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Exploration of the evidence supporting and opposing the role of *Clostridium botulinum* in the pathogenesis of Equine Grass Sickness

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Budapest, Hungary 2020

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1. Abbreviations

AGS: Acute grass sickness

BoNT/A: Botulism neurotoxin type A

BoNT/C: Botulism neurotoxin type C

BoNT/D: Botulism neurotoxin type D

CCG: Cranial cervical ganglion

CFV: Cresyl fast violet

CGS: Chronic grass sickness

C. botulinum: Clostridium botulinum

C. novyi: Clostridium novyi

C. perfringens: Clostridium perfringens

C. difficile: Clostridium difficile

DSI: Distended small intestines

EGS: Equine Grass Sickness

GI: Gastrointestinal

HE: Haematoxylin and eosin

MC: Matched controls

MGG: May Grunwald Giemsa

OTU: operational taxonomical unit

PCR: Polymerase chain reaction

SGS: Subacute grass sickness

SNAP: Synaptosomal-associated 25

SNARE: Soluble N-ethylmaleimide sensitive fusion attachment receptor

Syb: Synaptobrevin

Syn: Syntaxin

TetSA: *Clostridium tetani* surface antigen

UK: United Kingdom

2. Introduction

Equine Grass Sickness (EGS) also known as Equine dys-autonomia, is a polyneuropathy disease, causing neurodegeneration of the autonomic nervous system, most commonly resulting in fatality. It primarily effects the gastrointestinal tract with evidence suggesting that the ileum is the main location of damage.

Equine grass sickness was first described in eastern Scotland in 1904, most commonly occurring in Great Britain, with the exception of some other countries in western Europe, such as Sweden, Denmark and Germany, where the disease is well recognised. Fewer cases have been seen in Ireland, France, Italy, Belgium, Holland, Norway and Finland (McGorum et al., 2006). There was notably an outbreak in Hungary in 2001 (Schwarz et al., 2012). An identical disease has been described as 'Mal Secco' in Argentina, the Falkland's, Columbia and Chile (Robles et al., 1993). Equine grass sickness still currently is thought to be the cause of death of up to 2.3% of equine deaths in the United Kingdom (Newton et al., 2004; Ireland et al., 2011).

EGS presents clinically as acute, subacute and chronic, and shows clinical and histopathological evidence of disruption of the automated nervous system, particularly in the gastrointestinal tract (Obel, 1955; Barlow, 1969; Scholes et al., 1993; Doxey et al., 2000; John et al., 2001). Most cases result in death although horses with mild chronic form may be nursed to health, although rarely return to their normal working life (Doxey et al., 1995).

Equine grass sickness appears to be more prevalent in horses who spend an increased amount of time grazing, hence the name 'grass sickness'. Research shows that almost all cases of EGS occur in horses that are grazing full time, or at least part of the day (McGorum et al., 2006), with the occasional case being reported in horses that are kept away from grass (Pirie et al., 2014). Other risk factors include the soil type, pasture disturbance, dietary change and body condition etc. Some other examples can be seen from table 1 (Wylie et al., 2009). With regards to age, there is a peak incidence of cases which occur between the ages of 2-7 (Pirie et al., 2014). Foals do not develop EGS, with some suggesting there may be to some degree, maternal protection from the colostrum. There appears to be less cases in older horses which again may suggest a resistance can develop over time. This became evident during studies

examining the antibody levels in affected animals, and horses that had grazed on high risk pastures (Hunter et al., 2001).

There is no strong evidence that EGS is more prevalent in certain breeds of horses, but some suggest Clydesdales could be more susceptible, and on the other hand Shetlands and thoroughbred may have a higher resistance; but recently this was disproved, when research showed cases of EGS are in correlation with population density of these breeds (McGorum et al., 2006)

The cause for equine grass sickness is still unknown, with the most widespread theory being of *Clostridium botulinum* type C, which was first described in 1923, where circumstantial evidence was demonstrated supporting the theory (Tocher et al., 1923). This included detection of the toxin from clinical samples during post mortem examinations, the presence of IgG antibodies in the serum and mucosal IgA antibodies. Although there is a lot of strong evidence pointing towards *C. botulinum* playing a role in equine grass sickness's pathogenesis, many experiments have presented results that contradict the theory, or provide no valid evidence supporting the hypothesis. This includes examinations of histological lesions, and more recently the very disappointing vaccine trial results.

