0.03 – 0.04	17 (11)	23 (30)	0.93	0.36 – 2.39	
	0.05 – 0.07	24 (15)	18 (24)	0.84	0.32 – 2.22	
	0.08 – 0.16	19 (12)	20 (26)	1.70	0.66 – 4.37	0.67
	>0.16	19 (12)	21 (27)	1.16	0.41 – 3.22	
Number of days to last worming	1 – 18	19 (12)	16 (21)	Ref		
	19 – 35	27 (17)	24 (30)	1.22	0.27 – 5.54	
	36 – 56	23 (15)	28 (35)	0.56	0.13 – 2.52	0.85
	57 – 84	11 (7)	13 (16)	0.69	0.13 – 3.77	
	>85	20 (13)	20 (25)	1.05	0.21 – 5.29	

Table 4.3: Types of feed change

Type of change	Cases % (n)	Controls % (n)
Change of feed type	6 (4)	0
Hay excluded from diet	2.5 (2)	2 (2)
Concentrates excluded from diet	5 (3)	2 (2)
Decrease in hay or concentrate.	0	1 (1)
Increase in hay or concentrate quantity.	5 (3)	0
Hay & concentrates excluded	6 (4)	2 (2)
Hay and concentrates added	1.5 (1)	1 (1)
Hay added	6 (4)	2 (2)

Tables 4.4 – 4.7: Final multivariable conditional logistic regression models of risk factors associated with equine grass sickness with odds ratio, 95% confidence limits (95% CI) of odds ratio and p-values.

Table 4.4: Horse-level risk factors

Variable		Coefficient	Standard error	Odds Ratio	95% CI	P-value
Age	Increase of one year	-0.228	0.069	-	-	<0.001
Hay in daily diet	Yes	-	-	0.29	0.09 – 0.83	0.02
Ivermectin as penultimate wormer	Yes	-	-	5.64	1.23 – 25.84	0.03
Feed change in previous 14 days	Yes	-	-	7.71	2.31 – 26.10	<0.001

Table 4.5: Horse-level risk factors and *C. novyi* type A

Variable		Coefficient	Standard error	Odds Ratio	95% CI	P-value
Age	Increase of one year	-0.223	0.085	-	-	0.009
Antibody level to *C. novyi* type A	Increase of one % positivity	-0.042	0.012	-	-	<0.001
Ivermectin as penultimate wormer	Yes	-	-	4.94	1.01 – 24.15	0.048
Feed change in previous 14 days	Yes	-	-	3.81	1.07 – 13.55	0.040
Grazed pasture less than 56 days	Yes	-		6.09	1.53 – 24.22	0.011

Table 4.6: Horse-level risk factors and *C. botulinum* type C

Variable		Coefficient	Standard error	Odds Ratio	95% CI	P-value
Age	Increase of one year	-0.222	0.082	-	-	0.007
Antibody level to *C. botulinum* type C	Increase of one % positivity	-0.042	0.014	-	-	0.003
Ivermectin as penultimate wormer	Yes	-	-	6.08	1.30 – 28.50	0.022
Feed change in previous 14 days	Yes	-	-	5.04	1.15 – 17.35	0.010
Grazed pasture less than 56 days	Yes	-	-	4.79	1.32 – 17.32	0.017

Table 4.7: Horse-level risk factors and BoNT/C toxoid

Variable		Coefficient	Standard error	Odds Ratio	95% CI	P-value
Age	Increase of one year	-0.227	0.081	-	-	0.005
Antibody level to BoNT/C toxoid	Increase of one % positivity	-0.022	0.009	-	-	0.011
Ivermectin as penultimate wormer	Yes	-	-	6.73	1.41 – 33.11	0.019
Feed change in previous 14 days	Yes	-	-	4.83	1.41 – 16.47	0.012
Grazed pasture less than 56 days	Yes	-	-	5.48	1.48 – 20.30	0.011

Tables 4.8 to 4.11: Sensitivity (EGS case) and specificity (control) of the 4 conditional logistic regression models at various cut-off points.

Table 4.8: Horse-level risk factors

Cut Off	Sensitivity	Specificity
0.1	0.969	0.415
0.2	0.923	0.615
0.3	0.815	0.769
0.4	0.677	0.846
0.5	0.615	0.915
0.6	0.538	0.923
0.7	0.4	0.946
0.8	0.308	0.985
0.9	0.154	1.000

Table 4.9: Horse-level risk factors and *C. novyi* type A

Cut Off	Sensitivity	Specificity
0.1	0.985	0.585
0.2	0.877	0.738
0.3	0.815	0.792
0.4	0.8	0.846
0.5	0.723	0.908
0.6	0.646	0.923
0.7	0.538	0.946
0.8	0.415	0.927
0.9	0.277	1.000

Table 4.10: Horse-level risk factors and *C. botulinum* type C

Cut Off	Sensitivity	Specificity
0.1	0.969	0.561
0.2	0.938	0.7
0.3	0.877	0.792
0.4	0.815	0.838
0.5	0.677	0.915
0.6	0.585	0.923
0.7	0.492	0.938
0.8	0.385	0.992
0.9	0.231	0.992

Table 4.11: Horse-level risk factors and BoNT/C toxoid

Cut Off	Sensitivity	Specificity
0.1	1.000	0.546
0.2	0.923	0.646
0.3	0.846	0.746
0.4	0.723	0.831
0.5	0.585	0.861
0.6	0.523	0.923
0.7	0.446	0.954
0.8	0.385	0.977
0.9	0.262	1.000

Table 4.12: Multivariable conditional logistic regression models of the association between age, faecal egg count (FEC) and ivermectin with equine grass sickness. Odds ratio, 95% confidence limits (95% CI) of odds ratio and p-values are reported.

Parameter estimates			95% CI	
Terms	**p-value**	**Odds ratio**	**Lower**	**Upper**
Model 1				
age	<0.001	0.8	0.7	0.9
FEC c.f zero	0.03			
low		1.1	0.4	3.3
moderate		0.3	0.1	1.2
high		0.03	0.00	0.48
Ivermectin last	0.31	2.1	0.5	9.2
Model 2				
age	<0.001	0.8	0.7	0.9
FEC c.f zero	0.04			
low		1.1	0.4	3.2
moderate		0.3	0.1	1.4
high		0.02	0.00	0.45
Ivermectin penultimate	0.03	6.8	1.2	38.5
Model 3				
age	<0.001	0.8	0.7	0.9
FEC c.f zero	0.06			
low		1.1	0.4	3.2
moderate		0.3	0.1	1.3
high		0.02	0.00	0.58
Ivermectin last and penultimate	0.03	11.9	1.2	118.2

PART B: FAECAL ANALYSIS

Descriptive analysis

Faecal samples were analysed from 48 EGS cases and 124 control horses. A number of EGS cases had little or no faeces in the rectum on clinical examination. A significant ($p = <0.001$) difference was found between EGS cases and controls for dry matter (%), total nitrogen (%) and ammoniacal nitrogen (%) content of faecal material. EGS cases had a significantly higher dry matter (figure 4.1a), total nitrogen (figure 4.1b) and ammoniacal nitrogen content in faecal samples (figure 4.1c).

Univariable analysis

Results of univariable analysis of faecal data are shown in table 4.21. Univariable analysis of faecal data revealed a significant ($p = <0.001$) association between EGS and the dry matter (DM), total nitrogen and ammoniacal nitrogen content of faeces. Univariable analysis revealed an increased risk of disease per % increase in dry matter, an increased risk of disease per % increase in total nitrogen (% DM) and an increase in risk per % increase ammoniacal nitrogen (% DM). The significant association between EGS and total nitrogen was not confirmed in the multivariable analysis.

Multivariable analysis

Two variables were included in the final multivariable model (table 4.13). Coefficients and standard errors have once again been quoted. These were dry matter and ammoniacal nitrogen content of the faeces. EGS was associated with

an increased dry matter content of faecal material (coefficient 0.39, standard error 0.119, P = <0.001) and an increased ammoniacal nitrogen content (coefficient 18.31, standard error 6.10, P = 0.027). After adjusting for confounding, the estimated odds ratio for dry matter remained very close to the univariable model whilst the odds ratio for ammoniacal nitrogen increased when compared to the univariable model. Similarly, the P value for dry matter remained the same as the univariable model whereas the P value for ammoniacal nitrogen increased from <0.001 to 0.003. Linear transformation of continuous variables were considered prior to entry into multivariable models. Variables that categorised, centred or centre squared did not improve the fit of the final model.

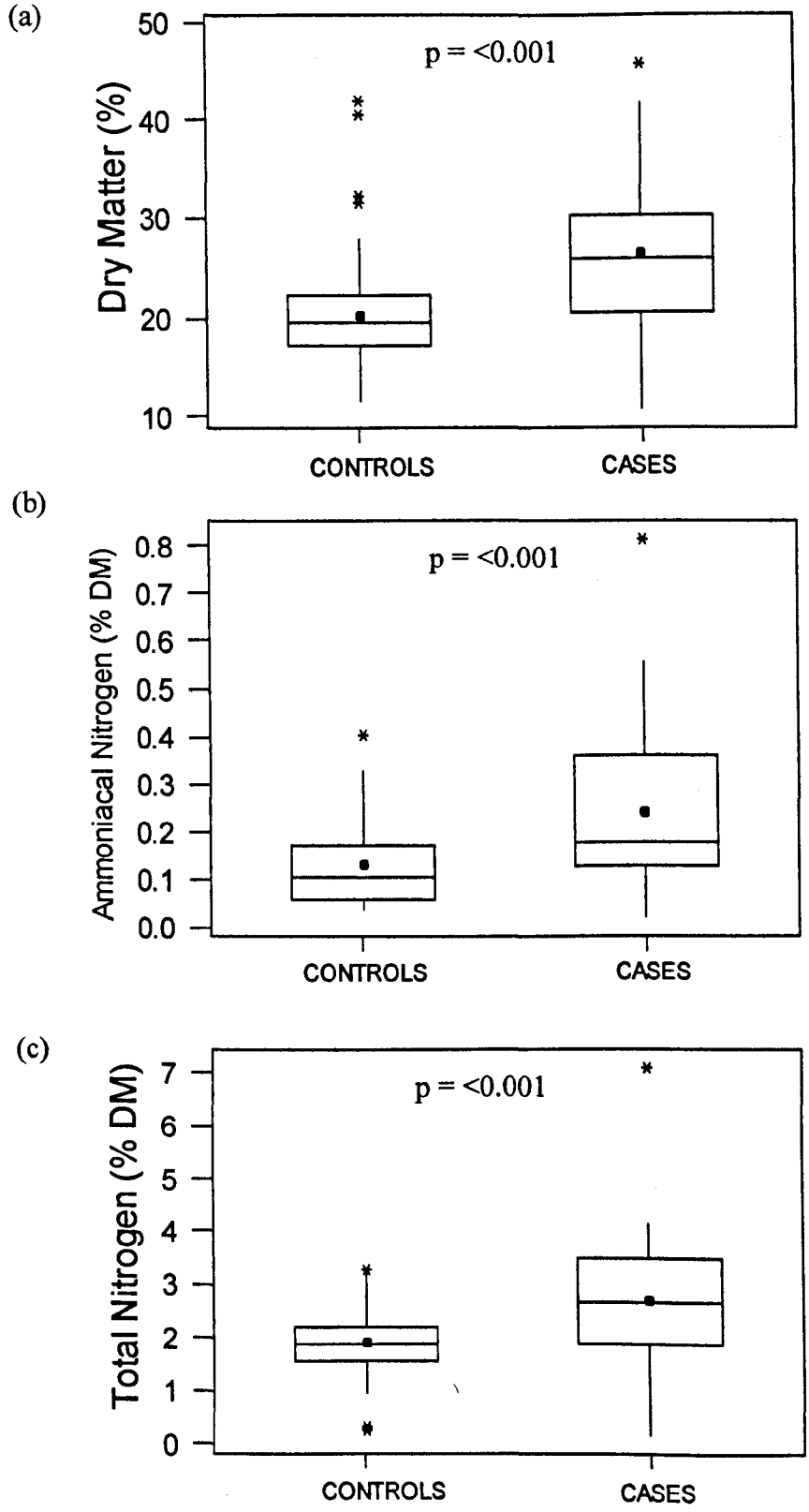


Figure 4.1 (a) Dry matter, (b) ammoniacal nitrogen and (c) total nitrogen content of case and control faeces. The bottom of the box is at the first quartile (Q1) and the top is at the third quartile (Q3). The mean value is represented by the red dot within the box. Outliers are points outside the lower and upper limits, plotted with asterisks (*).MINITAB considers any value lying between 1.5 and 3 times way from the middle 50% of the data as an outlier.

Table 4.12: Faecal Analysis; univariable analysis of continuous variables with coefficients, standard errors and P-values.

Variable	Unit of measurement	Coefficient	Standard error	P-value
pH	-	-3.46	0.33	0.29
Dry Matter (DM)	%	0.27	0.06	<0.001
Total Nitrogen	%	9.31	2.44	<0.001
Total Nitrogen	% DM	1.39	0.38	<0.001
Ammoniacal Nitrogen	%	131.59	27.31	<0.001
Ammoniacal Nitrogen	% DM	10.84	3.03	<0.001

Tables 4.13: Final multivariable conditional logistic regression model of faecal analysis values associated with equine grass sickness with coefficients, 95% confidence limits (95% CI) of odds ratio and p-values.

Variable		Coefficient	Standard error	P-value
Dry Matter	Increase in one %	0.39	0.12	<0.001
Ammoniacal Nitrogen	Increase in one % per unit DM	18.31	6.10	0.003

PART C: PREMISES LEVEL VARIABLES.

Information and samples were collected from 60 EGS premises and 120 control premises. Cases occurred in every month of the year except for December and January. The majority of cases (64%) occurred during the months of April, May and June (Figure 4.2). EGS cases were recruited from 18 different counties within England and Wales. Cheshire, Lancashire, Greater Manchester and Yorkshire gave rise to the majority of cases recruited to the study. Matched control premises were visited, on average, 2 days after the case premises had been visited (range 0 – 8 days).

Descriptive analysis.

1. Pasture management

Questionnaire data referring to pasture management was collected from EGS case premises and time matched control premises. The information related to the particular pasture that the EGS case was grazing during the onset of disease. For control premises, information was collected from a pasture that horses were actively grazing at the time of the field visit. Information was collected relating to management procedures carried out during the current year only. None of the pastures sampled had been ploughed, whilst 23% of both case and control fields had been harrowed, 3% had been re-seeded and 32% had been fertilised. 14% of case and control pastures were grazed by other species, 65% of other species being sheep and 30 % cattle.

2. *Soil Analysis*

Soil samples were collected from 60 EGS case premises and 120 time matched control premises. Soil samples from EGS case premises had a significantly (students t-test p = 0.049) higher dry matter content (figure 4.3a) and a significantly higher (Mann Whitney p = 0.008) levels of total nitrogen (figure 4.3b). There were no significant differences between case and control premises for soil pH (p = 0.50), extractable ammonium (p = 0.74) and extractable nitrate (p = 0.50).

A monthly variation in total nitrogen was observed in soil samples from case and control premises (figure 4.4a). The lowest levels of soil total nitrogen was observed in samples taken in the months of March (mean 0.51% DM) June (0.59% DM), July (0.53% DM) and October (0.52% DM). The highest levels of soil total nitrogen content were observed in May (0.67% DM), September (0.82% DM) and November (0.71% DM). The monthly difference in soil total nitrogen was not found to be statistically significant (p = 0.18). As expected, a monthly variation in soil dry matter content was observed (figure 4.4b). The lowest dry matter content was found in samples taken in November (66.02%) whilst the highest dry matter content was found in samples taken in July (77.39%). The monthly variation in dry matter content was statistically significant (p = 0.01). An inverse relationship between dry matter and total nitrogen content of soil samples was observed in this study (figure 4.4c). Regression analysis revealed a significant association between soil dry matter content and total nitrogen content of case and control soil samples (p = <0.001).

3. Pasture analysis

Pasture samples were collected from 60 EGS case premises and 120 time matched control premises. However, due to financial limitations only samples from 26 EGS case premises and 52 matched control premises were analysed. There were no significant differences between case and control premises for pasture dry matter ($p = 0.41$) and total nitrogen ($p = 0.46$).

4. Meteorological data.

Meteorological (Met.) office data were collected for 60 EGS case premises from 64 individual outbreaks of EGS. Data were also collected from 128 time matched control premises. Minimum daily temperature, maximum daily temperature and minimum daily grass temperature was collected from 53 different Ordinary Climatological stations and daily rainfall totals were collected from 103 different Daily Rainfall stations. On average, the Ordinary Climatological stations were 11km (range: 1 – 23km) from either the EGS or control premise whereas the Daily Rainfall stations were on average 6km (range 1 –22km) from the case or control premises. The mean and range values for the 5 Met. Office variables for case and control premises are shown in table 4.14. Mean values were calculated over both a 7-day and a 14-day period up to and including the day of disease recognition. There was no significant difference between case and control premises for i) average daily minimum temperature over 7 days ($p = 0.79$) or 14 days ($p = 0.96$), ii) average daily maximum temperature over 7 days ($p = 0.92$) or 14 days ($p = 0.98$), iii) average daily temperature range over 7 days ($p = 0.58$) or 14 days ($p = 1$), iv) average daily rainfall over 7 days ($p = 0.97$) or 14 days ($p = 0.11$) and v) average daily minimum grass temperature over 7 days ($p = 0.36$) or

14 days (p = 0.4). Using data from case premises only, figures 4.5, 4.6 and 4,7 show the average daily minimum temperature, the average daily maximum temperature and the average daily rainfall totals for 64 outbreaks of EGS. In addition, figure 4.8, 4.9 and 4.10 show the mean daily change in rainfall, minimum temperature and maximum temperature for the seven days prior to disease outbreak. There were no consistent patterns for each of the 3 weather parameters. All plausible interactions between weather parameters were tested and revealed no significant associations.

Univariable analysis

1. Pasture management

Results of univariable analysis of binary premises level variables are shown in table 4.15. Univariable analysis revealed a significant (p = 0.011) association between EGS and whether a pasture had been disturbed in the previous 12 months. Twenty-two per cent of case pastures had been disturbed compared to 8% of control pastures. A pasture that had been disturbed was found to be over 3 times more likely to give rise to a case of EGS when compared to pastures that had not been disturbed. Examples of how case and control pastures have been disturbed is shown in table 4.16. A significant (p = 0.035) association was also found between EGS and premises that had previously been affected by EGS. Twelve (20%) of the EGS case premises had been reported to have given rise to a case of EGS prior to the current outbreak whilst only 3 (2.5%) of control premises had given rise to a case. A premises that had previously given rise to a case of EGS was found to be 21 times more likely to give rise to the disease

again. Of the EGS case premises, 42% of the previous reports of EGS had been diagnosed by veterinary surgeon on the premises whilst another 42% had been referred to a veterinary hospital. 83% of the previous cases had been subjected to euthanasia whilst the remaining 17% had died. Previous outbreaks of EGS were reported to have occurred between 17 days and 10 years before the current outbreak.

5. Soil analysis

Results of univariable analysis of soil variables are shown in table 4.17. Coefficients and standard errors have been quoted due to the difficulty in interpreting the huge odds ratios produced in the univariable analysis. Univariable analysis revealed a significant ($p = 0.029$) association between EGS and the dry matter content of soil. Premises were 10 times less likely to give rise to a case of EGS per 10 percentage increase in dry matter content of the soil. This variable was not found to be significant in the multivariable model. Univariable analysis also revealed a significant ($p = 0.003$) association between EGS and the total nitrogen content of the soil. Premises were over 6 times more likely to develop grass sickness per percentage of dry matter increase in total nitrogen.

3. Pasture analysis

Results of univariable analysis of pasture analysis variables are shown in table 4.18. Univariable analysis revealed no significant associations between EGS and the dry matter and nitrogen content of the pasture.

4. Meteorological data.

Taking into account the matching variable, there were no significant associations between EGS and any of the 5 Met. Office variables when compared to control premises.

Multivariable analysis

Three variables were included in the final model (table 4.19). These were EGS on the premise before, pasture disturbed and total nitrogen content of the soil (% DM). EGS was associated with having EGS on the premise prior to the current outbreak (OR 45.06, 95 per cent CI 3.73-543.99, P = 0.002), a pasture that had been disturbed (OR 3.38, 95 per cent CI 1.15–9.90, P = 0.027) and soil with higher total nitrogen content (OR 18.82 for an increase 1 % DM, 95 per cent CI 3.22-110.16, P = 0.001). After adjusting for confounding, the estimated OR's for all the variables in the final model were lower than the univariable model and the P values for EGS previously on premises, and pasture disturbed, were increased. Linear transformation of the total nitrogen variable was considered prior to entry into the final multivariable model. Variables that categorised, centred or centre squared did not improve the fit.