It is thought that EGS is a toxico-infectious form of botulism (Hunter et al., 1999). This is considered to be similar to visceral botulism as seen in cattle (Bohnel et al., 2001). This is where the botulism toxin is produced in the intestines, affecting the autonomic nervous system of these internal organs directly, eliminating the evidence of flaccid paralysis of the locomotor muscles, as exhibited in classic botulism. This could explain some of the differences in the clinical signs and the post mortem lesions between EGS and botulism.

Clostridium botulinum produces neurotoxins A-G. A, B and E are capable of causing disease in humans, and A and B are often used as a human treatment for muscle spasms. Types C and D can cause disease in animals. Each toxin affects different SNARE proteins, which play a main role in the mediation and fusion of vesicles in the pre-synaptic neurons. Types A, C and E, cleave Synaptobrevin (syb), type C cleaves Synaxin (syn) and types B, D, F and G cleave SNAP- protein.

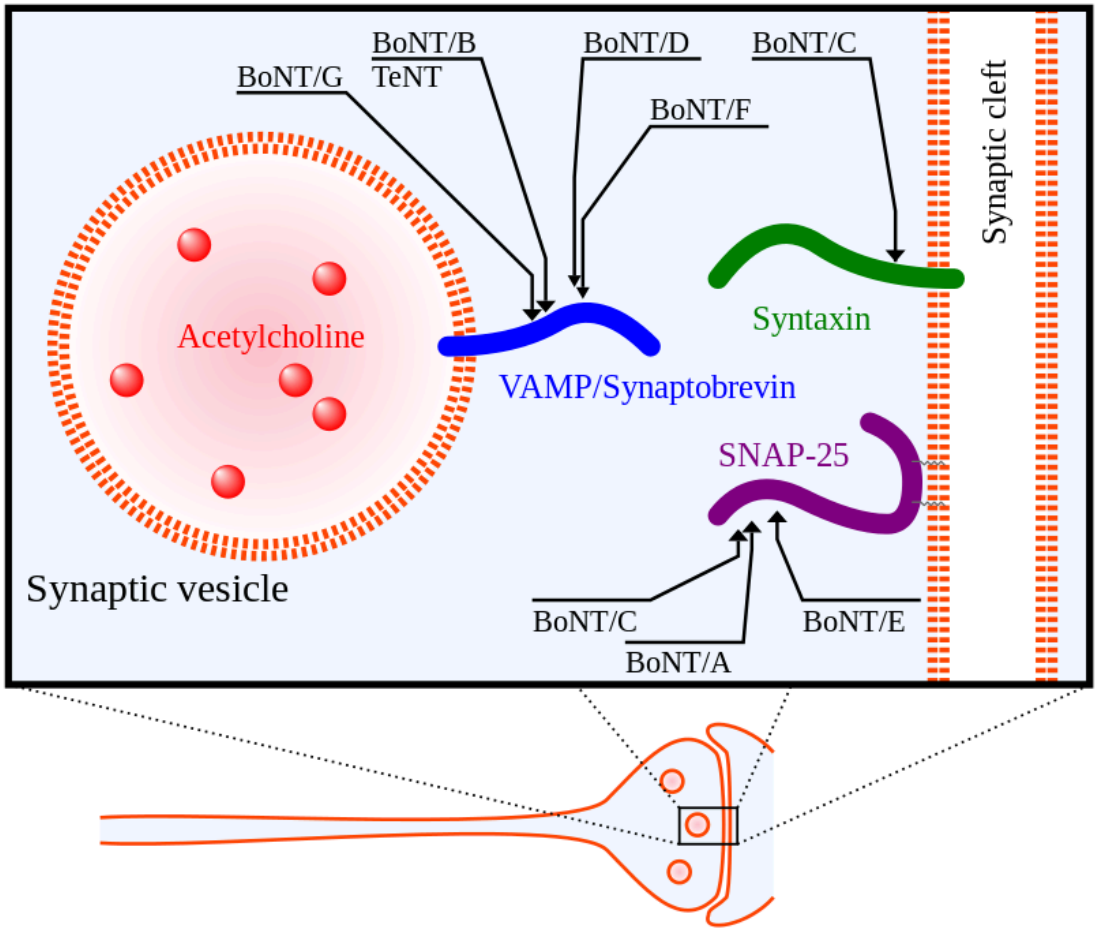

Figure 1: Target molecules of the botulinum neurotoxin (abbreviated BoNT) and tetanus neurotoxin (TeNT), toxins are acting inside the axon terminal (Barr et al., 2005).

These SNARE proteins are now unable to mediate the vesicles in the nerve terminals to move to the intracellular membrane, which in turn, prevents the release of Acetyl-choline neurotransmitter (Dressler et al., 2005). This is shown in figure 1. It is thought that these effects are more localised to the gastrointestinal tract due to the toxin being produced in the gut.

Throughout this thesis I will outline the experiments that concluded evidence over the last 100 years, that support and oppose the *C. botulinum* theory in the pathogenesis of equine grass sickness.

3. Aims and Goals

The overarching goal of this paper is to examine the theory that *C. botulinum* plays a major role in the pathogenesis of Equine Grass Sickness. The theory was first described by Tocher et al. in 1923 when he isolated a bacterium that resembled *Bacillus botulinum* as it was then known (now *Clostridium botulinum*), from the gastrointestinal tract of horses suffering from EGS (Tocher et al., 1923).

I plan to look at the evidence established by experiments undertaken by veterinarians and other professionals in the field, ranging over the last one hundred years, in a hope to illustrate the evidence that supports the theory and opposes the theory, thus summarising where we stand in determining the cause of equine grass sickness, and if the *C. botulinum* theory still presents as a valid hypothesis.

4. Materials and Methods