Sensitivity and Specificity of multivariable model

The fit and predictive ability of the premises level multivariable model was assessed by calculating the sensitivity and specificity at various cut-off points (table 4.20). When a cut-off point of 0.3 was selected (i.e. if the predicted probability of becoming an EGS case premises is above 0.3 and the premises is

predicted to give rise to a case of EGS), the sensitivity was 55% and the specificity was 57 %.

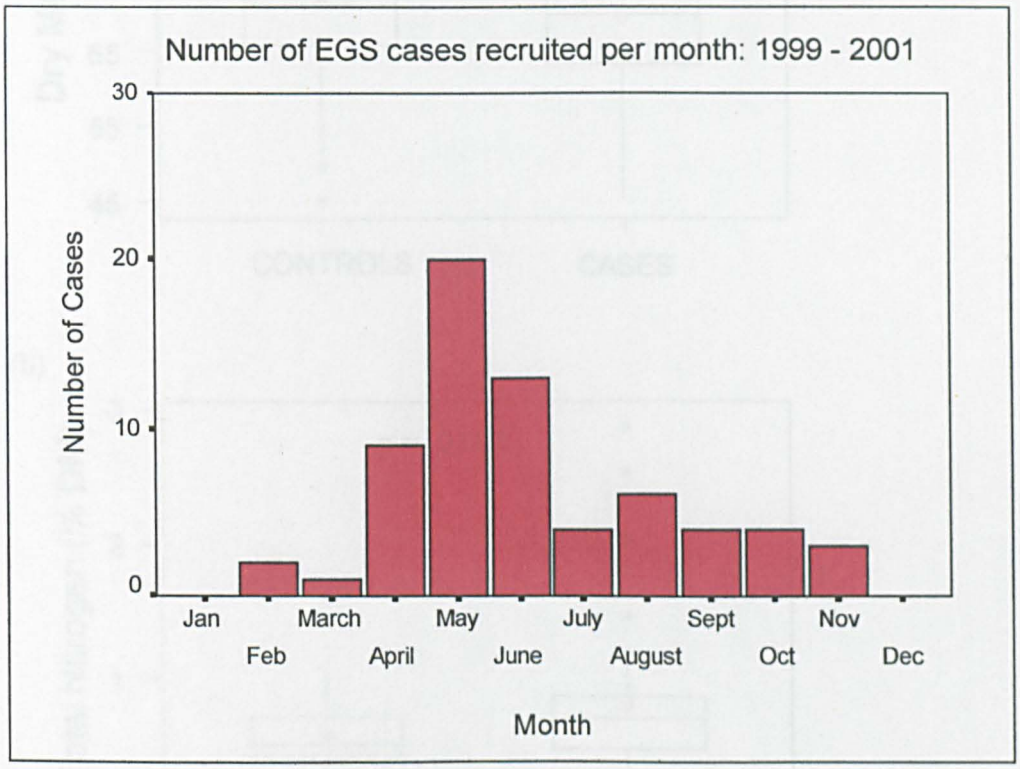


Figure 4.2. Annual distribution of EGS cases recruited to the study. The graph represents the month of disease occurrence for 66 cases of EGS.

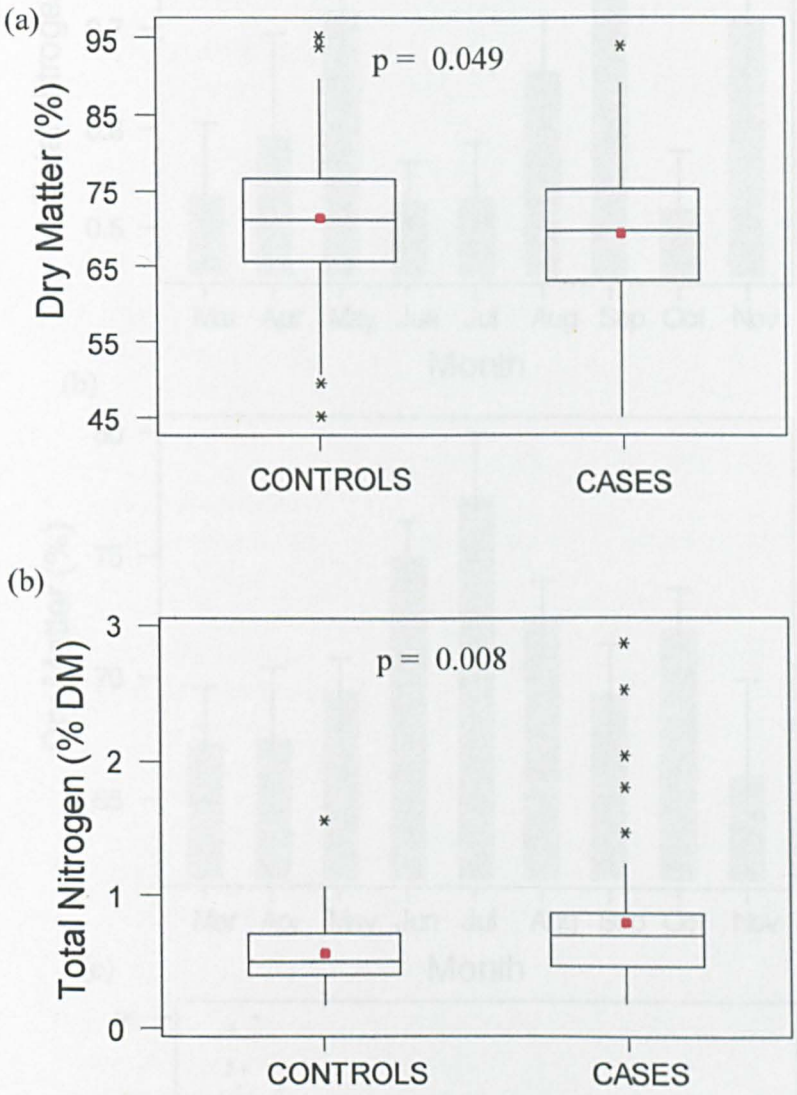


Figure 4.3 (a) Dry matter and (b) total nitrogen content of case and control soil samples. The bottom of the box is at the first quartile (Q1) and the top is at the third quartile (Q3). The mean value is represented by the red dot within the box. Outliers are points outside the lower and upper limits, plotted with asterisks (*).

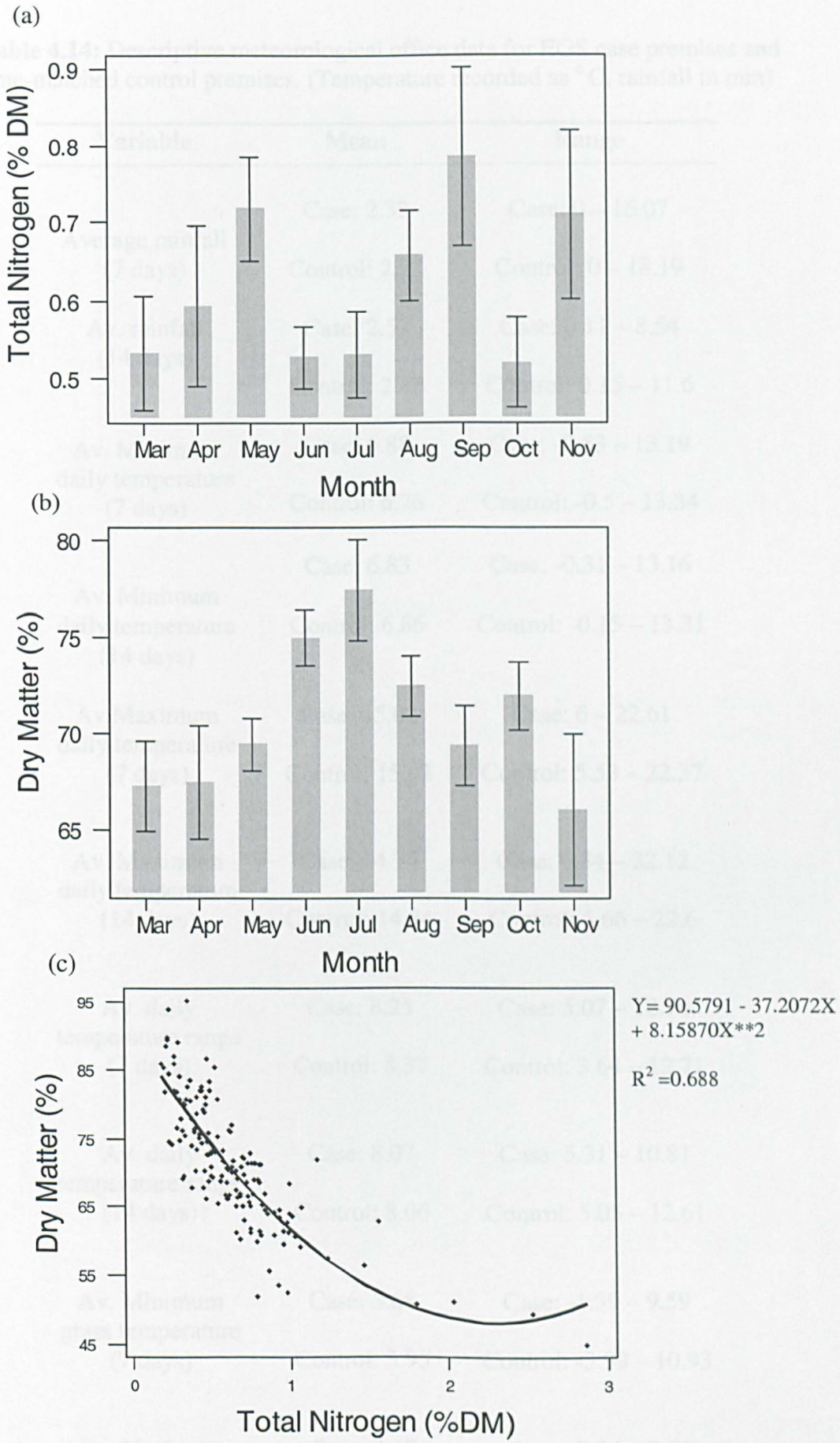


Figure 4.4: Monthly variation in (a) total nitrogen and (b) dry matter content of case and control soil samples. (c) Non-linear regression analysis to examine the relationship between soil total nitrogen and dry matter content.

Table 4.14: Descriptive meteorological office data for EGS case premises and time-matched control premises. (Temperature recorded as °C, rainfall in mm)

Variable	Mean	Range
Average rainfall (7 days)	Case: 2.33	Case: 0 – 16.07
	Control: 2.23	Control: 0 – 18.19
Av. rainfall (14 days)	Case: 2.57	Case: 0.11 – 8.54
	Control: 2.42	Control: 0.15 – 11.6
Av. Minimum daily temperature (7 days)	Case: 6.88	Case: -0.53 – 13.19
	Control: 6.76	Control: -0.5 – 13.34
Av. Minimum daily temperature (14 days)	Case: 6.83	Case: -0.31 – 13.16
	Control: 6.86	Control: -0.15 – 13.31
Av.Maximum daily temperature (7 days)	Case: 15.02	Case: 6 – 22.61
	Control: 15.02	Control: 5.53 – 22.37
Av. Maximum daily temperature (14 days)	Case: 14.76	Case: 6.34 – 22.12
	Control: 14.84	Control: 5.66 – 22.6
Av. daily temperature range (7 days)	Case: 8.25	Case: 5.07 – 12.74
	Control: 8.37	Control: 3.64 – 12.71
Av. daily temperature range (14 days)	Case: 8.07	Case: 5.31 – 10.81
	Control: 8.06	Control: 5.05 – 12.61
Av. Minimum grass temperature (7 days)	Case: 3.68	Case: -1.39 – 9.59
	Control: 3.95	Control: -3.99 – 10.93
Av. Maximum grass temperature (14 days)	Case: 3.67	Case: -2.24 – 9.66
	Control: 4.01	Control:-4.14 – 11.39

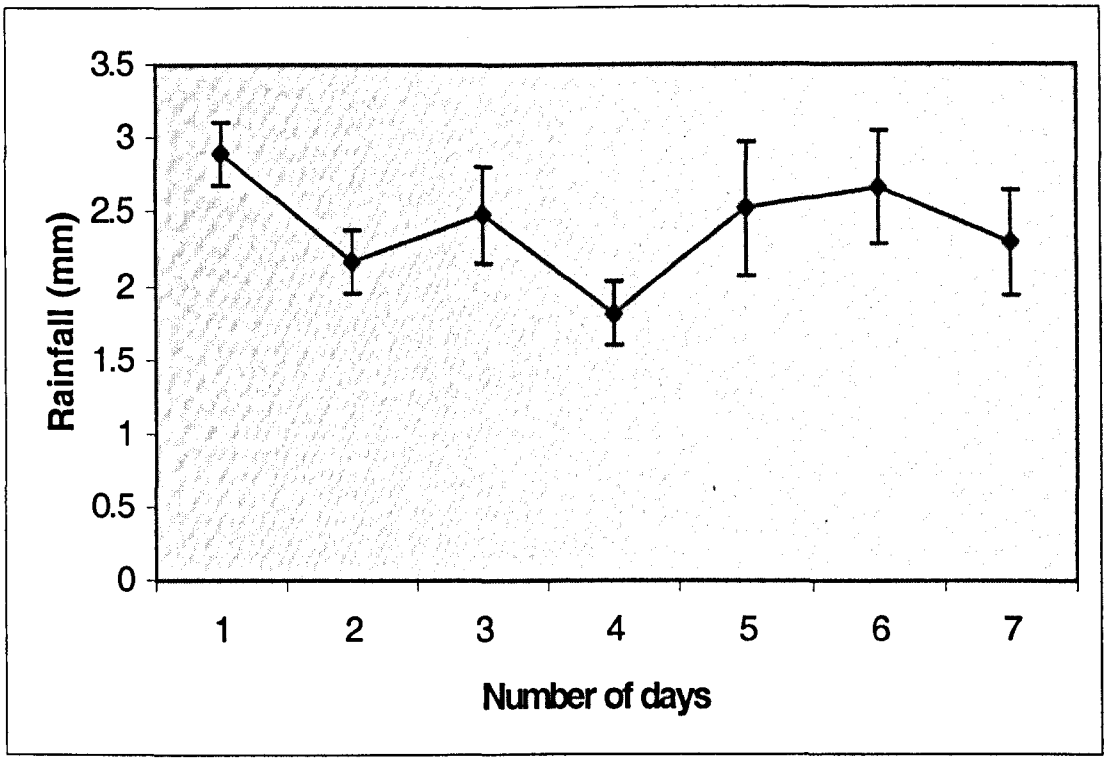


Figure 4. 5 Mean rainfall (± standard error) 7 days prior to disease recognition. Daily values taken from 64 outbreaks of EGS. Day 1 = Day of disease recognition.

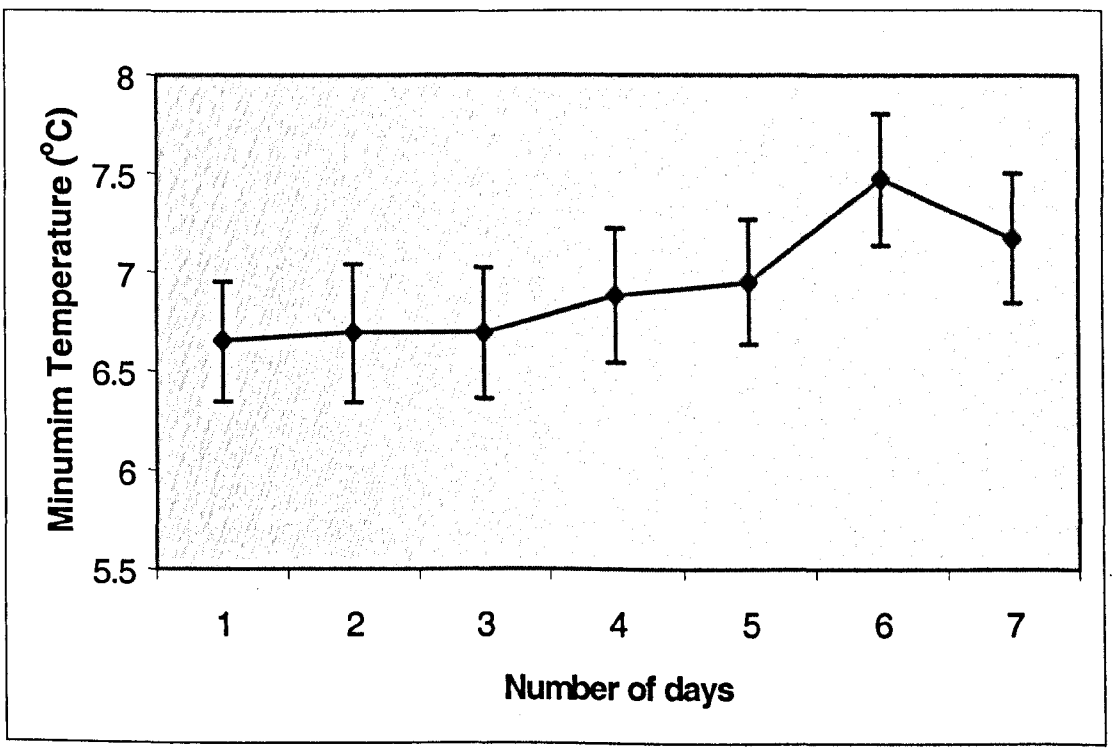


Figure 4. 6 Mean minimum temperature (± standard error) 7 days prior to disease recognition. Daily values taken from 64 outbreaks of EGS. Day 1 = Day of disease recognition.

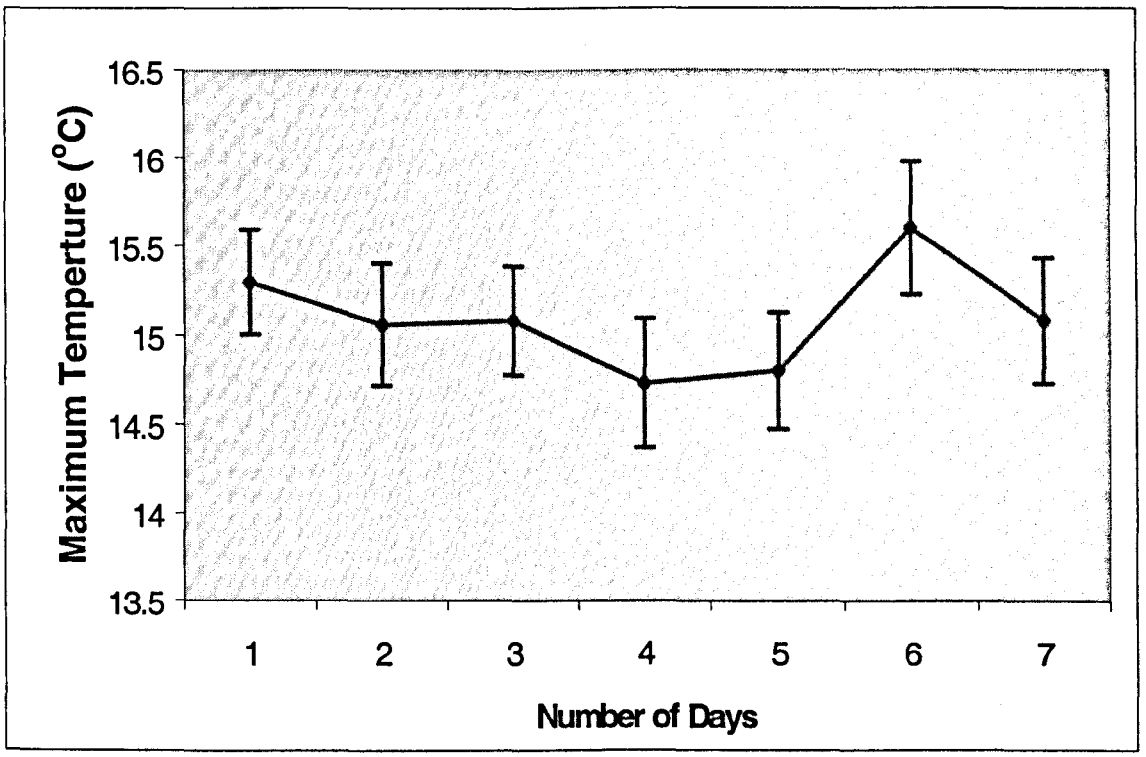


Figure 4.7 Mean maximum temperature (± standard error) 7 days prior to disease recognition. Daily values taken from 64 outbreaks of EGS. Day 1 = Day of disease recognition.