To acquire the evidence for this thesis, I used online research sites such as PubChem, acedmia.edu as well as the online college library, to find access to online papers and articles from journals.

I also got in contact with some equine hospitals in the UK, asking them for any case studies of EGS that they would be willing to share with me. Cinderhill equine clinic in the South of England, contacted me with a compiled document of all their cases over the last 20 years, after removing the owner's names to allow the cases to remain confidential. This included information on the horses, such as age of the horses, diet, body score condition, gender, breed, as well as clinical signs which they presented, laboratory results and post mortem lesions if examined. Although this data was interesting, and validated the certain risk factors associated with the disease (Table 1), it unfortunately did not provide any usable information regarding *Clostridium botulinum* as a cause for EGS.

I have also been in contact with the equine grass sickness fund in the UK, where I was given further access to more papers and research studies.

5. Results and Discussion

The most researched and debated theory for the cause of EGS, is a toxico-infection of *Clostridium botulinum*, a soil born bacteria, where the bacteria's spores are produced in the gut of the infected animal, as opposed to a consumption of spores which were produced in the environment (Hunter et al., 1999). The *C. botulinum* theory draws comparisons to the botulism disease as a whole.

In general, *Clostridia* species of bacteria are large, spore forming, anaerobic and gram positive bacteria, who's natural habitats include the soil, dust and the gastrointestinal tract of humans and animals (Bush et al., 2019). They have the highest production of exotoxins of any other bacterial species, and their spores are extremely resistant (Auwaerter et al., 2019). *C. botulinum* may infect the animal in one of three ways. The first being described as 'classic botulism', an infection via the ingestion of already formed toxin. The second 'wound botulism', which, as the name suggested, direct contact of the bacterium or spores through a wound. The third, a toxico-infection, where the toxin is produced locally in the gastrointestinal (GI) tract, after ingestion of the bacteria itself (Wylie et al., 2009).

In horses, the botulism disease occurs more often in foals around 2-5 weeks old, and is also known as 'Shaker foal syndrome". Shaker foal syndrome infects via a toxico-infection, but presents similar to botulism in other animals, with as the name suggests clinical signs surrounding muscle tremors and flaccid paralysis (Thomas et al., 1988).