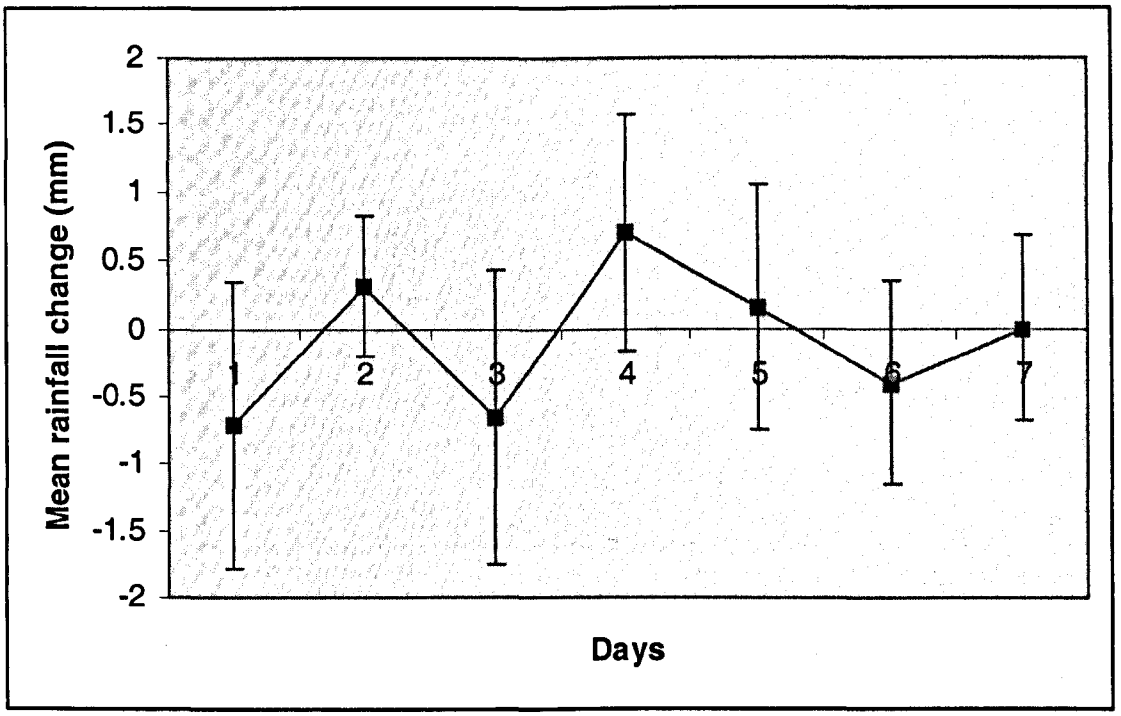


Figure 4. 8: Daily rainfall change (± standard error) 7 days prior to disease outbreak. Day 1 = Day of disease occurrence.

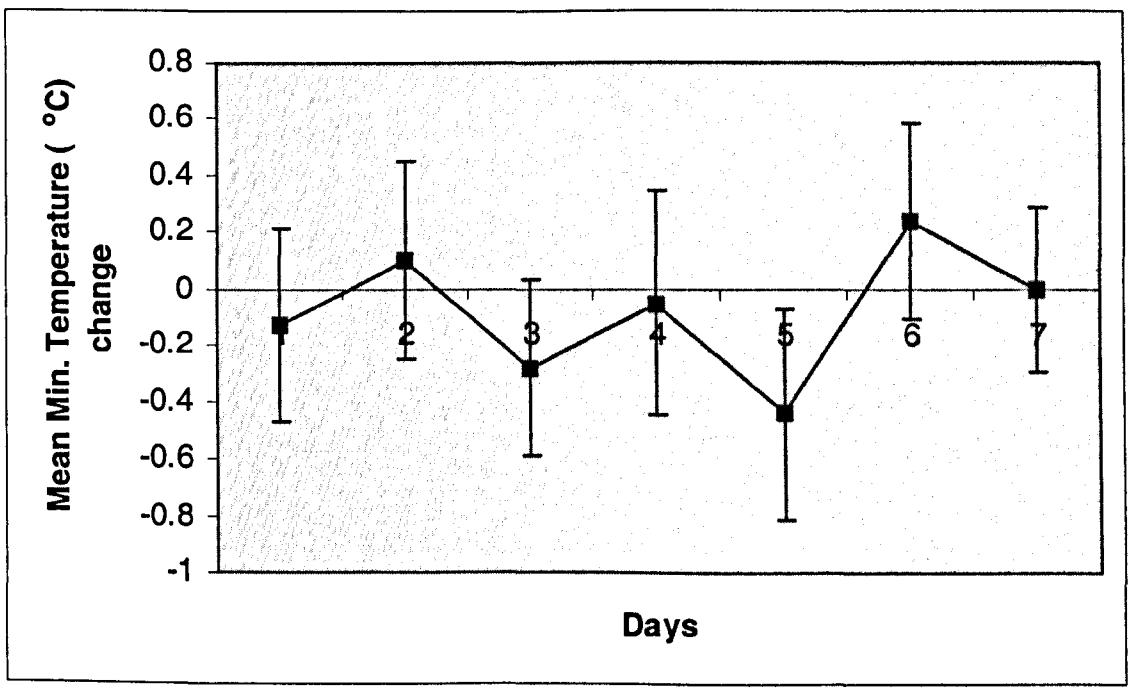


Figure 4. 9: Minimum daily temperature change (± standard error) 7 days prior to disease outbreak. Day 1 = Day of disease occurrence.

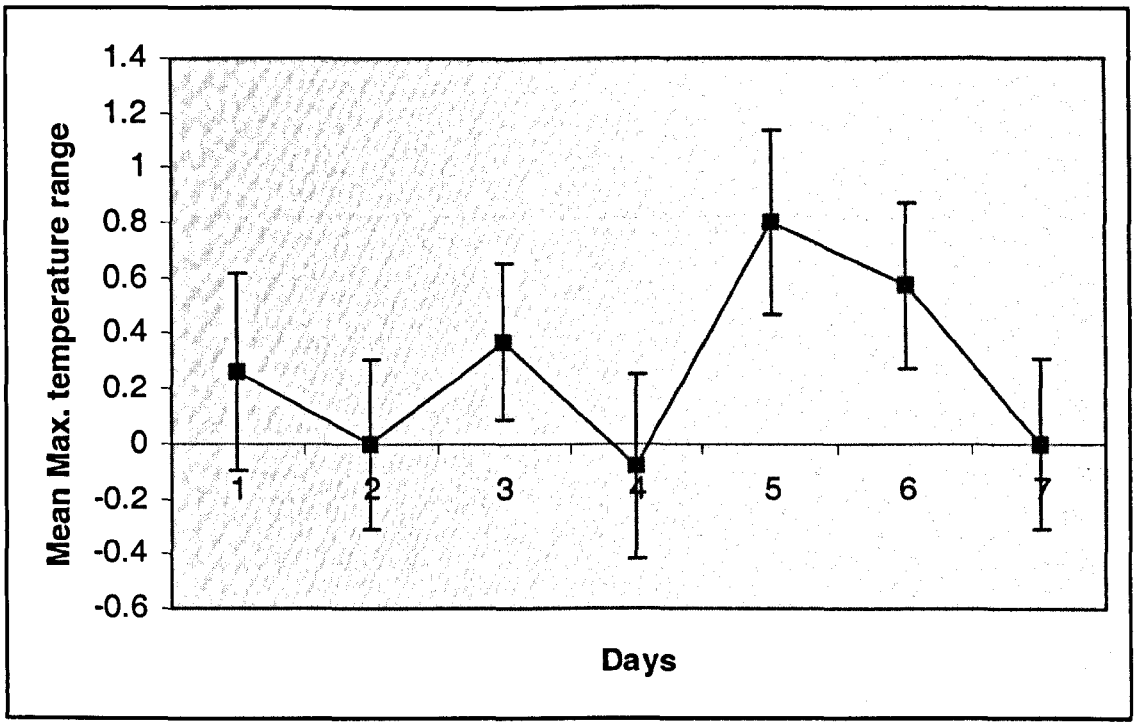


Figure 4. 10: Maximum daily temperature change (± standard error) 7 days prior to disease outbreak. Day 1 = Day of disease occurrence.

TABLE 4.15: Univariable analysis of binary premises level variables with odds ratio, p- values and 95% confidence intervals (95% CI)

Variable		Case % (n)	Control % (n)	Odds ratio	95% CI	P-value
Pasture Management						
Disturbed	Yes	22 (13)	8 (9)	3.34	1.32 – 8.47	0.01
	No	78 (47)	92 (11)			
EGS before	Yes	20 (12)	2.5 (3)	21.07	2.72 – 163.28	0.004
	No	80 (48)	97.5 (117)			
Harrowed	Yes	20 (12)	25 (30)	0.70	0.30 – 1.63	0.40
	No	80 (48)	75 (90)			
Fertilised	Yes	30 (18)	33 (39)	0.88	0.43 – 1.78	0.72
	No	70 (42)	67 (81)			
Re-seeded	Yes	5 (3)	2.5 (3)	2.0	0.40 – 9.91	0.39
	No	95 (57)	97.5 (117)			
Other Species	Yes	15 (9)	13 (15)	1.25	0.50 – 3.12	0.64
	No	85 (51)	87 (105)			

Table 4.16 Type of disturbance reported for EGS case and control pastures.

Type of disturbance	Cases % (n)	Controls % (n)
Access to or construction of underground drains/pipes	23 (3)	67 (6)
Construction (e.g. menage)	38 (5)	11 (1)
Mole hills	31 (4)	11 (1)
Horse burial	8 (1)	11 (1)

Table 4.17: Soil analysis: Univariable analysis of continuous variables with coefficients, standard errors and p-values.

Variable	Coefficient	Standard error	P-value
pH	0.32	0.31	0.29
Dry Matter (DM) (%)	-0.04	0.02	0.029
Extractable Ammonium	-0.0008	0.006	0.89
Extractable Nitrate (mg/kgDM)	0.03	0.02	0.15
Total Nitrogen (mg/kg)	0.0003	0.0001	0.018
Total Nitrogen (%DM)	1.83	0.62	0.003

Table 4.18: Pasture analysis: Univariable analysis (26 matched sets) of continuous variables with odds ratio, p-values and 95% confidence intervals (95% CI)

Variable	Odds Ratio	95% CI	P-value
Dry Matter	1.03	0.93-1.15	0.484
Nitrogen (% DM)	1.13	0.51-2.27	0.732

Tables 4.19: Final multivariable conditional logistic regression model of premises level risk factors associated with equine grass sickness with odds ratio, 95% confidence limits (95% CI) of odds ratio and p-values.

Variable		Odds Ratio	95% CI	P-value
Pasture disturbed	Yes	3.37	1.15-9.90	0.027
EGS before	Yes	45.06	3.37-543.99	0.003
Soil total nitrogen content	Increase in one % DM	18.83	3.21-110.16	0.001

Table 4.20: Sensitivity (EGS case) and specificity (control) of premises level risk factors logistic regression model with various cut off points.

Cut Off	Sensitivity	Specificity
0.1	0.830	0.217
0.2	0.66	0.339
0.3	0.547	0.566
0.4	0.377	0.726
0.5	0.264	0.849
0.6	0.226	0.925
0.7	0.17	0.943
0.8	0.15	0.962
0.9	0.07	1

DISCUSSION

Equine grass sickness is a largely fatal disease that continues to kill hundreds of horses a year. There is no specific treatment and no prophylactic vaccine available. The identification of risk factors for the disease currently offers the only method of disease prevention.

This study has identified new horse-level and premises level risk factors for EGS. The study has also supported the findings from previous epidemiological studies. In contrast to previous reports, this study has used information from EGS cases that were confirmed histologically rather than relying on a diagnosis from clinical signs alone. This has prevented information being collected from horses that could potentially be suffering from another form of colic or related disease. Matched case-control methodology was another strength of this study. Matching on pasture or premise enabled adjustment for the potentially confounding effects of environmental factors that would otherwise be difficult to control.

The outcome of this study produced 4 different horse-level models. One model was constructed using horse-level risk factors only and three others were produced using horse-level risk factors plus serological data for antibodies to i) *C. novyi* type A, ii) *C. botulinum* type C and iii) BoNT/C toxoid.

The main feature of this study was the association made with EGS and *C. botulinum* type C. Antibody levels to *C. novyi* type A, *C. botulinum* type C and *C. botulinum* type C toxoid was used to make this association. Interestingly, no association was made between EGS and the tapeworm ELISA antibody level. This finding suggests the response to the three clostridial antigen is a specific rather than a general immune response.

The association between EGS and antibody level to *C. novyi* type A, *C. botulinum* type C and *C. botulinum* type C toxoid was significant in univariable analysis but also remained highly significant when entered into multivariable analyses. Hunter and Poxton (2001) showed a basic statistical association between EGS and *C. botulinum* type C without taking into account other risk factors for the disease. It is a novel feature of this study that we have identified horses with low level of antibodies to *C. botulinum* type C to be at greatest risk of EGS when also allowing for and identifying other horse-level risk factors. The associations made here provide further evidence for the role of *C. botulinum* in EGS.

The study design matched cases and controls on the same or a nearby pasture. As a result all animals in this study had access to pasture. A suggestion for further a investigation might be that horses with no access to pasture are sampled to determine whether only grazing horses have measurable exposure to *C. botulinum* type C and its C1 neurotoxin.

This study identified several horse-level risk factors for EGS including age. This variable was consistent throughout all 8 of the final multivariable models. Univariable analysis of age as a categorised variable identified horses that were aged 4 and 5 years old to be most at risk from disease. However, in the final models age remained as a continuous variable even though univariable analysis revealed a non-linear relationship. In most cases, when age was fitted as a categorical variable in the final models it resulted in a monotonic decline in odds ratios. As a result there appears to be no advantage in fitting age as a categorical variable rather than a continuous variable. It is possible that once other risk factors along the causal pathway have been accounted for, there truly is a monotonic decline in risk as the age of the animal increases.

This study suggests that hay exerts a protective effect against EGS. It is possible that horses receiving hay in their daily diet consume a smaller proportion of pasture in their diet than horses receiving no hay. It has already been mentioned that it is possible that haylage is exerting the protective effect rather than the wet or dry hay. In the univariable analysis, the odds ratio for haylage was less than one whereas the odds ratio for wet hay and dry hay was one or above. This is an interesting finding that requires further investigation.

Three of the final multivariable models showed that being on a pasture for less than 56 days was a risk factor for EGS. Time on pasture is not a new risk factor for EGS as it has been previously described by Wood *et al,* 1998. However, Wood *et al,* suggest that horses on a pasture for less than 14 days were most at risk.

A change in feed type or quantity in the previous 14 days was a risk factor identified all final multivariable models. It is hypothesis that a change in feed type or quantity contributes to a change of environment within the gastrointestinal (GI) tract of the horse. When a horse undergoes a change in routine there is often a feed change that occurs at the same time. For example, when a horse changes from being stabled at night and being at grass during the day to being turned out at grass for 24 hours a day, it is not uncommon for hay and/or concentrates to be decreased or excluded from the diet if the grass is of good enough quality. However, change of routine was not significant in univariable or multivariable analysis.

One of the weaknesses with the study questionnaire was that the actual amount of feed change was not measured. It is therefore unknown whether a

relatively small or large change in feed quantity contributes to an animal being at increased risk of EGS.

A novel finding of this study is the association between certain anthelmintics and EGS. Ivermectin was associated with a 5 to 7 fold increase in the likelihood of disease when administered at the penultimate worming episode. It is unclear why the use of ivermectin at the penultimate worming episode rather than the final worming episode should appear as a risk factor. It is possible at the time of disease that owners had wormed most recently with the relatively new anthelmintic moxidectin whereas they had previously used ivermectin. Moxidectin was introduced onto the market in May 2000 and was not therefore in use during the first year of the study. There is no obvious reason why ivermectin should contribute to the development of EGS. I hypothesise that the drug may have an adverse effect on gut motility or gut flora that may subsequently make the horse more susceptible to disease. Ivermectin itself is a macrolide antibiotic produced from a soil dwelling fungus (American Board of Veterinary Toxicology, 2001). The drug acts as an agonist for the neurotransmitter gamma-amminobutyric acid (GABA) which is a major inhibitory neurotransmitter. GABA-containing neurones and receptors are found in the CNS in mammals while in arthropods and nematodes GABA is primarily found in the peripheral nervous system. There is no literature reporting the effects of ivermectin on the mammalian gastrointestinal tract and ivermectin does not readily cross the blood brain barrier when given at the recommended dose (Veterinary Technical Services, Merial, UK; personal communication). Ivermectin does not have any antibacterial, antifungal or antiprotozoal activity and no direct effects would therefore be expected on gut flora (Veterinary Technical Services, Merial, UK; personal communication).

There does however remain the possibility of indirect effects on gut motility or gut flora through removal of gastrointestinal parasites.

The association between EGS and ivermectin was strengthened when further analysis involving just age, faecal egg count and the use of ivermectin revealed nearly a 12 times increase in risk of disease when ivermectin was used on both the penultimate and final occasion

In conclusion, the study of horse-level risk factors has identified young horses, with low level of antibodies to *C. botulinum* type C that have had a recent change in pasture and feed and have been wormed with ivermectin during the penultimate episode to be at most at risk of EGS.

Analysis of faecal material has implicated increasing dry matter content and increasing ammoniacal nitrogen content as risk factors for EGS. It is highly likely however that EGS cases have higher dry matter content of faecal material as a result of disease rather than this being causal. EGS cases often find difficulty in swallowing and are unable to drink water. Horses have the ability to resorb water from the large colon. In the early stages of disease, EGS cases will draw on the water available in the gastrointestinal (GI) tact and this will decrease the dry matter content of faecal material. Due to gut stasis, the contents of the GI tract are not re-hydrated.

Ammoniacal nitrogen is a non-protein nitrogenous compound. The amount of ammoniacal nitrogen in herbage in usually negligible (Whitehead, 2000). Therefore, the ammoniacal nitrogen content in faeces arises from the microbial degradation of dietary nitrogen (Obara *et al.*, 1991). The contribution of nitrogen is dependent on the nitrogen content of the diet.

It appears to be more difficult to interpret the higher ammoniacal nitrogen content found in faecal samples obtained from EGS cases compared to the higher dry matter content. Ammoniacal nitrogen is reported as a percentage of dry matter content so the result has no bearing on the latter result. It is possible that gut stasis leads to an overgrowth of bacteria in the large intestine, especially the caecum, and it is this excessive microbial activity which produces higher ammoniacal nitrogen levels and leads to the results found in the analysis. However, it is unclear whether gut stasis precedes or succeeds disease development. It is also possible that the increased ammoniacal nitrogen content reflects the nitrogen content of the diet. In addition, the increased ammoniacal nitrogen could favour the survival of the aetiological agent within the gut. As cause versus effect could not be determined, it was decided that the variables associated with faecal analysis should not be entered into the final horse-level multivariable model.

The results of the faecal analysis in this study could have been strengthened by the addition a third population of horses. It may have been valuable to analyse faecal samples from horses that have had some level of GI stasis similar to that experienced by EGS cases. Suitable candidates would be horses that have suffered post-operative ileus. The results of this study would therefore be more specific to EGS cases and may infer a causal association with disease rather than a result of disease.

In addition to the identification of horse-level risk factors, this study identified several premises level risk factors. Premises were at increased risk of giving rise to a case of EGS if they had EGS before, if the pasture horses were grazing had been disturbed and if the soil had an increased level of total nitrogen. The latter two risk factors are novel findings whereas the former has been reported

in the literature previously. It is unclear why a pasture that has been disturbed should be at increased the risk of giving rise to a case of EGS. It is hypothesised that pasture disturbance could provide conditions more favourable for the survival of the aetiological agent in the soil. Smith and Young (1980) list disturbance of soil as one of the factors that affect the prevalence of *C. botulinum* in soil. It is also interesting that the type of disturbance appears to be different in case and control premises. It could be possible that access to underground drains and pipes can increase drainage on pastures and exert a protective effect. EGS is more associated where construction has occurred on a pasture. In this study examples of construction include the building of ménages, erecting of telephone masts and construction of a cess tank. Whatever this effect, the type of disturbance is important in interpreting pasture disturbance as a risk factor for EGS.