EGS is thought to be a combination of a toxico-infection of *C. botulinum*, along with one or more risk factors which are outlined in table 1 (Wylie et al., 2009). Evidence also points towards low antibody levels of *C. botulinum* possibly increasing the risk of EGS, which in turn supports the theory that EGS is caused by a form of *C. botulinum* infection.

During this discussion I will examine the evidence that both supports and opposes the theory that *C. botulinum* plays a role in equine grass sickness. I will outline the basic methods used in the experiment, the results, and how they relate to the theory, thus drawing a conclusion about how reliable the *C. botulinum* theory is.

Manageable risk factors (and references) for equine grass sickness

Risk Factor	Findings
Access to grazing	Part-stabling results in EGS occurring almost as infrequently as in horses with no access to fresh grass.[40]
Grazing type	Horses grazing on sand or loam soil types are at an increased risk for EGS, whereas horses grazing on chalk soils are at decreased risk.[68] Soils high in nitrogen have also been found to be a risk for EGS.[69]
Previously affected premises/paddocks	EGS tends to recur on previously affected grazing,[40,42,68,69] with the incidence on previously affected premises estimates at two cases per 100 horse-years at risk.[68] There is evidence of space-time clustering with cases occurring more frequently within 20 days of the last case.[44] Cases are most likely to occur within 2 years of a previous case.[40,42]
Pasture disturbance	Recently disturbed pasture (construction work or mole activity) increases the risk for EGS development threefold.[69] One study found that the use of machinery on the pasture (mechanical feces removal, harrowing) increased the risk for recurrence, possibly because of pasture disturbance.[68]
Premises type	Stud farms and livery yards are at increased risk, possibly associated with increased horse numbers.[68]
Pasture feces removal	In one study the mechanical removal of feces was found to increase the risk for recurrence, whereas manual removal of feces was protective.[68] In the same study grass cutting was found to be protective, and it was postulated these features may be reflective of reduced overgrazing.[68]
Other grazing stock	In one study on EGS recurrence on previously affected premises the risk increased with the presence of domesticated and game birds and decreased when co-grazing with ruminants was used.[68]
Previously affected horses	Reports of the disease occurring in the same horse more than once are rare, and there are no reports of histopathologic diagnosis being made in the same horse more than once. Contact with a previous case reduces the risk for EGS development 10-fold in co-grazers.[42]
Recent movement	Movement from one premises or paddock to another increases the risk, with occurrence commonly within 2 to 4 weeks.[41,42]
Dietary change	Inconsistent evidence that absence of supplementary feed increases risk[40,42] with the provision of preserved forage found to be protective.[45] Change in the quality and quantity of feeding increases the risk.[39,45]
Anthelmintic strategy	Increased frequency of worming, especially the use of ivermectin-based wormers in succession, may increase the risk.[42,45]
Body condition	Inconsistent evidence that horses in good-fat body condition are at increased risk.[41,42]

Table 1: Manageable risk factors for equine grass sickness (Wylie et al., 2009).

5.1 Clinical signs

Equine grass sickness appears to present clinically as either one of the three clinical forms, usually relating to the diseases duration. These forms are acute equine grass sickness (AGS), which usually lasts 1-2 days before resulting in mortality, subacute equine grass sickness (SGS), which is usually fatal after 2-7 days, and chronic grass sickness (CGS) which usually lasts greater than 7 days, with chronic equine grass sickness not always resulting in death. Although the clinical presentations can be separated this way there are often overlaps between these sub-classifications (Pirie et al., 2014).

All classifications are associated with a dysfunction of the autonomic nervous system, in particular the enteric nervous system, and often with some somatic neuron damage, with the main clinical signs being depression, tachycardia, anorexia, colonic impactions and colic signs. The colic signs are thought to be related to motor and secretary changes as well as a possible displacement and abdominal distention (Cottrell et al., 1999). Dysphagia is thought to be due to either oesophageal dysfunction or due to neuronal lesions present in cranial nerves V, VII, X, and XII, which are all the cranial nerves associated with swallowing and

mastication, with the exception of IX where no lesions have been found. The severity of the clinical signs depends on the clinical presentation of the disease (Pirie et al., 2014). In AGS there is often a decrease in oesophageal peristalsis, and in turn an observed mega-oesophagus (Cottrell et al., 1999).