This study failed to identify any meteorological parameters significantly associated with EGS. This may be due to overmatching of case and control premises on time (weather during the period in question was similar for case and control premises). However, the study offers no evidence that climatological factors are associated with occurrence of EGS. Any such differences are likely to have been detected by this time-matched study. There is still however a need for further exploration of the association between EGS and meteorological variables.

This study found that premises that had given rise to a case of EGS had significantly higher levels of soil total nitrogen when compared to time matched unaffected control premises. The main route nitrogen enters the soil is through plant residues either directly or after passage through the animal. This nitrogen is in the form of proteins and amino acids and must be converted by symbiotic and free-living bacteria to ammonium or nitrate before it becomes available to plants

(Simpson, 1983). Usually, more than 95% of the soil total nitrogen occurs in the soil organic matter, and less than 5% occurs in the inorganic forms, ammonium and nitrate (Whitehead, 2000). The application of fertiliser nitrogen often produces no increase in soil nitrogen because the fertiliser enhances the concentration of nitrogen in plant material, and therefore promotes mineralization rather than accumulation in organic matter (Whitehead, 2000). This could explain why no association has been found between EGS and fertiliser application. Results from several field investigations suggest that fertiliser nitrogen, even when applied to grassland regularly for a number of years, has no significant effect on soil nitrogen unless the soil is extremely low in organic matter (Whitehead, 1995). It has also been reported that when sewerage sludge is applied to grassland, there is often an increase in soil nitrogen content (Whitehead, 2000). Three EGS case premises had recently had slurry (pig, human, cattle) spread on the affected field whereas there were no reports of such activity on control premises.

Most cases of EGS occur during the spring. It is known that young herbage is rich in amino acids and nitrates that are components of non-protein nitrogen and levels are highest during the period of rapid leaf growth in the spring and during re-growth in the early autumn. Generally, the more favourable the growth conditions, the higher is the non-protein nitrogen content as well as the total nitrogen value of pasture, and as the plants mature the content of both decreases (McDonald *et al* 1988). It was thought that pasture levels of non-protein and total nitrogen would reflect those found in the soil. The peak levels of soil total nitrogen during the months of May and September that were found in this study support such a statement. However, the study did not find any differences between

EGS case premises and control premises when pasture samples were analysed for total nitrogen.

The supply of nitrogen from the soil might be expected to exert a major influence on the concentration of nitrogen in grass herbage but apparently often this is not so (Whitehead, 2000). Variations in the supply of nitrogen from the soil, though influencing yield, often have little effect on herbage nitrogen concentration.

This study suggests that increased levels of soil total nitrogen play a key role in the development of EGS and this finding should be explored further. It is hypothesised that increased levels of soil total nitrogen influence the amount of herbage available to horses through increased growth and yield (rather than influencing nitrogen levels found in herbage). Subsequently, this could play a role in the alteration of the digestive chemistry of the gastrointestinal tract, which could contribute to disease development in susceptible horses.

In conclusion, this study has provided valuable information on both horse-level and premises level risk factors for EGS. The major finding of this study is evidence for the association between low antibody levels against *C. botulinum* type C and the development of EGS. The study has identified risk factors such as the use of ivermectin, that can be used to develop disease avoidance strategies, whilst other risk factors, such as soil nitrogen content, need further exploration to understand its role in disease development. In order to understand the disease more fully it is imperative that relationships between horse-level and pasture-level risk factors are fully understood and their place on the causal pathway of disease is determined.

CHAPTER 5

THE USE OF SPACE-TIME K-FUNCTION ANALYSIS TO DETECT AND DESCRIBE CLUSTERING OF EQUINE GRASS SICKNESS CASES IN THE UK

INTRODUCTION

Equine grass sickness (EGS) is a largely fatal, pasture associated dysautonomia with an unknown aetiology. The disease is characterised primarily by failure of normal alimentary function. The highest incidence of EGS is believed to occur in Scotland where the disease was first identified in 1907. The disease also occurs throughout England and Wales with anecdotal reports suggesting it has higher prevalence in particular areas. The temporal pattern of EGS has been well described, but to date, little has been done to explore the spatial patterns of EGS in England and Wales. In addition, no studies have considered the joint distribution of EGS cases in space and time.

Many diseases, both in humans and animals, have shown evidence of clustering in space, time or both. Clustering of disease outbreaks in space or time may provide clues to the aetiology of disease. Some disease may be clustered in time, so that most cases occur at a particular time of year. Other diseases, like those caused by a point release of toxic chemicals, may be clustered in space, so that most cases occur in the same place. An example of spatial clustering in a human disease occurred in Woburn, USA where it was found that two contaminated wells were responsible for an outbreak of childhood leukemia (Lagakos *et al.*, 1986). In veterinary literature, significant spatial and temporal clustering of horses with *Corynebacterium pseudotuberculosis* infection has been detected (Doherr *et al.*, 1999). The results indicated that the disease has a 3 to 4 week incubation period and could be transmitted through horse-to-horse contact or from infected to susceptible

horses via insects, other vectors, or contaminated soil. The spatial and temporal patterns of disease were considered independent in this study.

Clustering of disease outbreaks in space and time can provide evidence of contagion or an underlying spatially and temporally localised process. Describing the nature and extent of space-time clustering can assist in the formulation of disease control and prevention.

In human disease, Birch *et al.*, (2000) showed highly significant evidence of space-time clustering of childhood leukemia based on place of birth and time of diagnosis. The results of the analysis are consistent with an infectious hypothesis. Significant space-time clustering of dengue cases was identified within individual households in an outbreak in Puerto Rico (Morrison *et al.*, 1998). Appropriate disease control measures were implicated as a result of the investigation. In veterinary medicine, French *et al.*, (1999) identified strong evidence of space-time clustering of sheep scab outbreaks in Great Britain between 1973 and 1992. Space-time clustering revealed a pattern of very local spread (less than 12 km) of sheep scab outbreaks within a time period of 5 months from an arbitrary outbreak. Space-time clustering has also been identified when investigating an outbreak of acute respiratory disease in Norwegian cattle herds (Norstrom *et al.*, 2000). The results supported the authors hypothesis that one common source of infection was involved in the outbreaks.

There are many approaches to the exploration of space-time clustering in human and animal diseases. The Mantel test, Barton's method, nearest-neighbour test and Knox's test are techniques useful for investigating space-time interaction (Bailey and Gatrell, 1995). Most of these tests concentrate solely on testing the null

hypothesis of no space-time interaction and do not consider the scale or nature of space-time interaction. An alternative approach, which estimates and describes the extent of space-time clustering, was first suggested by Diggle *et al.*, 1995. This method extends the use of K-functions (Ripley 1976), previously used to describe purely spatial point patterns, to spatial-temporal processes. To date the technique has been used to describe sporadic cases of human disease but to our knowledge has rarely been used in veterinary epidemiology. In this chapter, the use of K-function analysis has been combined with the use of Geographical Information Systems (GIS), to examine the spatial patterns of EGS in England and Wales and to detect and describe the extent of space-time clustering. We therefore aim to answer the question: Are EGS cases that are close in space also close in time?

MATERIALS AND METHODS

Source of data

Data from histologically confirmed cases of equine grass sickness in England and Wales was collected from UK veterinary diagnostic centres. Cases that were diagnosed from clinical signs only were excluded from the study due to the possibility of misdiagnosis. The date used in this analysis was the date the horse first showed clinical signs or the date the horse was admitted to a veterinary hospital (if applicable). The former was used in preference. The geographical location where the case occurred was identified as the nearest village or town to the premises where the horse originated. Patient confidentiality restricted the authors' access to the exact premises location. The location was converted into a 4 figure Ordinance Survey grid reference using The Ordnance Survey Gazetteer of Great Britain (third edition). Information was collected on 133 histologically confirmed cases of grass sickness in 1999 and 2000. Two datasets were used in the analysis; first all cases were included, including multiple cases from the same premises. The second was a slightly smaller (119 cases) database comprising only the first cases to be reported on each premises.

Spatial and temporal patterns of EGS

Prior to investigating the space-time interaction of EGS cases both spatial and temporal patterns were examined separately. The temporal pattern of EGS was visually examined using a chart produced in Microsoft Excel 97. The spatial patterns of EGS outbreaks were visually examined using GIS (Geographical Information Systems) software (Mapinfo Professional version 6.5).

K-function analysis

K-function analysis emphasises estimation of the extent of space-time clustering as a function of spatial and temporal separation. The technique is fully described by Diggle *et al.*, (1995). Briefly, the observed spatial-temporal point process are compared with a pattern that has the same temporal and the same spatial properties as the original data, but with no space-time interaction. This involves the estimation of three component processes or K-functions:

$K_{(s,t)} = \lambda^{-1}$ E [number of further events occurring within distance *s* and time *t* of an arbitrary event of the process]

$K_{(s)} = \lambda_1^{-1}$ E [number of further events occurring within distance *s* of an arbitrary event of the process]

$K_{(t)} = \lambda_2^{-1}$E [number of further events occurring within time *t* of an arbitrary event of the process]

where λ is the expected number of events per unit space per unit time (intensity of the disease process), λ_1 and λ_2 are the are the spatial and temporal intensities respectively.

In addition to providing an edge-corrected Monte-Carlo test for space-time interaction, the method can be used to estimate and describe proportional and absolute increases in disease risk, attributable to space-time interaction, for a range of spatial and temporal separations. There are three diagnostic functions that can be

estimated and plotted to analyse possible dependence between the spatial and temporal components of the underlying spatial-temporal point process. The three diagnostic functions are:

$\hat{D}_{(s,t)}$ (absolute differences) (1)

$\hat{D}_{o\,(s,t)}$ (proportional differences) (2)

Residual plot (R) (3)

(1) $\hat{D}_{(s,t)} = K_{(s,t)} - K_{(s)}\ K_{(t)}$

A contour plot of $\hat{D}_{(s,t)}$ gives information on the scale and nature of the dependence between the spatial and temporal components. This function is proportional to the increased numbers of cases within distance s and time t by comparison with a process with the same spatial and temporal structures but no space-time interaction. It is analogous to the risk difference in epidemiology.

(2) $\hat{D}_{o\,(s,t)} = D_{(s,t)} / \{K_{(s)} K_{(t)}\}$

A three-dimensional plot gives an indication of the proportional increase in cases that can be attributable to space-time clustering. This is the proportional increase in risk attributable to space-time interaction and is analogous to the relative risk.

(3) $R = D_{(s,t)} / \sqrt{\{V_{(s,t)}\}}$

In this diagnostic, $V_{(s,t,)}$ is the variance of $\hat{D}_{(s,t)}$. When plotted against $K_{(s)} K_{(t)}$ this two-dimensional plot is analogous to a plot of the standardised residuals against fitted values. If the spatial and temporal processes were independent (i.e. there was no space-time interaction) then we would expect approximately 95% of the values to lie between the values plus and minus two. A disadvantage of this plot is that the spatial and temporal scales are no longer explicit.

All analyses were performed using the SPLANCS library (Rowlingson and Diggle, 1993) in S-PLUS (Insightful corporation).

RESULTS

Temporal pattern of EGS

Figure 5.1 shows the temporal pattern of the 133 EGS cases used in this study. Even though cases were recorded in every month of the year there is a strong seasonal pattern with a peak number of cases recorded in May.

Spatial pattern of EGS

Figure 5.2 shows the spatial distribution of the 133 histologically confirmed cases of EGS used in this study. EGS cases were recorded in a large number of counties during the two years of data collection. There appears to be spatial clustering of EGS cases in several parts of England and Wales including Anglesey, the counties of Cheshire and Lancashire as well as the Newmarket area. It is likely however, that spatial clustering of cases in the North West of England was biased by the project location.

Space-Time Cluster Analysis

When all cases were considered together there was strong evidence of space-time clustering of EGS cases. The residual plot (Figure 5.3) reveals almost all of the residuals are above 2 indicating space-time interaction over wide spatial and temporal separations. The $\hat{D}$ plot (figure 5.4) shows a sharp rise over the first 5 km and 20 days and this is demonstrated further in the $\hat{D}_o$ plot (figure 5.5) which shows the greatest proportional increase within these separations. High values on the Z axis of

the $\hat{D}_0$ plot indicates that there are many more outbreaks within the given spatial and temporal separation than would be expected if there was no space time clustering. It can be seen from figure 5.5 that a large proportion of the cases within 5km and 20 days of each other can be attributed to space-time clustering. There are many times more outbreaks within this spatial and temporal separation that could be attributed to a pattern that has the same temporal and spatial properties as the observed pattern, but no space time interaction. The Monte Carlo test for space-time interaction computes a P value of <0.0001.

When multiple cases were excluded from the analysis this left 119 EGS outbreaks. Multiple cases were defined as more than one case of EGS occurring within a week of another on a single premises. For example, if 3 cases occurred during 7 days of each other on one premises, this was regarded as one single outbreak. The $\hat{D}_0$ plot (figure 5.6) of the 119 EGS outbreaks also shows, as in figure 6, that a large proportion of cases within 5 km and 20 days of each other can be attributed to space time clustering. The exclusion of multiple cases still reveals significant evidence of clustering. The Monte Carlo test for space-time interaction computes a P value of <0.02.

Figure 5.1: Seasonal distribution of 133 histologically confirmed cases of grass sickness during 1999 (blue bars) and 2000 (red bars) in England and Wales.

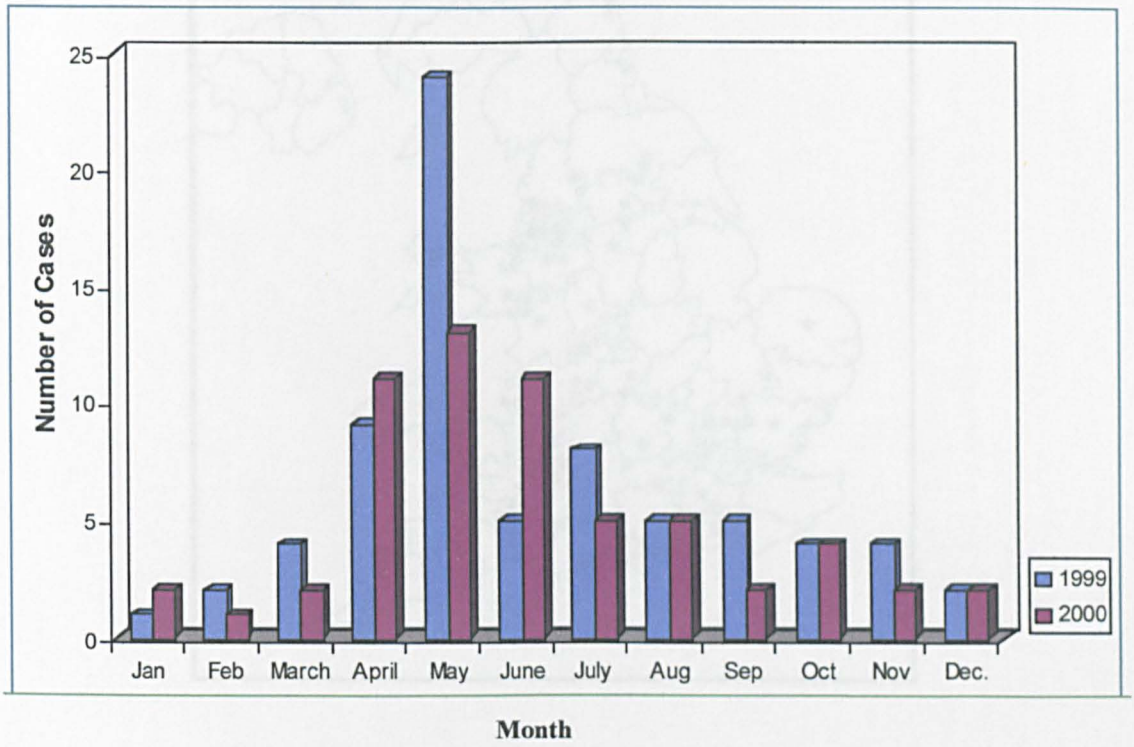


Figure 5.2. The spatial distribution of grass sickness cases used in this study (1)

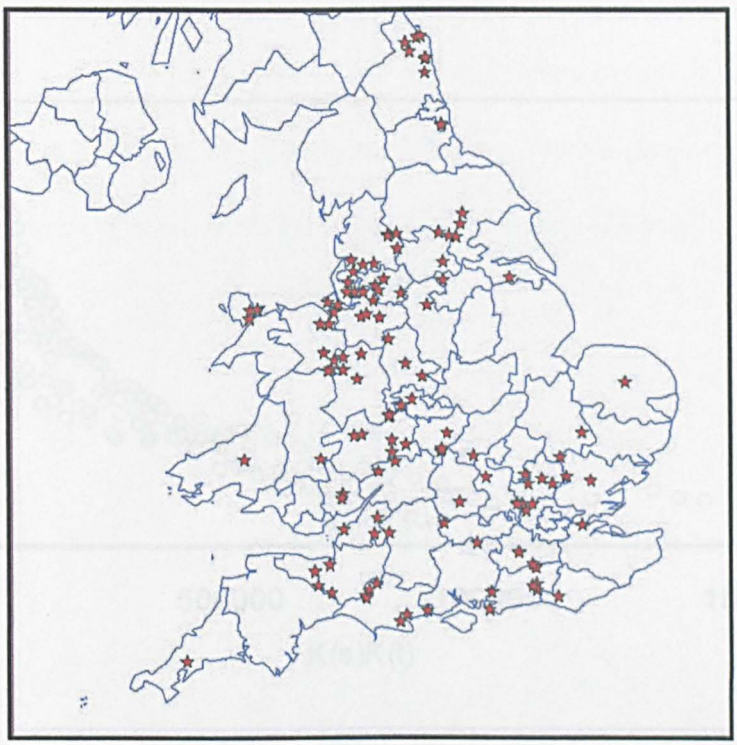


Figure 5.3: A residual plot of $R_{(s,t)}$ against $K_{(t)}K_{(s)}$ for 133 cases of EGS

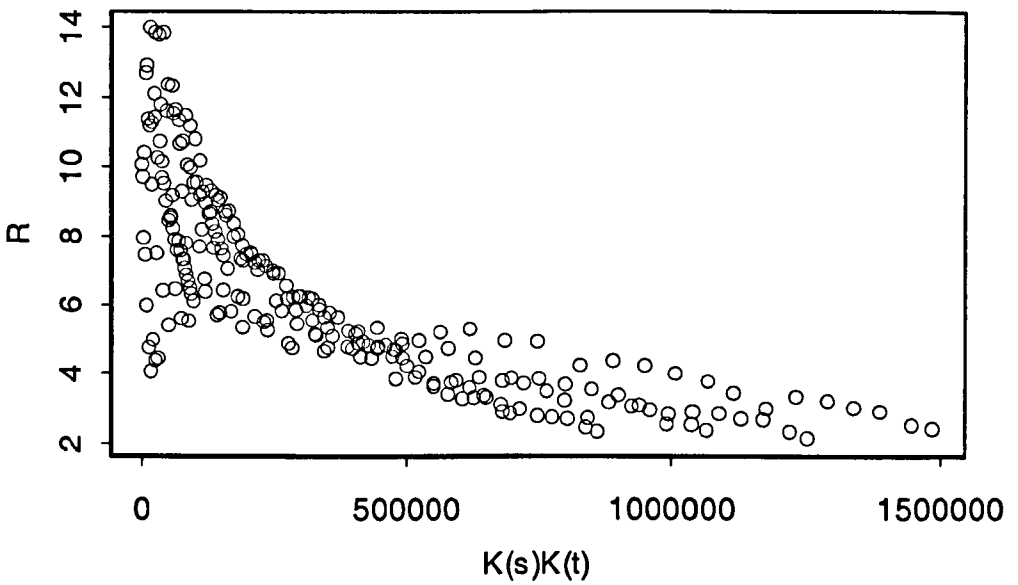


Figure 5.4: $\hat{D}$ plot $(K_{(s,t)} - K_{(s)}\ K_{(t)})$ for for 133 cases of EGS

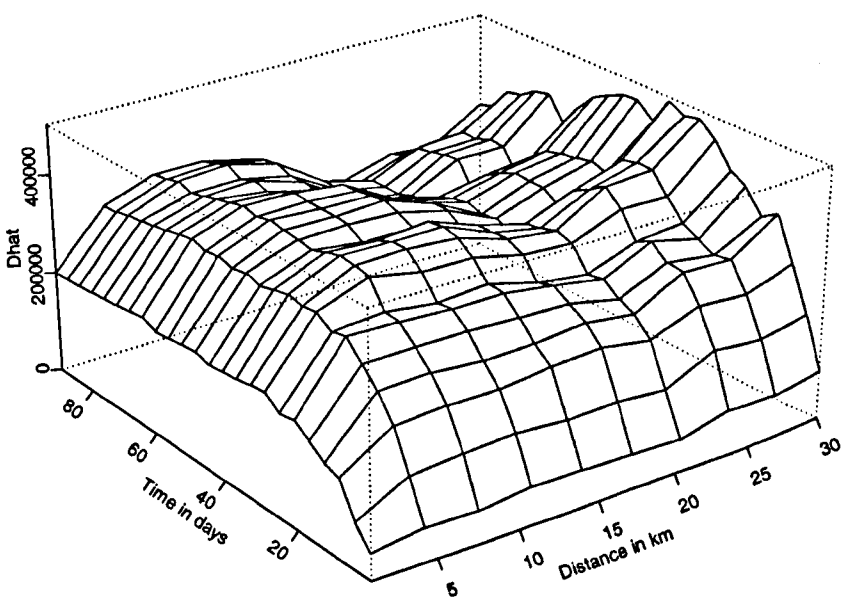


Figure 5.5: A 3 dimensional plot of the $\hat{D}_o(s,t)$ function (all 133 cases). The spike peaks and tails off rapidly at 5km and 20 days where a proportional increase in EGS cases is attributable to space-time clustering.