Other typical clinical signs include muscle fasciculation's, often seen over the flank, patchy sweating and often ptosis which can be seen variably in acute, subacute and chronic grass sickness. Specific to AGS are signs such as hyper-salivation, possibly related to dysphagia and large volumes of nasogastric reflux, which may be present even without gastric intubation (Wylie et al., 2009). With regards to chronic grass sickness the most common sign is rhinitis sicca, as well as remarkable weight loss, and a tucked up posture, although this could just be due to the longer duration of the disease.

During further investigations, for a diagnosis of EGS, blood parameters commonly show results such as an increased packed cell volume, total protein and increased urea and creatinine concentrations which is all relevant to a hypovolemia (Collins, 2008). The sudden reduction in circulatory volume in the acute cases may explain the sudden cardiac failure, and the increased luminal capacity of the gastric and enteric compartments may cause visceral pain, explaining the colic signs, as well as causing electrolyte imbalances (Cottrell et al., 1999). Other diagnostic approaches include a rectal exam, which in AGS often shows relative increased distended small intestine (DSI), and often small colonic impactions. In a chronic case the only finding here may be simply just a reduced intestinal content. These findings can be supported by an abdominal ultrasound scan, which again can show DSI, as well as a decrease in intestinal motility (Collins, 2008). As ptosis is a common sign of EGS, we can use a phenylephrine test as a way to diagnose the disease. In grass sickness cases, the ptosis should be dramatically reduced in the medicated eye within 20-30 minutes. The only definite ante-mortem diagnosis includes an ileal biopsy, taken either in surgery or via a laparotomy, where the histological examination shows marked degenerative changes of the enteric neurons (Collins, 2008).

Clinical signs	Botulism	AGS	CGS
Depression	+++	+++	+++
Dilated pupils	+++		
Distended abdomen		+++	
Drooping eyelids	++	+++	+++
Dysphagia	+++	+++	+++
'Elephant on tub' stance		+	+++
Hyper-salivation	++	+++	
Ileus, colic, colonic impactions	+++	+++	++ (varying and often mild)
In-appetence	+++	+++	++
Mega-oesophagus and oesophageal peristalsis inhibition.	+++	+++	
Muscle tremors	+++	+++	+++
Nasogastric reflux	++ (Possible green milky nasal discharge)	+++ (high volumes)	
Progressive muscle weakness, flaccid paralysis	+++		
Ptosis	++	++	++
Rhinitis sicca			+++ (most common clinical sign in CGS)
Sweating	+ (sometimes present)	+++ (patchy)	+ (patchy)
Tachycardia	+ (Possible increased heart rate followed by Cardiac paralysis)	+++	++
'Tucked up' abdomen			+++

Table 2: Comparison of the clinical signs of EGS and equine Botulism.

5.2 Post mortem investigation

5.2.1 Macroscopic appearances

Pathological gross lesions of EGS cases differ between acute, subacute and chronic cases, although there is often an overlap between these sub-classifications. Many investigations have been undertaken examining the gross lesions in EGS, all of which provided similar results meaning the lesions are reliable (Obel, 1955).

In 1995, Obel carried out an investigation on 9 acute and 5 chronic grass sickness cases, during a post mortem exam, she looked at the macroscopic changes and the microscopic changes.

In regards to AGS, the most significant sign was the distention of the stomach and upper small intestine, with large amounts of fluid, amounting to 30 litres, being collected from here (Obel, 1955). This followed the similar results collected in a previous study in 1940, where the volume of stomach content and small intestinal content was collected from 36 AGS cases and 13 healthy controls. Results showed on average 22 litres and 32 litres collected from the stomach and the small intestine respectively in the AGS cases, and 7 and 9 litres of the stomach and small intestine of the healthy controls (Stewart et al., 1940).
Other evidence in previous years has also noted a distended stomach and increased content as a typical gross pathological lesion of Acute equine grass sickness. This is due to the paralysis of the autonomous and enteric nervous systems, causing a cessation of peristalsis in the GI tract, which in turn prevents the passing of the stomach content, increasing its volume (Begg, 1936).
In the large intestine, a typical black coating of the intestinal content and the mucosa was seen in acute and subacute cases (Obel, 1955), which represented the adhesion of blood products (Wylie et al., 2009).
Another typical post mortem lesion for AGS, is reflux oesophagus, which results in small ulcers in the oesophagus and a possible aspiration pneumonia (Obel 1955, Wylie et al., 2009). Extensive intestinal haemorrhages were also seen in some cases (Obel, 1955).