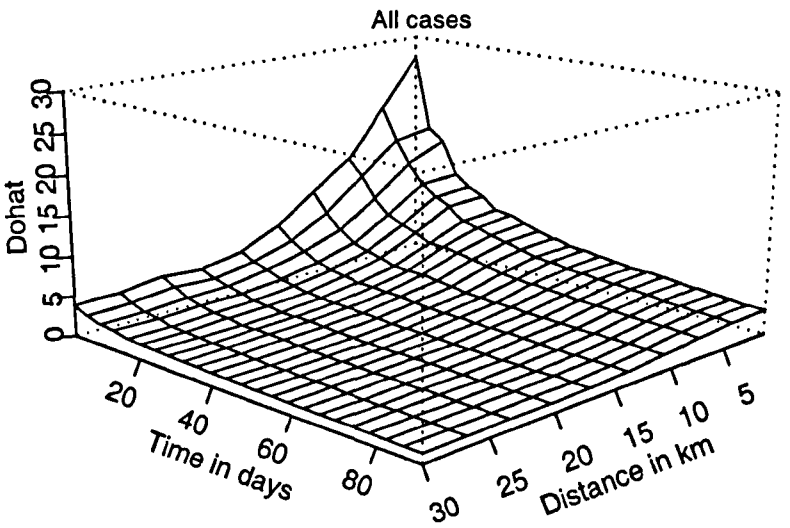
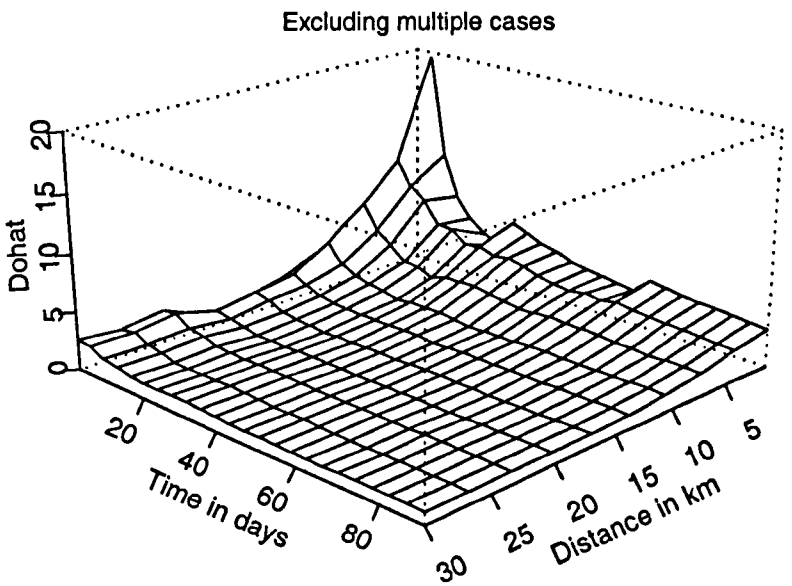


Figure 5.6: A 3 dimensional plot of the $\hat{D}_o(s,t)$ function (119 outbreaks)



DISCUSSION

This study provides strong evidence for space-time clustering of grass sickness cases in the UK; particularly within 5km and 20 days of an arbitrary case. Evidence of space-time clustering occurs when individual EGS cases are investigated or individual outbreaks of EGS. Although the temporal pattern of EGS in England and Wales has been reported previously, the exploration of the spatial pattern and the investigation of space-time interaction is novel.

For many years was widely perceived that EGS was a Scottish disease. This study has provided evidence for its occurrence in both England and Wales. Others however, believed that if the disease does occur in England and Wales it occurs 'sporadically'. This study has shown that a number of EGS cases in England and Wales can be clustered in particular areas.

During data collection, the authors were initially concerned about bias in spatial reporting. Bias could occur by underreporting of EGS cases by vets and pathologists in certain areas of England and Wales. K-function analysis however has the advantage that is unaffected by both random and non- random spatial thinning (P. Diggle, personal communication). K-function analysis also considers all cases in the study, not just localised clusters. In addition, one of the many strengths of the test is that although the K-function is motivated by the theory of stationary point processes, the test remains valid even if the true underlying process is non-stationary (Diggle *et al.*, 1995).

There are several other forms of bias that should be taken into consideration. Temporal bias could occur where a vet might be less inclined to suspect a horse has EGS outside the months of April, May and June. A form of spatial bias could occur where a horse has moved premises immediately prior to disease. It is difficult to answer the question; did the horse develop EGS prior to or after its move? Even though we cannot adjust for these potential sources of bias it is important to be aware of their existence.

Compared to other techniques of investigating space-time clustering, K-function analysis allows a scale to be determined. This is how the analysis can provide strong evidence for space-time clustering of EGS cases within a given distance and time; 5km and 20 days of an arbitrary case. K-function analysis was used by French *et al.*, (1999) who reported that a large proportion of sheep scab cases within 12km and 5 months of each other. This was attributed to space-time clustering.

This study has shown that the spatial and temporal clustering of EGS cases in England and Wales do not act independently. Identification of space-time clustering can provide evidence of contagion, an underlying infectious process or a spatially and temporally localised process. It is extremely unlikely however that EGS is a contagious disease. It has been reported often that only one horse in a field of many develops the disease and the occurrence of disease does not follow the typical spread of an infectious disease. It is more likely however, that EGS results from a spatially and temporally localised process. These processes could

be environmental, seasonal, geological or geographical. Tocher *et al.*, (1923) mapped the spread of disease in Scotland and reported that no theory could explain the spread of disease and it was undecided whether the distribution of cases resembled that of a contagious epidemic disease or a disease arising from the spread of a sporing organism. It is hypothesised here that an environmental factor, possibly meteorological, in particular geographical areas, triggers the development of disease in susceptible individuals. The causal agent of EGS has yet to be determined but there has been further evidence for the role of *C. botulinum* type C (Chapter 3). It is possible that *C. botulinum* type C is more prevalent in certain areas of the UK and that disease is triggered in susceptible animals under conditions that stimulate toxin production *in vivo*. This could ultimately explain the space-time clustering of EGS cases found in this study. Once the causal pathway of EGS is determined, it will be clearer how important the factors governing space-time clustering are.

Future work investigating the spatial and temporal pattern of EGS would be aided by the improved reporting of EGS cases in the UK. At present, we have no idea of the exact number of cases that occur in the country. Such knowledge would enable us to determine if the prevalence of the disease was increasing, decreasing or relatively stable. This information would also increase the power of detecting space-time clustering of EGS cases and establish a clearer picture of EGS in the UK.

ACKNOWLEDGEMENT

The assistance of Dr. Nigel French with the analysis in this chapter is gratefully acknowledged.

CONCLUDING DISCUSSION

This thesis has provided further evidence for the role of *C. botulinum* type C in EGS. In addition, the thesis has identified new horse-level and pasture-level risk factors for EGS and supports the findings from previous epidemiological studies. As a result of the findings of this thesis, the proposed causal pathway that was put forward in Chapter 1 has been amended (Figure 6.1). There are still a number of steps in the causal pathway that are speculated, but established risk factors have been highlighted in the diagram.

A clear relationship has been made between disease and an increased level of soil nitrogen. Concentrations of nitrogen in harvested herbage can be influenced by the supply of available nitrogen in the soil (Whitehead, 2000). The level of nitrogen in soil is relatively stable whereas the nitrogen level in pasture is much more volatile (K. Miller, personnel communication). It is possible that we did not find a significant association with EGS and pasture nitrogen for this reason. During a field visit we would have only taken a 'snap-shot' herbage sample that might well have different levels of nitrogen compared to the time when EGS was first recognised. Cold and wet conditions cause slow penetration of the soil by roots and restricts the amount of nitrogen taken up (Simpson, 1983). This effect is temporary and a change in weather conditions from the time of disease recognition to the time of field visit could explain the lack of association between EGS and pasture nitrogen. As a result of the explanation here, it is decided to keep increased pasture nitrogen in the proposed causal pathway for EGS.

A novel finding of this thesis is the association between EGS and pastures that have been disturbed. It is unclear why such an association exists and as a result it is difficult to place on the causal pathway.

It has been suggested that growth and toxin formation by *C. botulinum* is regulated by nutritional and environmental factors. This study has shown that most horses have some level of exposure to the organism and its toxin. It is likely that the organism is spread through animal faeces, including birds. However, at present the prevalence of *C. botulinum* type C in the GI tract of the equine population is unknown. It is likely that competition for nutrients affects GI colonisation of *C. botulinum* type C and may influence toxin production (Patterson-Curtis & Johnson, 1989). It has also been suggested that *C. botulinum* can only multiply in the GI tract of humans when its competitors are absent (Sugiyama, 1986).

This study hypothesised that alteration of the GI environment, due to an increased level of nitrogen from a dietary origin, provides ideal environment for *C. botulinum* type C proliferation and toxin production. As identified in this study, the alteration in the GI environment could arise from a change in pasture conditions or a change in feed type or quantity. It has been demonstrated that the expression of botulinum neurotoxin is controlled by nutritional metabolism. For growth, the organism *C. botulinum* requires the amino acids tryptophan, threonine, valine, leucine, isoleucine, methionine, arginine, phenylalinine and tyrosine with the last three being required in unusually high concentration (Smith, 1977). Patterson-Curtis and Johnson (1989) demonstrated the availability of

nitrogen, particularly in the form of arginine, influences toxin production. High levels of arginine markedly decreased neurotoxin titres in cultures of certain strains of *C. botulinum* types A and B. In addition, Leyer and Johnson (1990) demonstrated that nitrogen availability, particularly tryptophan, decreased toxin formation of *C. botulinum* type E. These two examples show how an increased level of nitrogen decreases toxin production *in vitro*. The authors state that the strains may have altered genetic or physiological characteristics that has resulted in toxin depression. There is no literature available on nitrogen levels and toxin production of *C. botulinum* type C so it is still possible that certain amino acids increase or trigger the start of toxin production of *C. botulinum* type C *in vivo*.

Another possibility for the trigger for toxin production could be a phage, carrying a toxin gene that is introduced into a microorganism that is normally non-toxigenic. It has already been mentioned in chapter 3 that bacteriophages can convert non-toxigenic cultures of *C. botulinum* type C to a toxigenic state. Both *C. botulinum* types C and D can no longer produce toxins when cured of their phage but can become toxigenic once again by reinfecting the organism with the phage isolated from the corresponding toxigenic strain. The use of PCR would be a more sensitive technique for determining the presence of these group III organisms and their relevant phages in the equine GI tract, soil or pasture. Testing of soil samples could demonstrate whether there is an environmental reservoir for the organism.

This study demonstrated an association between EGS and an increase in faecal ammoniacal nitrogen. It is likely that the ammoniacal nitrogen is of microbial origin rather than dietary origin. Ammoniacal nitrogen normally arises

from microbial degradation of dietary non-ammonia nitrogen (Obara *et al.*, 1991). An opportunity for bacterial overgrowth arises during GI stasis that occurs during EGS. During ileus the lack of normal fermentable fibre reaching the colon diminishes volatile fatty acid production which may permit the overgrowth of organisms such as *Salmonella* spp, *Clostridium difficile* and/or *Clostridium perfringens* (Divers, 2002). More suitable controls for this part of the study would be horses with non-EGS associated ileus. This group of horses would include those with post-operative ileus or anterior enteritis, for example.

The association with ivermectin and EGS is an unexpected finding of this project. It is unknown at this stage whether this anthelmintic could have an effect on gut motility, growth of *C. botulinum* type C, toxin production by *C. botulinum* type C or some other effect. It is also unclear whether the drug has an immediate effect or is more long lasting. Due to the length of time between worming and the time of disease recognition it is possible that drug administration has an effect that persists for several weeks. Whatever the effect, it is worthy of further investigation

The relationship between age and antibody level to *C. botulinum* type C, *C. novyi* type A and *C. botulinum* type C that is demonstrated in chapter 3 goes some way to explain why younger animals are more susceptible to EGS. It appears that older horses with higher antibody levels have been subclinically exposed to *C. botulinum* type C and its neurotoxin and have made a systemic immune response. As demonstrated in the causal pathway, if a horse is exposed to the other risk factors identified in this study they will only develop EGS if they

have a low antibody level to *C. botulinum* type C. There have been some instances where older animals develop EGS (e.g. horses older than 10 years). It is possible that these horses have been immunocompromised in some way or have not received sufficient exposure to the organism when they were younger.

The relationship between EGS and *C. botulinum* type C and the relationship between *C. botulinum* type C antibody level and age provides a strong case for vaccination. In theory, if EGS is caused by toxicoinfection with *C. botulinum* type C then it should be preventable by vaccination against both the organism and its toxins. A suitable study design would involve vaccinating young horses (less than 8 years, for example) that have a low antibody level to *C. botulinum* type C (an ELISA OD cut-off point would be decided) that are grazing pastures that have given rise to a case of EGS previously. Other studies have demonstrated that horses with EGS have a significantly higher level of mucosal IgA to *C. botulinum* type C toxin in GI contents when compared to non-EGS controls (Hunter, 2001). Further investigation of the mucosal immune response is required to establish whether a potential vaccine should also stimulate mucosal immunity (Hunter, 2001).

Chapter 5 provides evidence of space-time clustering of EGS cases in the England and Wales. The chapter discusses a spatially and temporally localised process that could occur to give rise to a case of EGS. In light of the causal pathway developed here and the discussion surrounding it, it is possible that certain areas of England and Wales have increased levels of soil nitrogen or higher prevalence of *C. botulinum* type C or its phage.

To conclude, the findings of this thesis support the hypothesis that toxicoinfection with *C. botulinum* type C is involved in the aetiology of EGS. Whilst the results support the involvement of a toxicoinfection with *C. botulinum* type C, they also support the multifactorial aetiology of EGS. This thesis establishes the basis for further research into the role of *C. botulinum* type C in the pathogenesis of EGS. The toxoid used in this study was an inactivated *C. botulinum* C_1 toxin and further investigations are required to determine the role of the C_2 and C_3 toxin types. Additionally, on the basis of both the horse-level and pasture-level risk factors identified in this thesis, it would be plausible to develop an intervention study to investigate the potential protection afforded by intervening to modify managemental risk factors.

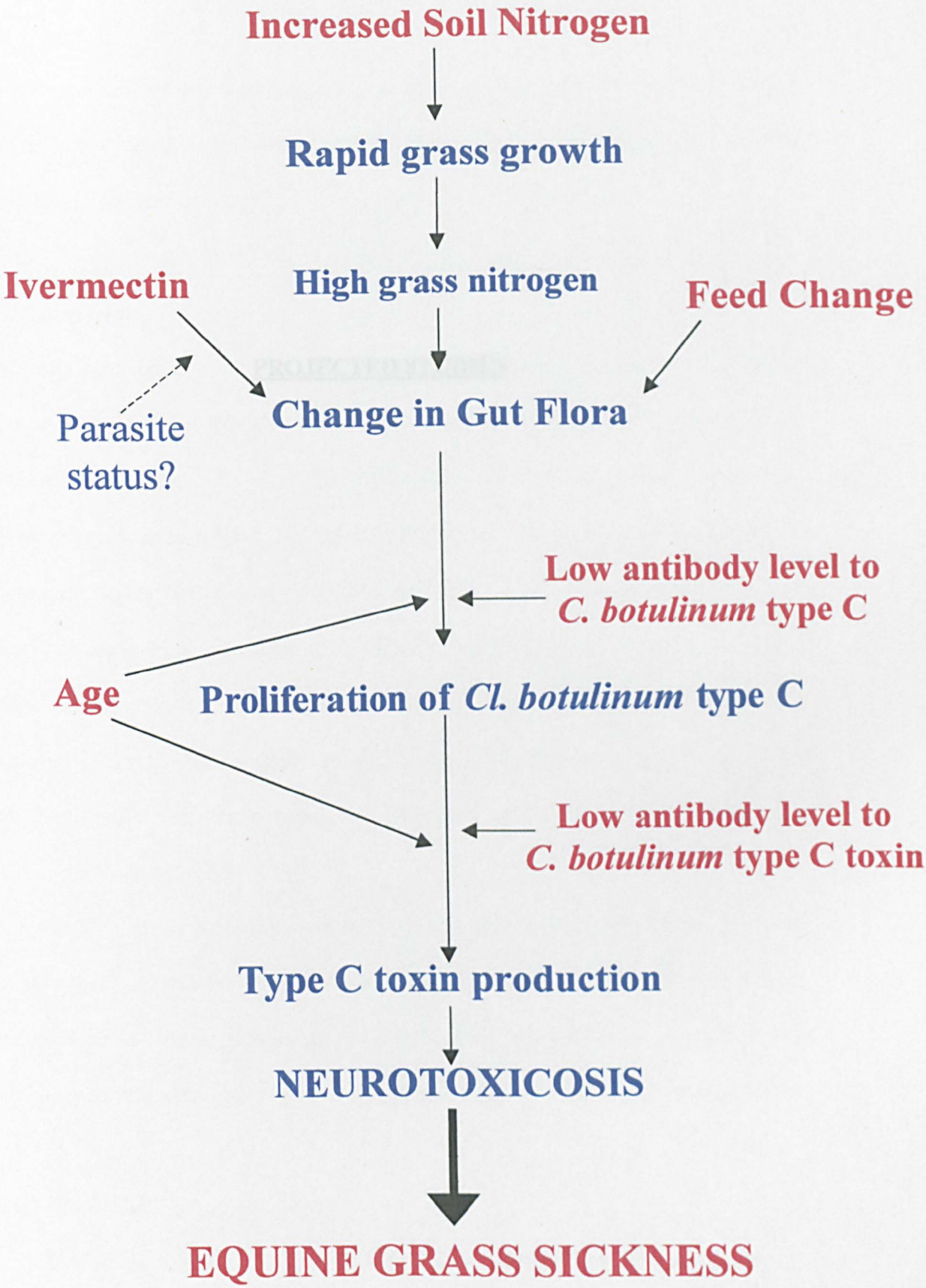


Figure 6.1: Proposed causal pathway by which horse-level and pasture-level variables lead to toxicoinfectious botulism resulting in clinical grass sickness. Thesis findings are in red font whilst speculated steps are in blue.

PROJECTED STUDIES

Projected Studies

The results of this study suggest that there are two possible paths of research that can follow on from the current project. The first is a vaccination trial and the second is an intervention study.

Vaccination trial

The serological results from the work presented in this thesis offers further evidence to support the hypothesis that EGS is associated with *C.botulinum* type C and its C1 neurotoxin. The work further suggests that vaccination is an attractive preventive measure especially for use in horses less than 10 years old. Clostridial vaccines are highly effective at preventing clostridial diseases in the horse and other domestic animals. However, there is no approved type C vaccine available for use in equines and one may take several years to develop. In addition to the development of an approved type C vaccine challenge experiments would be necessary to detect the level of antibodies that are protective. At present, a type C vaccine is only available for use in mink and cattle.

An ideal study would involve the vaccination of horses on high risk premises that have been identified in this study. A suitable control group would also be recruited from a similar population. A reduction in disease in vaccinated animals would suggest that vaccination would be a suitable disease prevention method.

Intervention study

Due to the lack of an approved vaccine, an intervention study may provide a more immediate method of risk reduction. A number of managemental risk factors have been identified in this study and an intervention study could determine whether

altering the management of horses could significantly reduce their risk of suffering from EGS. Based on the findings of this thesis, alterations in management will involve both diet and anthelmintic use. More specifically, i) forage (haylage in particular) is to be fed constantly, ii) concentrate type is to remain constant, iii) changes in feed type or quantity are to be avoided and iv) ivermectin should not be used between February and September. Until vaccines are available, managemental manipulation offers the prospect of cheap, easily implemented disease prevention.

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APPENDIX i

Visualisation of proteins on SDS-PAGE gels.

1) Coomassie blue staining

Reagents

Coomassie Stain: 1g Coomassie, 300ml Methanol, 100ml acetic acid made up to 1 litre with distilled water and filtered with Whatman no. 1 filter paper.

Coomassie destain: 400ml Methanol, 70 ml acetic acid, made up to 1L with distilled water.

Protocol

i) Gel placed in Coomassie stain for 1 hour, with rocking.

ii) Following staining, gel placed in destain overnight.

iii) Rinse in distilled water and air-dry

2) Silver staining

Reagents and Protocol

* = make up reagents fresh.

i) Gel placed in distilled water for 5 minutes

ii) Two sets of 30 minutes in fixer (45% Methanol, 10% acetic acid)

iii) 2 minutes in sensitiser* (0.02% Sodium Thiosulphate in distilled water).

iv) 1 minute in distilled water

v) 30 minutes in Silver Nitrate* (0.1% Silver Nitrate in distilled water. Store at 4°C).

vi) Develop* (2% Sodium Carbonate and 0.04% formaldehyde (added immediately before use) in distilled water.

vii) Stop (39.2 MM EDTA (1.459g in 100ml distilled water)

viii) Rinse in distilled water and air-dried.

APPENDIX ii

Multiple R-squared values to assess the fit of positive power curves to the data.

Response variable	Explanatory variable	*Cl .novyi* OD	*C. botulinum* OD	BoNT/C toxoid OD
OD	Age	0.085	0.088	0.133
OD	Age + Age^2	0.104	0.131	0.184
OD	Age + Age^2 + Age^3	0.107	0.147	0.184
Log OD	Age + Age^2	0.112	0.154	0.200
Log OD	Age + Age^2 + Age^3	0.121	0.184	0.144
Log OD	Log Age	0.138	0.191	0.229

APPENDIX iii

QUESTIONNAIRES

Project Case Number: ❑ ❑ ❑ ❑

EQUINE GRASS SICKNESS QUESTIONNAIRE

TODAY'S DATE: ..... DATE GS CASE ADMITTED.....

GRASS SICKNESS CASE

Name of Horse: ..... Owner: .....

Address of premises .....

.....

Postcode ..... OS Grid reference .....

Owner Address.....

.....

Postcode .....

Contact telephone number of primary carer: .....

Contact telephone number of yard owner/manager: .....

Referring vet: .....

Practice address: .....

..... Tel: .....

PART 1: HORSE DETAILS

- **SEX**: Mare (M),Gelding (G), Stallion (S).....
- **AGE** (in years) .....
- **BREED** :

Thoroughbred.....❑ Thoroughbred X.....❑ Welsh mountain.....❑

Miniature/Shetland....❑ Heavy horse.....❑ Arabian ..... ❑

Anglo Arab.....❑ Warmblood.. ..... ❑ Native breed..... ❑

Hunter/mixed breed....❑ Other ..... ❑

- **PRESENT USE:**

Competing ..... ❑ *Circle*: Racing Eventing Dressage Showjumping Driving Showing

Hunter/GP .....❑

Breeding .....❑

Retired/not in use/UB.....❑

Other .....❑

PART 2: MANAGEMENT PROCEDURES

◆ How long has this horse been on these premises? ..... Years

..... Months

..... Weeks

..... Days

◆ Daily routine.

For the 2 weeks prior to disease, was the horse:

Stabled full time? .....❑

Grass during day & stabled at night?.....❑

Grass at night & stabled during day?.....❑

Grass-kept full time?.....❑

Other? .....❑

◆ How long has the horse been kept on this particular routine?

..... Years

..... Months

..... Weeks

..... Days

◆ If this routine (over the past2 weeks) involves time spent at grass:

On average, how many hours per day/night did the horse spend at grass? .....

◆ How long has the horse been grazing its current pasture?

..... Years

..... Months

..... Weeks

..... Days

◆ How many horses share the same pasture? .....

◆ Has the horse changed routine within the past two weeks?

Yes ..... ❑ No..... ❑

If yes, what was the routine prior to the current one?:

Stabled full time? ..... ❑

Grass during day & stabled at night ..... ❑

Grass at night & stabled during day..... ❑

Grass-kept full time? ..... ❑

Other ..... ❑

◆ On this routine how many hrs per day/night did the horse spend at grass? .....

◆ How long was the horse kept on the previous routine?

..... Years ..... Weeks

..... Months..... Days

◆ Has there been a change of pasture over the past 2 weeks? ?

Yes ..... ❑ No..... ❑

If yes, how long was the horse on the previous pasture?

..... Years ..... Weeks

..... Months..... Days

◆ FEEDING

Over the two weeks was the horse fed: ('Hay'=Hay or hay alternatives)

Grass only ..... ❑

Grass supplemented with hay?..... ❑

Grass supplemented with concentrates ..... ❑

Grass supplemented with hay & concentrates? ..... ❑

Hay and concentrates?..... ❑

Hay only? ..... ❑

Other? ..... ❑

If concentrates are fed,

What type of concentrates are fed, and how many scoops per day:

FEED	N° Scoops	Weight(kg)	FEED	N° Scoops	Weight(kg)
Course mix			Bran		
Cubes			Sugar beet		
Oats			Mollichop		
Barley			Alfalfa		
Maize			Hi-Fi		
Other			Other		

If 'hay' is fed, in what form is it fed

Dry hay ..... ❑

Wet hay ..... ❑

Big bale haylage..... ❑

Horsehage..... ❑

Silage... ..... ❑

How much is fed per day? .....Bale(s)

or.....Slices

or.....Nets

Total weight of forage per day per day (kg).....

◆ Is this horse's feed supplemented with additives? (e.g. vitamin & mineral supplements, electrolytes, probiotics). Yes..... ❑ No ..... ❑

If the answer is yes, what is the type of additive and its brand name?

Type: .....

Brand name: .....

Type: .....

Brand name: .....

Type: .....

Brand name: .....

◆ Have there been any changes in feed type within the last two weeks?

If yes: Yes..... ❑ No ..... ❑

What was this change: .....

.....

When did this change take place? .....days ago

◆ Have there been any changes in feeding routine within the last two weeks? Yes ..... ❑ No ..... ❑

If yes, What was this change: .....

When did this change take place?..... days ago

◆ **PARASITE CONTROL**

Over the past twelve months have wormers been used?

Yes ..... ❑ No ..... ❑

If Yes: Over the past twelve months, how often has the horse wormed?

Every : ..... months / weeks.

♦ When was the horse last wormed? ..... (date)

♦ When was the time before? ..... (date)

♦ Which brand of anthelmintic was used when the horse was last wormed (1) and which brand was used the time before that (2)?

	(1)	(2)
EQVALAN.....	❑	❑
EQUEST	❑	❑
FUREXEL	❑	❑
PANACUR.....	❑	❑
PANACUR GUARD	❑	❑
STRONGID P	❑	❑
TELMIN	❑	❑
EQUIVERM PLUS	❑	❑
OTHER(state brand)	❑	❑

♦ Was the horse wormed:

At the same time as other horses ..... ❑

More often than the other horse ..... ❑

Less often than the other horses ..... ❑

♦ Are any of the following worm control procedures also carried out?

Regular removal of droppings from pasture? Yes ..... ❑ No ..... ❑

If yes, how often? .....

Rotating of horses on different pastures? Yes ..... ❑ No ..... ❑

If yes, how often? .....

PART 3: *HEALTH & DISEASE*

■ Within the past 3 months has this horse suffered any illness/disease?

YES ..... ❑ NO..... ❑

If yes, what was the illness:

Colic ..... ❑

Unexplained weight loss..... ❑

Diarrhoea..... ❑

Lameness..... ❑

Infectious disease..... ❑ State.....

Other. ..... ❑ State.....

♦ When did this illness occur?..... (date)

♦ Was this illness/disease treated by a vet? YES .....❑ NO..... ❑

■ Do you know of any of your horses' relatives suffering from Grass sickness?

YES .....❑ NO.....❑

If yes:

♦ What was the relationship?.....

♦ When did this occur? .....

■ Do you know of any grass sickness cases occurring on these premises previous the current one? YES ....❑ NO..... ❑

If yes:

♦ When was the last case of grass sickness?.....

♦ How was this case diagnosed?

Veterinary surgeon on the premises?..... ❑

Referral to practice surgery? .... ❑

Referral to veterinary hospital? .... ❑

♦ What was the outcome?

Full recovery?..... ❑

Recovered but never returned to original work?..... ❑

Euthanasia? ..... ❑

Died?..... ❑

♦ Has this horse ever grazed the same pasture as a grass-sickness case?

Yes ..... ❑ No ..... ❑

If Yes;

Can you give dates of when your horse grazed the same pasture as the grass sickness case?

.....

.....

PART 4: PREMISE DETAILS

- Has the pasture, on which the horse was kept, been ploughed this year ?

  Yes ..... ❑ No ..... ❑ Don't know ..... ❑

  If yes, when did this take place?.....

- Has the pasture, on which the horse was kept, been harrowed this year ?

  Yes ..... ❑ No ..... ❑ Don't know ..... ❑

  If yes, when did this take place?.....

- Has the pasture, on which the horse was kept, been re-seeded this year ?

  Yes ..... ❑ No ..... ❑ Don't know ..... ❑

  If yes, when did this take place?.....

  What was it re-seeded with? .....

● Has the pasture, on which the horse was kept, been fertilised this year?

Yes ..... ❑ No ..... ❑ Don't know ..... ❑

If yes, What has the pasture been fertilised with?

Manure ..... ❑

Lime ..... ❑

Manure & lime ..... ❑

Nitrates ..... ❑

Nitrates & other ..... ❑

Other ..... ❑

♦ When was the pasture last fertilised? ..... (date)

♦ What is the brand of fertiliser used? .....

If Nitrates are used, what % of nitrogen is contained in the fertiliser?

5% ..... ❑ 10% ..... ❑

11-20% ..... ❑ 21-30% ..... ❑

31-40 ..... ❑ 41-50% ..... ❑

■ Has the pasture been disturbed within the last 12 months (eg, excavation, pipe laying) Yes ..... ❑ No ..... ❑

If yes, how was the pasture disturbed? .....

When was the pasture disturbed? .....

● Do any other species share this pasture? Yes ..... ❑ No ..... ❑

If yes, which Species? .....

Project Case Number: ❑ ❑ ❑ ❑

EQUINE GRASS SICKNESS QUESTIONNAIRE

TODAY'S DATE: ..... DATE GS CASE ADMITTED.....

HORSE CONTROL

Name of Horse: ..... Owner: .....

Address of premises .....

.....

Postcode ..... OS Grid reference .....

Owner Address.....

.....

Postcode .....

Contact telephone number of primary carer: .....

Contact telephone number of yard owner/manager: .....

Referring vet: .....

Practice address: .....

..... Tel: .....

PART 1: HORSE DETAILS

- **SEX**: Mare (M),Gelding (G), Stallion (S).....
- **AGE** (in years) .....
- **BREED** :

Thoroughbred.....❑ Thoroughbred X.....❑ Welsh mountain.....❑

Miniature/Shetland....❑ Heavy horse.....❑ Arabian ..... ❑

Anglo Arab.....❑ Warmblood..... ❑ Native breed..... ❑

Hunter/mixed breed...❑ Other ..... ❑

- **PRESENT USE:**

Competing ..... ❑ *Circle*: Racing Eventing Dressage Showjumping Driving Showing

Hunter/GP .....❑

Breeding .....❑

Retired/not in use/UB.....❑

Other .....❑

PART 2: MANAGEMENT PROCEDURES

- How long has this horse been on these premises? ..... Years
  ..... Months
  .....Weeks
  ..... Days

- Daily routine.

  For the 2 weeks prior to disease, was the horse:

  Stabled full time? ..... ❑

  Grass during day & stabled at night?..... ❑

  Grass at night & stabled during day?..... ❑

  Grass-kept full time?..... ❑

  Other? ..... ❑

◆ How long has the horse been kept on this particular routine?

..... Years

..... Months

..... Weeks

..... Days

◆ If this routine (over the past2 weeks) involves time spent at grass:

On average, how many hours per day/night did the horse spend at grass? .....

◆ How long has the horse been grazing its current pasture?..... Years

..... Months

..... Weeks

..... Days

◆ How many horses share the same pasture? .....

◆ Did this horse share the same pasture as the EGS case?

Yes ..... ❑ No.....❑

◆ Has the horse changed routine within the past two weeks?

Yes ..... ❑ No.....❑

If yes, what was the routine prior to the current one?:

Stabled full time? ..... ❑

Grass during day & stabled at night ..... ❑

Grass at night & stabled during day..... ❑

Grass-kept full time? ..... ❑

Other ..... ❑

On this routine how many hrs per day/night did the horse spend at grass? .....

How long was the horse kept on the previous routine?

..... Years ..... Weeks

..... Months..... Days

◆ Has the horse changed pasture over the past 2 weeks? ?

Yes ..... ❑ No.....❑

If yes, how long was the horse on the previous pasture?

..... Years ..... Weeks

..... Months..... Days

◆ FEEDING

Over the two weeks was the horse fed: ('Hay'=Hay or hay alternatives)

Grass only ..... ❑

Grass supplemented with hay?..... ❑

Grass supplemented with concentrates ..... ❑

Grass supplemented with hay & concentrates? ..... ❑

Hay and concentrates?..... ❑

Hay only? ..... ❑

Other? ..... ❑

If concentrates are fed,

What type of concentrates are fed, and how many scoops per day:

FEED	N° Scoops	Weight(kg)	FEED	N° Scoops	Weight(kg)
Course			Bran		
Cubes			Sugar beet		
Oats			Mollichop		
Barley			Alfalfa		
Maize			Hi-Fi		
Other			Other		

If 'hay' is fed, in what form is it fed

Dry hay ..... ❑

Wet hay ..... ❑

Big bale haylage..... ❑

Horsehage..... ❑

Silage... ..... ❑

How much is fed per day? .....Bale(s)

or.....Slices

or.....Nets

Total weight of forage per day per day (kg).....

◆ Is this horse's feed supplemented with additives? (e.g. vitamin & mineral supplements, electrolytes, probiotics). Yes..... ❑ No ..... ❑

If the answer is yes, what is the type of additive and its brand name?

Type: .....

Brand name: .....

Type: .....

Brand name: .....

Type: .....

Brand name: .....

◆ Have there been any changes in feed type within the last two weeks?

If yes: Yes..... ❑ No ..... ❑

What was this change: .....

.....

When did this change take place? .....days ago

◆ Have there been any changes in feeding routine within the last two weeks?

Yes ..... ❑ No ..... ❑

If yes, What was this change: .....

When did this change take place?..... days ago

◆ **PARASITE CONTROL**

Over the past twelve months have wormers been used?

Yes ..... ❑ No ..... ❑

If Yes: Over the past twelve months, how often has the horse wormed?

Every : ..... months / weeks.

♦ When was the horse last wormed? ..... (date)

♦ When was the time before? ..... (date)

♦ Which brand of anthelmintic was used when the horse was last wormed (1) and which brand was used the time before that (2)?

	(1)	(2)
EQVALAN.....	❑	❑
EQUEST	❑	❑
FUREXEL	❑	❑
PANACUR.....	❑	❑
PANACUR GUARD	❑	❑
STRONGID P	❑	❑
TELMIN	❑	❑
EQUIVERM PLUS	❑	❑
OTHER(state brand)	❑	❑

♦ Was the horse wormed:

At the same time as other horses ..... ❑

More often than the other horse ..... ❑

Less often than the other horses ..... ❑

♦ Are any of the following worm control procedures also carried out?

Regular removal of droppings from pasture? Yes ..... ❑ No ..... ❑

If yes, how often? .....

Rotating of horses on different pastures? Yes ..... ❑ No ..... ❑

If yes, how often? .....

PART 3: HEALTH & DISEASE

■ Within the past 3 months has this horse suffered any illness/disease?

YES .....❑ NO..... ❑

If yes, what was the illness:

Colic ..... ❑

Unexplained weight loss..... ❑

Diarrhoea..... ❑

Lameness..... ❑

Infectious disease..... ❑ State.....

Other. ..... ❑ State.....

- ♦ When did this illness occur?..... (date)
- ♦ Was this illness/disease treated by a vet? YES .....❑ NO..... ❑

■ Do you know of any of your horses' relatives suffering from Grass sickness?

YES .....❑ NO.....❑

If yes,

- ♦ What was the relationship?.....
- ♦ When did this occur? .....

■ Do you know of any grass sickness cases occurring on these premises previous the current one? YES ..... ❑ NO..... ❑

If yes:

- ♦ When was the last case of grass sickness? .....
- ♦ How was this case diagnosed?

Veterinary surgeon on the premises?..... ❑

Referral to practice surgery? .... ❑

Referral to veterinary hospital?..... ..... ❑

♦ What was the outcome?

Full recovery?..... ❑

Recovered but never returned to original work?..... ❑

Euthanasia? ..... ❑

Died?..... ❑

♦ Has this horse ever grazed the same pasture as a grass-sickness case?

Yes ..... ❑ No ..... ❑

If Yes; Can you give dates of when your horse grazed the same pasture as the grass sickness case?

.....

.....

Project Case Number: ❑❑❑❑

EQUINE GRASS SICKNESS QUESTIONNAIRE

TODAY'S DATE: ..... DATE GS CASE ADMITTED.....

PREMISE CONTROL

Name of Horse: ..... Owner: .....

Address of premises .....

.....

.....

Postcode ..... OS Grid reference .....

Contact telephone number of primary carer: .....

Contact telephone number of yard owner/manager: .....

(If different from above)

Referring vet: .....

Practice address: .....

..... Tel: .....

PART 1: PREMISE DETAILS

- How many horses share *the same* pasture as your horse .....
- Do other species of animals share the pasture?

Yes ..... ❑ No ..... ❑

If yes, which species?..... .....

- Has the pasture, on which the horse was kept, been ploughed this year?:

Yes ..... ❑ No ..... ❑ Don't know ..... ❑

If yes, when did this take place?.....

- Has the pasture, on which the horse was kept, been harrowed this year?:

Yes ..... ❑ No ..... ❑ Don't know ..... ❑

If yes, when did this take place?.....

- Has the pasture, on which the horse was kept, been reseeded this year ?:

Yes ..... ❑ No ..... ❑ Don't know ..... ❑

If yes, when did this take place?.....

What was it re-seeded with? .....

- Has the pasture, on which your horse is kept, been fertilised this year ?:

Yes ..... ❑ No ..... ❑ Don't know ..... ❑

If yes, when was the pasture fertilised? .....

What has the pasture been fertilised with

Manure ..... ❑

Lime ..... ❑

Manure & lime ..... ❑

Nitrates ..... ❑

Nitrates & other ..... ❑

Other ..... ❑

What is the brand of fertiliser used?.....

If Nitrates are used, what percentage of nitrogen is contained within the fertiliser?

5% ..... ❑ 10%..... ❑

11-20%..... ❑ 21-30%..... ❑

31-40 ..... ❑ 41-50% ..... ❑

- Has the pasture been disturbed within the last 12 months (eg, excavation, pipe laying)

Yes ..... ❑ No ..... ❑

If yes, How was the pasture disturbed?.....

When was the pasture disturbed? .....

PART 2: HEALTH & DISEASE

■ Do you know of any grass sickness cases occurring on these premises?

YES ..... ❑ NO..... ❑

If yes, when was the last case of grass sickness?

.....

How was this case diagnosed?

Veterinary surgeon on the premises?..... ❑

Referral to practice surgery? .... ❑

Referral to veterinary hospital? .... ❑

Where?.....

What was the outcome?

Full recovery? ..... ❑

Recovered but never returned to original work? ..... ❑

Euthanasia?..... ❑

Died?..... ❑

Appendix iv: Definition of horse-level variables

Variable	Definition
The horse	
Age	Age of horse (years).
Sex	Sex of horse; i) Mare, ii) gelding or ii) stallion/colt
Breed	Breed of horse; i) Thoroughbred (TB), ii) TB cross, iii) Hunter or mixed breed, iv) Warmblood, v) Arab or Anglo Arab, vi) Native breed (eg Shetland or Fell pony), vii) Welsh Mountain/Cob or vii) other (eg Appaloosa).
Use	Use of horse; i) Not in work (includes unbroken, retired or unused) ii) General riding horse or hack, or iii) competition horse.
Horse Management	
Time on premises	Number of days on horse premises.
Recent Routine	During the past 14 days has the horse been stabled for 24 hours a day? Out at grass for 24 hours a day? Stabled at night, at grass during the day? Or, Stabled during the day and out at grass during the night?
Time on routine.	Number of days on recent routine.
Time on pasture	Number of days on recent pasture.
Change of routine in the last 14 days	Has the horse changed from one routine to another during the past 14 days? Yes or No.
Inconsistent routine	Has the horse had an inconsistent routine during the past 14 days? Yes or No.
Hours grazed per day	Number of hours at pasture per day.
Hours grazed in previous week	Number of hours at pasture during the past week.

Grazing pasture < 14 days	Has the horse grazed its current pasture for 14 days or less? Yes or No.
Grazing pasture < 21 days	Has the horse grazed its current pasture for 21 days or less? Yes or No.
Grazing pasture < 56 days	Has the horse grazed its current pasture for 56 days or less? Yes or No.
Feeding	
Hay	Has the horse been fed hay (haylage, wet hay or dry hay) in its daily diet during the past 14 days? Yes or No.
Concentrates	Has the horse been fed concentrated feed (Course mix, cubes or oats etc.) in its daily diet during the past 14 days? Yes or No?
Course Mix	Has the horse been fed course mix in its daily diet during the past 14 days? Yes or No?
Cubes	Has the horse been fed cubes in its daily diet during the past 14 days? Yes or No?
Oats	Has the horse been fed oats in its daily diet during the past 14 days? Yes or No?
Barley	Has the horse been fed barley in its daily diet during the past 14 days? Yes or No?
Bran	Has the horse been fed bran in its daily diet during the past 14 days? Yes or No?
Sugar Beet	Has the horse been fed sugar beet in its daily diet during the past 14 days? Yes or No?
Mollichop	Has the horse been fed mollichop in its daily diet during the past 14 days? Yes or No?
Alfalfa	Has the horse been fed alfalfa in its daily diet during the past 14 days? Yes or No?
Hi-Fi	Has the horse been fed Hi Fi in its daily diet during the past 14 days? Yes or No?
Dry Hay	Has the horse been fed dry hay in its daily diet during the past 14 days? Yes or No?

Wet Hay	Has the horse been fed wet hay in its daily diet during the past 14 days? Yes or No?
Haylage	Has the horse been fed haylage in its daily diet during the past 14 days? Yes or No?
Supplements	Has the horse been fed supplements (eg Garlic, cod liver oil etc.) in its daily diet during the past 14 days? Yes or No?
Change of feed type/quantity in the last 14 days.	Has the horse had a change in feed type or quantity during the past 14 days? Yes or No?
Intermittent feeding	Has the horse been fed less than once a day?
Worming	
Ivermectin (1)	Was the horse administered ivermectin on its last worming occasion? Yes or No?
Moxidectin (1)	Was the horse administered moxidectin on its last worming occasion? Yes or No?
Benzimidazole (1)	Was the horse administered benzimidazole on its last worming occasion? Yes or No?
Pyrantel (1)	Was the horse administered pyrantel on its last worming occasion? Yes or No?
Ivermectin (2)	Was the horse administered ivermectin on its penultimate worming occasion? Yes or No?
Moxidectin (2)	Was the horse administered moxidectin on its penultimate worming occasion? Yes or No?
Benzimidazole (2)	Was the horse administered benzimidazole on its penultimate worming occasion? Yes or No?
Pyrantel (2)	Was the horse administered pyrantel on its penultimate worming occasion? Yes or No?
Faecal egg count	Number of strongyle eggs per gram of faeces (McMasters technique)

Tapeworm ELISA OD	Tapeworm (*Anaplocephala perfoliata*) ELISA OD
Number of days to last worming	Number of days to last administration of an anthelmintic.
Exposure to EGS	
Relative with EGS	Has any of the horses (known) relatives suffered from grass sickness? Yes or no?
Grazed an EGS pasture? (previous outbreak)	Has the horse grazed the same pasture, at the same time, as a grass sickness case during a previous outbreak to this one.
Grazed an EGS pasture? (current or previous outbreak)	Has the horse grazed the same pasture, at the same time, as a grass sickness case during the current outbreak.

(1) = Last worming event
(2) = Penultimate worming event

widespread in the environment and most animals are exposed to it, only 12·3 per cent of the farms had clinical listeriosis in their animals, possibly owing to differences in farm practices. The relationships between the prevalence and incidence of clinical listeriosis and farm-related factors are being investigated.

ACKNOWLEDGEMENTS

The authors wish to thank the Faculty of Veterinary Medicine, Kafkas University, Turkey, for sponsoring H. M. Erdogan and Dr N. French for his comments on this manuscript.

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Epidemiology of equine grass sickness: a literature review (1909-1999)

H. E. McCARTHY, C. J. PROUDMAN, N. P. FRENCH

The geographical spread of grass sickness between 1909 and 1999, particularly in England and Wales, is described, and the experimental investigations to identify a causal agent are summarised. The epidemiological techniques used to investigate grass sickness vary from clinical observations, to more advanced methods such as case-control studies using logistic regression analyses. Several risk factors for grass sickness have been reported consistently (age, time of year and recent movement to new pasture or premises) and several others have been reported for which the findings remain inconsistent (weather, pasture type, breed, supplementary feeding and use of anthelmintics).

Veterinary Record (2001) **149,** 293-300

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GRASS sickness is a usually fatal disease of horses, ponies and donkeys that was first recognised early in the 20th century. The disease is a dysautonomia, characterised by severe and extensive lesions in neurons of the autonomic and enteric nervous systems (Pollin and Griffiths 1992). The clinical signs include sweating, muscle fasciculations and tachycardia (Greig 1942) and the biochemical changes include increases in the plasma concentrations of catecholamines (Hodson and others 1984); both changes are compatible with overactivity of the sypathoadrenal system. The only definitive way of diagnosing grass sickness is histologically either from an ileal biopsy or from sympathetic ganglia and/or ileal tissue taken postmortem (Scholes and others 1993).

The causal agent of grass sickness is unknown although associations between the disease and a number of infective or toxic agents have been investigated. Until recently, none was convincingly associated with the disease. The search for a single causal agent has recently made way for epidemiological investigations of a multifactorial aetiology.

This paper reviews over 80 years of epidemiological research on grass sickness, summarises current knowledge of the disease and identifies gaps in that knowledge which could be investigated epidemiologically.

GEOGRAPHICAL SPREAD OF GRASS SICKNESS

There have been several reports of the first occurrence of grass sickness in different regions of the UK since 1900. The 'erratic' occurrence of the disease is one of its main features (Guthrie 1940). Its distribution varies from year to year and often only one animal in a grazing group is affected. Greig (1942) speculated that 'there is little doubt that these are not chance occurrences but that there is some fundamental law, as yet not understood, which governs their manifestation'.

Scotland

The first outbreak of grass sickness in Scotland occurred in 1909, in army horses at Barry Camp near Broughty Ferry, Angus (Tocher and others 1923). It is possible that sporadic cases occurred earlier, but the clinical syndrome had not been recognised or described (McKay 1958). These early cases were diagnosed by veterinary surgeons as 'obstinate cases of impaction' of the large intestine (Guthrie 1940).

Between 1911 and 1922 outbreaks of grass sickness occurred in the county of Angus and also in the east of Perthshire with the distribution of cases being irregular. In 1913, in Angus, many horses died of the disease, and the disease then gradually spread to other parts of Scotland. Until 1918, grass sickness was apparently confined to the east of the River Tay, but it then spread to the west of the Tay and continued to spread westwards and northwards to Perth and Aberdeen (Tocher and others 1923, Greig 1942). The disease also spread south to parts of the Lothians, Berwick, Wigtown, Dumfries, Kirkcudbright, Ayrshire and Lanark, but the West Highlands remained relatively free (Guthrie 1940). By 1928, grass sickness had been described in every county of north-east Scotland but it was still the south eastern part of the country which had most recorded cases (Pool 1928).

Grass sickness was first reported in Orkney in 1938, and 13 cases were recorded between 1938 and 1940; these were the first cases recorded on any of the Scottish islands (Greig 1942).

In 1923, an effort was made to plot the geographical distribution of cases of grass sickness in the east of Scotland (Tocher and others 1923). Following the outbreak at Barry Camp in 1909, cases next occurred at Broughty Ferry in 1911, and in the following two years a further eight farms were affected in the area immediately north of Barry Camp and Dundee. The disease became more widely distributed by 1917 moving out towards Blairgowrie, Kirriemuir and Forfar (Fig 1). There was a high mortality from the disease in 1919 near Dundee and Coupar Angus, and during 1919 and 1922 its spread was mapped in Morayshire, Banffshire and Aberdeenshire. The first case in these areas occurred around the Elgin area. In Morayshire during this period 128 of 888 horses (14·4 per cent) died. Tocher reported at the time that no theory could explain the spread of the disease and it was undecided whether the distribution of cases resembled that of a contagious epidemic disease or a disease arising from the spread of a sporing organism.

England

Since 1911, grass sickness has not only been recorded in every county in Scotland, but also in most counties of England and Wales. There are some discrepancies in the dates recording when these cases first occurred but the reports emphasise the fact that grass sickness has occurred in England and Wales for almost as long as it has occurred in Scotland. Guthrie (1940) described the occurrence of grass sickness in many counties of England and Wales (Fig 2) and gave the dates when the disease first occurred (Table 1). From 1911, the cases recorded in England were initially confined to the northern counties of Northumberland and Cumberland (Greig 1942). A report by the Agricultural Research Council in 1939 reported that in the previous few years several hundred cases had occurred in England (Greig 1942), outbreaks having been recorded in various areas including the Severn Valley, Essex and the east and north Ridings of Yorkshire.

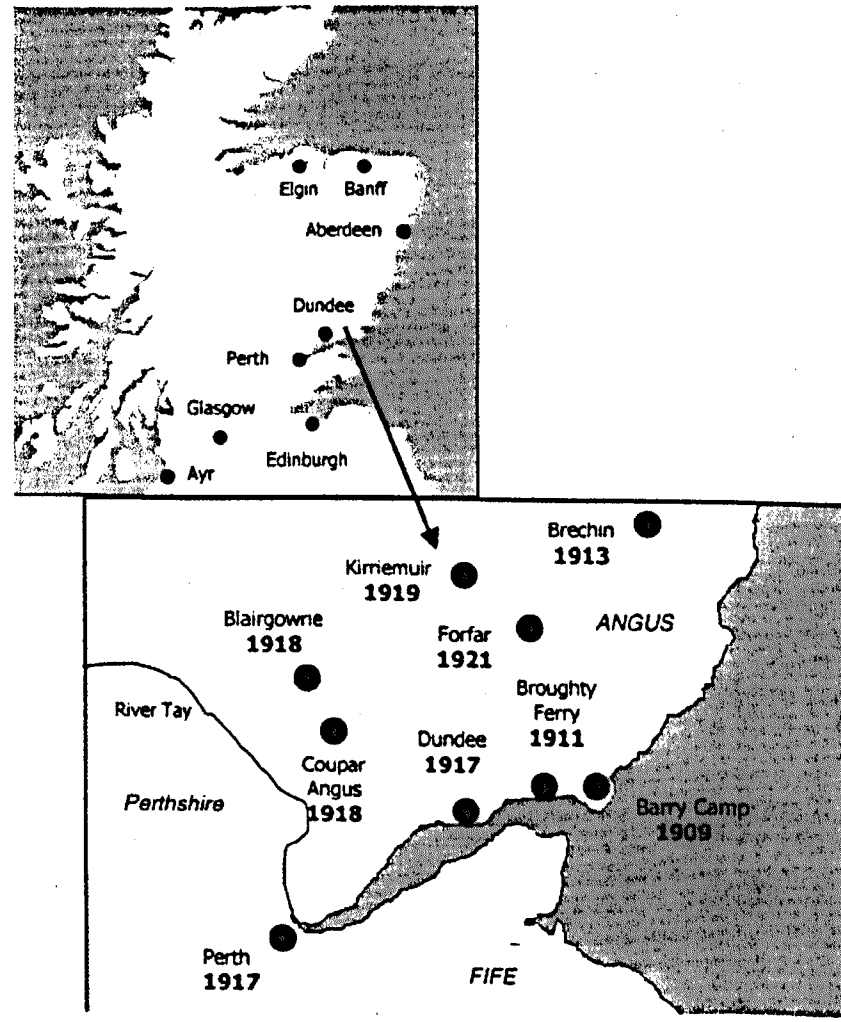

FIG 1: Origin and spread of grass sickness in Scotland (fatal cases). (After Tocher and others 1923.) Maps not to scale

Begg (1936) reported the occurrence of grass sickness in Hampshire, and Wylie (1936) reported a case in Essex. During October and November 1939 two suspected cases were reported in Wiltshire and a third in Hampshire (Wallace 1940). A three-year-old shire mare from the Midlands was diagnosed clinically with grass sickness in May 1941, and it was thought that in the same part of the country the deaths of two horses with acute abdominal pain might have been due to the disease (Lee 1941). There was further evidence of the spread of the disease in 1945, with the report of two cases in Northamptonshire (Ellis and Cooper 1945).

Three cases occurred on the same premises in Leigh, Lancashire in 1963 (Greer and Bardgett 1963), but in the same report it was mentioned that the staff at the University of Liverpool veterinary field station had seen only two other cases in the previous 10 years. In 1971, two cases were reported at a thoroughbred stud in Surrey (Limont 1971).

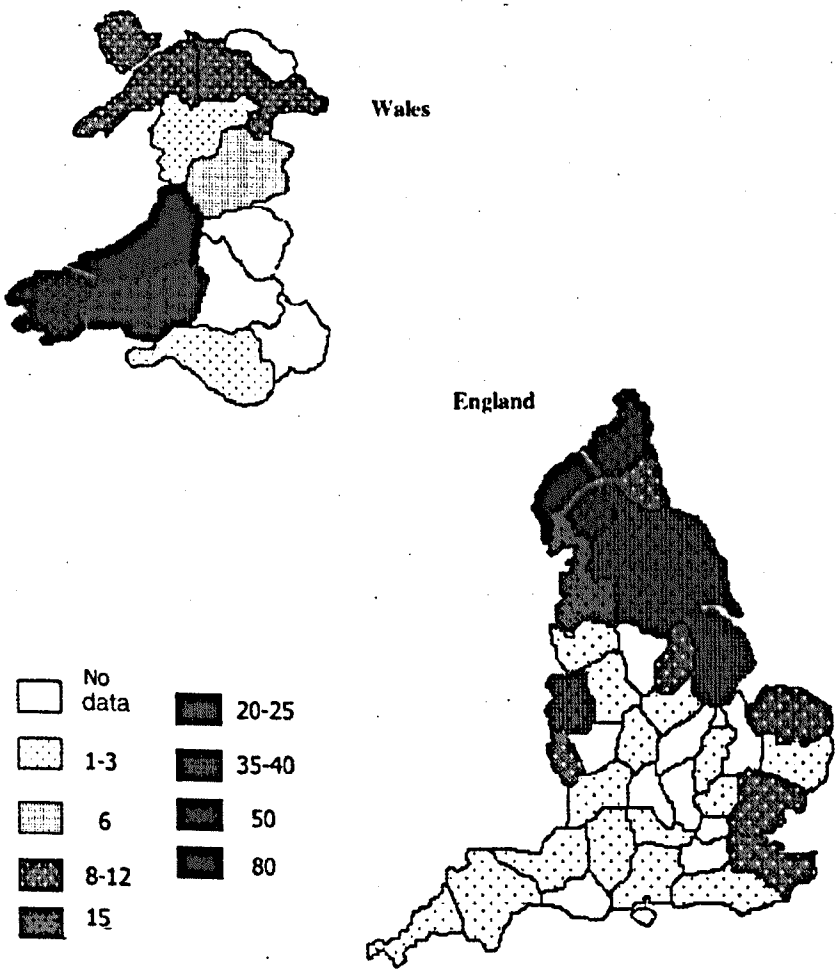

FIG 2: Approximate annual numbers of cases of grass sickness in England and Wales (pre-1974 county boundaries). (After Guthrie 1940.) Maps are not to scale

TABLE 1: First records of grass sickness in England and Wales as reported by Guthrie (1940)

County (pre-1974 county boundaries)	Place and year disease first recognised
Cumberland	Brampton, 1915
Northumberland	Alnwick, 1915
Westmoreland (Cumbria)	Kendal, 1935
Pembroke	Pembroke, 1925
North Riding (Yorkshire)	Harem, 1915
Shropshire	Severn Valley, 1930
Carmarthen	Kidwelly and Pembrey, 1897
West Riding (Yorkshire)	Blackburn, 1900
Lancashire	Liverpool, 1919
Kent	Eltham, 1915
Denbighshire	Denbigh, 1939
Caernarfon and Anglesey	Anglesey, 1936

Wales

Grass sickness is believed to have occurred first in Wales in 1897 (Guthrie 1940). However, other reports claim that the disease did not occur until the early 1920s (Lloyd 1934, Thomas 1943). The first case apparently occurred at St Dogmeals, Pembrokeshire, in 1922 and grass sickness was also reported in Carmarthenshire in 1928 (Lloyd 1934). In 1934, the disease occurred almost exclusively to the south of the river Teifi, and it was most prevalent in the St Dogmeals, Moylgrove and Eglwyswrw districts. By 1940, grass sickness was described as 'rife' in south-west Wales, and it was widespread throughout the counties of Carmarthen, Cardigan and Pembrokeshire (Guthrie 1940, Thomas 1943). It was reported that acute cases were more common in the spring with chronic cases being more common in autumn. On one occasion six cases were witnessed on one farm during one visit (Thomas 1943).

Northern Ireland

In June 1941, a three-year-old gelding from Belfast was diagnosed clinically with the chronic form of the disease. The horse was diagnosed by a veterinary surgeon who had just returned from Scotland where he had seen a number of cases of the disease (McFarlane 1941). A tentative diagnosis of grass sickness was made on a second horse in Northern Ireland in the same year (McColl 1941).

Ireland

Grass sickness is widely believed not to occur in Ireland but there is evidence to suggest that it has an extremely low incidence. Mullaney (1957) described a six-year-old hunter with clinical signs and postmortem findings that resembled grass sickness, and a tentative diagnosis of grass sickness was made on the basis of the clinical signs and the gross pathology.

Mainland Europe

Grass sickness is well recognised in Sweden and Denmark (Bendixen 1946, Obel 1955, Lannek and others 1961). The disease has also been reported in Germany (Mayer and Valder 1968, Schulze and others 1997), France (Lhomme and others 1996), Switzerland (Arnold and others 1981), Holland (Leendertse 1993) and Belgium (Christmann and others 1999).

Worldwide

A single case of grass sickness has been confirmed histologically in the Falkland Islands (Woods and Gilmour 1991) and also in Australia (Stewart 1977). A condition indistinguishable from grass sickness known as mal seco is recognised in Argentina, the Falklands and Chile. Both the clinical signs and postmortem findings of mal seco are similar to those of grass sickness, and the histological lesions in horses with mal seco are also very similar to those described in the coeliacomesenteric ganglia of horses with grass sickness; it has been suggested that mal seco and grass sickness are the same disease (Uzal and others 1992).

PUTATIVE AETIOLOGICAL AGENTS

Plants

In 1918, there was an attempt to identify plants, and particularly clovers, that might have a toxic effect on horses. A survey was made of the infected areas in Forfarthshire and Perthshire and a search for the following plants all proved negative; tormentil (*Potentilla tormentilla*), hemlock (*Conium maculatum*), wild chervil (*Chaerophyllum sylvestre*), rough chervil (*Chaerophyllum taenulum*) and stinking mayweed (*Matricaria suavolens*) (Guthrie 1940). Experiments were also conducted to test the toxicity of alsike clover (*Trifolium hybridum*). Two horses were grazed for an entire season on two acres of land sown with alsike clover, and alcohol extracts of the plant were fed to a four-year-old horse; no ill effects were observed (Tocher and others 1923).

Bacteria

***Clostridium botulinum* (*Bacillus botulinus*)** In 1919, a series of postmortem examinations of horses that had died from grass sickness suggested they had succumbed to an acute toxaemia of bacterial origin (Tocher and others 1923). A large anaerobic bacillus grown from portions of the

stomach and intestines had the morphological character and toxigenic properties of *Bacillus botulinus*. Horses were injected with *B botulinus* toxin obtained from culture (Buxton 1923, Tocher and others 1923), and the horses died after developing dysphagia, becoming recumbent and showing evidence of cardiac and respiratory failure. The postmortem findings were similar to those observed in horses that had died of grass sickness. It was concluded that the bacillus and its toxin produced lesions that were identical to those of horses with grass sickness (Buxton 1923, Tocher and others 1923). In 1921, a therapeutic antitoxin was produced from known strains of *B botulinus*. In horses which did not recover, the acute signs of the disease were ameliorated by the intravenous administration of the antitoxin (Buxton 1922). A vaccination trial was conducted with a toxin/antitoxin mixture in 1316 horses (Tocher and others 1923); 961 horses were immunised and 355 were left as untreated controls. The reduction in mortality of 6·5 per cent was significant ($P<0.0001$) (after Tocher and others 1923).

In 1924, the *B botulinus* theory came under intense criticism. The bacillus isolated from the spleens of grass sickness cases had not been shown by scientific methods to be identical to *B botulinus*. The anti-botulinus serum was not made from the grass sickness organism and some researchers felt that immunisation did not make any appreciable difference to mortality. Finally, an article in *The Lancet* concluded that there was no association between grass disease and botulism (Anon 1927).

In an attempt to resolve the issue, Walker (1929) stomach-tubed ponies with varying doses of cultures of either type A or type B toxin of *B botulinus*. He reported that the clinical signs and postmortem findings produced by botulism in horses were quite different from those produced by grass sickness.

More recently, Poxton and others (1997) investigated the role of *C botulinum* (type C) in grass sickness. Serum from grass sickness cases and control horses contained antibodies to the C1 toxin and to the cell-surface antigens, but no clear relationship was found between the disease and the antibody levels. Hunter and others (1999) demonstrated that *C botulinum* (type C) toxin was present in the ileum and faeces of horses with grass sickness.

Clostridium perfringens Ochoa and de Velandia (1978) investigated the role of *C perfringens* (type A) in recovered cases of grass sickness in Columbia. The horses, which had been diagnosed by clinical signs only, had higher serum neutralisation titres than horses that had no record of having the disease. They concluded that enterotoxic *C perfringens* (type A) is involved in clinical cases of grass sickness either as an aetiological agent or as a complicating organism. In contrast, Gilmour and others (1981) found a negative serological association between cases of grass sickness in Scotland and *C perfringens* (type A). The sera from acute and chronic cases of the disease failed to neutralise either crude or partially purified enterotoxin.

Insect vectors

In 1934, it was noticed that some of the areas with large populations of the springtail (*Sminthurus viridis*) were also affected by grass sickness. However, feeding springtails and other insects to horses failed to produce any definite signs of grass sickness (Lloyd 1934).

An extensive survey of the insect populations of infective and non-infective pastures in south Cardiganshire and Pembrokeshire failed to establish any relationship between the pastures and their insect populations (Anon 1936).

Fungi

A variety of non-endophytic, toxin-producing fungi have been isolated from the stomach, colon and rectum of healthy horses and horses with grass sickness (Doxey and others 1990). The survey did not associate any particular fungal species with grass sickness.

Robb (1996) investigated three groups of fungi (*Fusarium* species, endophytic fungi and *Penicillium* species) which are toxigenic and grow at the base of plants. Endophytic fungi were identified in less than 20 per cent of the plants examined on grass sickness premises and no *Penicillium* species were identified. The rarity of these organisms in fields where grass sickness has occurred make it unlikely that they were involved (Robb 1996). However, *Fusarium* species were found on all the samples examined but no conclusions could be drawn about their effect on nerve cells (John 1996, Robb 1996). Uzal and others (1996) failed to reproduce mal seco by feeding horses with *Festuca argentina* (a plant native to Argentina) which was infected by an endophytic fungus.

EPIDEMIOLOGICAL INVESTIGATIONS

The failure to reproduce the disease experimentally by feeding horses with infectious or poisonous materials has made it necessary to look elsewhere for an explanation for grass sickness (McKay 1958). The identification of predisposing causes of grass sickness may be assisted by identifying horses and premises that are at a higher than normal risk of the disease.

Since the 1930s, several epidemiological investigations have attempted to identify risk factors for grass sickness (Table 2). The risk factors that have been investigated are summarised in Table 3. In the following description, P values are given if there is a statistically significant relationship between the factor and the disease at a 5 per cent level. Odds ratios (OR) and 95 per cent confidence intervals (CI) are also cited if available.

TABLE 2: Epidemiological publications on equine grass sickness, 1937-1998

Year of Publication	Authors(s)	Country of study	Year(s) of study	Data collection method	Number of Cases	Number of Controls
1958	McKay	Scotland	1936	Interview questionnaire	Not given	None
1940	Guthrie	UK	1936-1939	Observations	Not given	None
1942	Greig	Scotland	1938-1940	Observations	Not given	None
1970	VCOU	Scotland	1968-1969	Various	105	None
1974*	Gilmour and Jolly	Scotland	1971	Interview questionnaire	90	2554
1991a*	Doxey and others	Scotland	1972-1989	Meteorological office premises	15	0
1991b*	Doxey and others	Scotland	1970-1987	Interview and postal questionnaire	60	1575
1992*	Barrett and others	Wales	1998	Telephone questionnaire	17	67
1998*	Wood and others	UK	1992-1995	Postal questionnaire	135	226

* Statistical analyses used
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TABLE 3: Epidemiological observations in grass sickness research

Level	Variable	Risk factor	Reference(s)*
Horse	Age	Young horses	VCOU (1970), Greig (1942), Gilmour and Jolly (1974), Barrett and others (1992), Doxey and others (1991a), Wood and others (1998)
	Sex	No consistent findings	Greig (1942), Wood and others (1998)
	Breed	No consistent findings	Wood and others (1998), Doxey and others (1991a)
	Body condition	No consistent findings	Wood and others (1998), Doxey (1998), Guthrie (1940), Greig (1942), McKay (1958), Gilmour and Jolly (1974)
	Grazing	Horses kept solely outdoors	Doxey and others (1991a), Wood and others (1998)
	Time on premises	Horses that have recently moved premises	Gilmour and Jolly (1974), Wood and others (1998), Greig (1942), McKay (1958)
	Time on pasture	Horses that have recently moved pasture	Gilmour and Jolly (1974), Wood and others (1998)
	Supplementary feeding	No consistent findings	Gilmour and Jolly (1974), Wood and others (1998)
	Change of diet	No consistent findings	Begg (1936), McKay (1958)
	Anthelmintic administration	No consistent findings	Guthrie (1940), Wood and others (1997, 1998)
Pasture		Pasture previously associated with the disease	Doxey and others (1991a), Wood and others (1998)
	Pasture type	No consistent findings	Guthrie (1940)
	Soil conditions	No consistent findings	Stewart (1941)
Environment	Season	Spring	VCOU (1970), Begg (1936), Guthrie (1940), Greig (1942), Doxey and others (1991b), Wood and others (1998)
	Weather conditions	No consistent findings	VCOU (1970), Pool (1928), Guthrie (1940), Greig (1942), McKay (1958), Doxey and others (1991b), Wood and others (1998)

* Gilmour and Jolly (1974), Doxey and others (1991a, b) and Wood and others (1998) are the only authors to cite statistical analyses of their results
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Other equids

Dysautonomias with a similar histopathology to grass sickness have been reported in equids other than the horse. At Whipsnade zoo in 1977 it was reported that acute grass sickness occurred in a Przewalski's horse, and chronic grass sickness in a common zebra (Ashton and others 1977). The disease had previously been reported only in the horse, pony and donkey. The histological examination of the autonomic ganglia from both cases revealed lesions consistent with a diagnosis of grass sickness (Ashton and others 1977).

Breed

Anecdotal reports suggest that heavy, draught horses are at a higher risk of grass sickness. In a univariate analysis of a case-control study, heavy horses were found to be at higher risk (OR=5·0, P=0·03) than all other breeds (Wood and others 1998). However, after adjusting for confounding by other variables, the relationship between breed and grass sickness was no longer significant.

Age

Age was one of the first risk factors to be identified. Guthrie (1940) found that the disease was most prevalent in four year olds, and Greig (1942) recorded that the prevalence of the disease was very high between the ages of three and six years. In 1939 and 1940, the percentage mortality and morbidity of horses of different ages were calculated by using data from the National Farmers' Union Insurance Society (Grieg 1942). In total, 6617 horses were insured, of which 3·49 per cent had died of grass sickness; mortality was highest (7·29 per cent) in four-year-old horses.

More recent investigations have also shown that younger animals are more likely to be affected by grass sickness than older animals (Gilmour and Jolly 1974, Doxey and others 1991a, Wood and others 1998). Gilmour and Jolly (1974) reported that grass sickness occurs more commonly among horses between the ages of two and seven years than among younger or older groups of horses.

In a telephone-based case-control study, Barrett and others (1992) reported that horses with grass sickness were significantly younger than horses with other types of fatal colic (P<0·02); all the grass sickness cases were between two and 12 years old. Wood and others (1998) reported that horses between three and five years of age were nearly six times more likely to contract the disease (OR=5·7) than horses which were 10 years old or older. Horses which were less than three years old had almost a five times higher risk (OR=4·8) and horses aged between six and nine had a three times higher risk (OR=3·1). After adjusting for the confounding effects of sex, time since grass sickness had last occurred on the premises, contact with previous grass sickness and time since a change of pasture, the relationship between age and disease was still significant. Horses between the ages of three and five years were at the greatest risk of grass sickness (OR=7·1, P<0·001, 95 per cent CI 3·1 to 16·3).

It has been suggested that older horses become resistant to grass sickness. The longer they spend in a field, and the older they get, the less likely they are to be affected (Wood and others 1998). It is possible that a non-fatal exposure to the causative agent of grass sickness induces resistance to the disease.

Grass sickness is rarely reported in foals and horses less than one year of age. The reasons for this are unknown and the fact is rarely referred to in the literature. In three publications in which precise age data are given on 212 cases of grass sickness, only 6 per cent were less than two years old (Veterinary Clinical Observation Unit [VCOU] 1970, Gilmour and Jolly 1974, Barrett and others 1992).

Pasture

As the name suggests, grass sickness is strongly associated with access to grass. Horses kept solely on pasture are at greater risk of developing the disease than those that are stabled for part of the day (Gilmour and Jolly 1974). There have been rare exceptions when entirely stable-kept horses have suffered the disease. Lannek and others (1961) reported seven horses dying of grass sickness in two stable yards in Sweden, and in 1927 the disease was reported in pit ponies that received no grass at all (Forsyth 1941).

Guthrie (1940) observed that in Scotland most cases occurred on temporary pastures, especially those which were two or three years old. However, in England and Wales, more than 90 per cent of cases have been reported on permanent pastures. More recent studies have reported that there is no

significant association between the nature of the pasture and grass sickness (Gilmour and Jolly 1974, Doxey and others 1991a). However, the disease is more likely to occur on previously affected pastures than on pastures where grass sickness has never been recorded (Gilmour and Jolly 1974, Wood and others 1998).

Low levels of pasture selenium have been implicated in grass sickness. Macpherson (1978) reported that the majority of grass sickness cases appeared on farms where muscular dystrophy in cows was a problem, and that blood selenium concentration was low in horses suffering from grass sickness. However, blood selenium levels were also low in apparently healthy horses on the same farms, thus providing little evidence to support the theory that selenium deficiency was associated with the disease.

Change of pasture or premises

It has frequently been observed that horses suffer from grass sickness shortly after moving from one pasture to another. Greig (1942) described 15 horses that had succumbed to grass sickness after changing pastures, and observed that 10 of them had changed pasture in the preceding two days.

Doxey and others (1991a) found that 45 per cent of the cases in their study had moved from either one premises or one field to another in the two weeks before they became ill. Only 3 per cent of the horses in the control population moved regularly to fresh pasture at less than four-week intervals.

Wood and others (1998) found that horses which had changed pasture in the two weeks before succumbing to the disease had a 17-fold higher risk than horses that had changed pasture in the last three months ($P=0{\cdot}021$, OR=17·4). There was a five-fold increase in risk if the horse had changed pasture within the last two to four weeks ($P=0{\cdot}004$, OR=5·3) and nearly a three-fold increase if the change had been made in the last month (OR=2·6). After adjusting for the confounding effects of sex, the time since grass sickness had last occurred on the premises, contact with previous grass sickness, and age, the relationship between the change of pasture and the disease was still significant. Horses that had changed pastures within the previous two weeks were at greatest risk of the disease (OR=29·7, $P<0{\cdot}001$, 95 per cent CI 6·7 to 130).

Diet

Gilmour and Jolly (1974) recorded that a higher proportion of the horses which experienced grass sickness were receiving either no supplement or only concentrates than of those receiving hay and concentrates. This difference could potentially be confounded by how the animals are kept, because most horses which receive only concentrates are kept outdoors whereas most of those receiving hay and concentrates are stabled. Wood and others (1998) found no evidence that the supplementary feeding of hay or forage alone was associated with a decreased risk of the disease.

A change of diet has been implicated in the onset of grass sickness. Begg (1936) stated that the disease was always associated with a change of diet. McKay (1958) suggested that winter feed might affect a horse's susceptibility to the disease owing to the lower levels of protein in winter feedstuffs compared with young grass.

Body condition

Doxey and others (1991a) found that horses in good body condition were more likely to contract grass sickness ($P<0{\cdot}005$). However, work by Wood and others (1998) did not support these findings.

Anthelmintics

The possibility that the administration of anthelmintics is related to the risk of disease requires further investigation. Preliminary work by Wood and others (1997) showed that the more frequently horses were given anthelmintics, the more likely they were to be affected by grass sickness (OR=2·0, $P=0{\cdot}002$, 95 per cent CI 1·1 to 3·8). However, this finding was not confirmed by multivariate analysis on the full dataset (Wood and others 1998). It has been suggested that the frequent removal of worms might interfere with gut function in some way that increases the absorption of a neurotoxin (Milne 1997).

Soil

Stewart (1941) studied soil samples taken from 63 farms within a few days of cases of grass sickness having occurred during the summer of 1938 in Aberdeenshire. No control soil samples were taken. The results showed that (i) grass sickness was not confined to soils of any particular geological origin, (ii) there was no relationship between the soil texture and the incidence of grass sickness, (iii) almost all the soils were acid (pH 5·5 to 6·2), (iv) there was no relationship between grass sickness and the soil levels of magnesium, manganese or potassium, and (v) the average values for carbon and nitrogen and for carbon:nitrogen ratios in the soils were higher than those recorded in cultivated soils in south-east England. The wide range in values makes the possibility of a relationship between the occurrence of grass sickness and the carbon:nitrogen ratio unlikely (Stewart 1941).

Season

It has consistently been reported that there is a seasonal variation in the incidence of grass sickness. Begg (1936) stated that 'the disease makes its first appearance for the season towards the last week in April, just as the grass is beginning to show the first signs of growth and when the weather is getting milder. However, if April is particularly warm following a wet March, then cases appear early and are greater in number. It is noticeable that a spell of warm weather following a wet period brings a large number of cases'. Guthrie (1940) reported that the great majority of cases occur in June, with the peak period in the early part of the month, although cases can occur all year round. The VCOU (1970), Doxey and others (1991b) and Wood and others (1998) have all reported May as having the largest number of cases.

Weather

At present there is no good evidence that meteorological factors are important in the occurrence of grass sickness. Pool (1928) observed cases in fine, dry, warm weather and suggested that frosty nights were a predisposing factor. Guthrie (1940), Greig (1942) and McKay (1958) have suggested that cases occurred in warm, dry, fine weather and that cold and wet conditions decreased the incidence. Early morning ground frosts were also implicated in an increased incidence of the disease. Greig (1942) reported the occasional case during periods of hard frost and even when the ground was covered in snow. Doxey and others (1991b) investigated local weather patterns associated with 15 premises with multiple cases of grass sickness in eastern Scotland between 1972 and 1988. The records of the meteorological stations closest to the affected premises were examined for the two weeks before an outbreak. On all 15 premises the mean daily air temperature was below 6°C on only 36 occasions (17 per cent) and the temperature exceeded 20°C on only 11 days. There was often ground frost, and 85 per cent of the pastures had grass temperatures lower than the previous 10-year average; 73 per cent of the premises had lower than average daily rainfall within two weeks of the first case. The conclusion drawn was that the majority of outbreaks occurred during cooler, drier weather (temperatures between 7 and 11°C) associated with irregular ground frosts. No data from control premises were collected.

It has been suggested that weather conditions influence an east/west split in the prevalence of grass sickness in Scotland, with eastern Scotland apparently having a higher prevalence of the disease. The weather pattern for eastern Scotland, compared to the west, shows a trend towards slightly cooler, drier weather with frosty mornings (Doxey and others 1991b).

Association of grass sickness with other animal diseases

Barrett and others (1992) investigated the possible association between grass sickness and scrapie. A telephone-based case-control study of fatal equine colics was made in Wales in 1988. Of a total of 84 fatal colics, 17 were due to grass sickness, and the other 67 were used as controls. It was concluded that there was no significant association between grass sickness and scrapie (P=0·06).

CONCLUSION

Several experimental studies have failed to identify the causal agent(s) of grass sickness. However, epidemiological studies have provided information that has helped a fuller understanding of the disease.

Several risk factors for grass sickness have been identified. Young horses with access to grass that have recently moved to new pasture or premises are at greatest risk of the disease, especially during the months of April, May and June. The disease is more likely to occur on premises which have previously experienced the disease. A better understanding of the risk factors associated with grass sickness is needed to help horse owners and veterinary surgeons to take measures to prevent the disease.

Case-control studies have been successful in identifying the factors that may prevent or cause the disease. This method involves the comparison of cases with control horses which are free of the disease. The comparison reveals exposures that may differ between the two groups and may explain the occurrence of the disease. Risk factors may either increase or decrease the risk of the disease. A case-control study can assess the change in risk associated with each factor either individually or in combination (Schlesselman 1982).

More focused epidemiological studies are needed to define more precisely the horses and premises that are at greatest risk, so that better advice on how to avoid the disease can be given. Studies of the interactions between recognised risk factors may shed further light on the causal pathway of the disease. This review has identified gaps in the knowledge that need further investigation. For example, more information is required about the local weather patterns that precede an outbreak of grass sickness, and about the factors in the pasture or soil that put premises at higher risk.

Perhaps the most important feature of epidemiology in the study of grass sickness is its ability to deal with multiple risk factors. The complex nature of the disease makes it unlikely that a single causal organism is responsible. On the basis of the studies described above, it is more likely that there is a complex causal pathway involving multiple factors. Epidemiology can describe and quantify these factors, and it may be possible in the future to develop mathematical models of the disease.

This review has highlighted the fact that grass sickness is not just a Scottish disease but one that occurs in England and Wales. There is scope to examine the spatial and temporal patterns of the disease by mathematical modelling techniques. The clustering of disease outbreaks in space, location or time may provide clues to its aetiology and can provide evidence of contagion or an underlying spatially and temporally localised process (Diggle and others 1995). Such an analysis may make it possible to predict and prevent outbreaks of the disease more successfully.

Future epidemiological studies should also investigate immunological and genetic risk factors. There is scope to examine the importance of the major histocompatibility complex in the susceptibility of horses to grass sickness. Population-based research is needed to study the possible involvement of major genes, and to determine whether certain breeding lines carry an increased risk of the disease.

Epidemiology has greatly assisted our current understanding of equine grass sickness, and further studies should make significant contributions towards the elucidation of a causal pathway. However, until this pathway is fully understood, epidemiology can help veterinary surgeons and horse owners to prevent the disease.

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