With regards to chronic grass sickness, the post mortem gross lesions present quite differently. The first notable sign is a massive decrease in body condition, with the carcass appearing emaciated and dehydrated, with signs of muscle atrophy. Unlike the acute cases,

the stomach was small with no or little content, with the colon and the rectum having very hard and dry faecal balls with increased amounts of mucus (Obel, 1955), of which can be used as a diagnostic tool and felt upon rectal examination (Wylie et al., 2009). Other post mortem signs of EGS that cannot be considered specific to the disease include pneumonia (Begg, 1936; Whitwell, 1997), hepatic changes (Obel, 1955; Whitwell, 1997; Mars, 2001), splenomegaly and haemorrhagic adrenal glands (Whitwell, 1997).

When comparing the post mortem lesions of EGS with those of botulism (table 3), we see almost no comparable signs. The gross post mortem examination of botulism cases is usually unremarkable, and the post-mortem examination is usually done as a methods of exclusion of other potential differential diagnosis's (Anniballi et al., 2013).

The most common post mortem lesions of botulism are pulmonary oedema and pericardial effusion, which usually containing free floating fibrin strands (Stämpfli, 2014), whereas the EGS cases have no macroscopic changes in the heart, and lungs. Aspiration pneumonia can be seen in EGS, and is also seen often in botulism cases, as both EGS and botulism cause dysphagia can lead to aspiration pneumonia. In the AGS cases, the aspiration pneumonia could also be due to the reflux oesophagus.
One comparable sign could be the evidence of diffuse intestinal haemorrhage in both EGS and botulism.

These very different macroscopic post mortem lesions could be considered to oppose the *C. botulinum* theory as the cause of grass sickness, although the theory suggests EGS is a toxico- infection of the botulism toxin, which remains localised to the gastrointestinal tract (Hunter et al., 1999). The macroscopic lesions alone are not enough to compare the impact of equine grass sickness and botulism.

5.2.2 Microscopic changes

Equine grass sickness is known for causing a degeneration in the autonomic and the enteric nervous system, leading to clinical signs focused around the gastrointestinal tract, something that equine botulism has not been described as causing. Obel (1955), was the first to discover these changes of the vertebral and paravertebral ganglia and the alimentary mural plexi of

Post mortem lesion	AGS	SGS	CGS	Botulism
Aspiration pneumonia	++			+++
Black coating of intestinal content and mucosa	++	+++		
Crusting ulcerative lesions of Rhinitis sicca			+++	
Decrease in body condition			+++	
Decreased intestinal content			+++	
Desiccation of the intestinal content	++	+++		
Diffuse intestinal haemorrhage	+			++
Enlarged mesenteric Lymph nodes with moist cut surface			+++	
Erosions in the oesophagus	+++	+		
Hard faeces in the colon and the rectum		+++	+++	
Large intestine impaction		+++		
Muscle atrophy			+++	
Oedema of the head and neck				+++
Pericardial fluid (often with fibrin)				+++
Pulmonary oedema				+++
Reflux Oesophagus	+++	+		
Stomach distention	+++	+		

Table 3: Comparison of the Macroscopic post mortem lesions of Acute, subacute and chronic grass sickness, with those of botulism.

the autonomic nervous system. Later investigations discovered some changes in the central nervous system (Hunter, 2001). Obel (1955) described that the effected neurons have a chromatolyis which is a word to describe the dissolution of the Nissl substance – a rough endoplasmic reticulum, as well as the nucleus being slightly enlarged and laying eccentrically, with karyopiknosis and a karyolis, and often cytoplasmic vacuoles (Obel, 1955). These changes are now considered pathognomonic for equine grass sickness (Wylie et al., 2009). Neuronal degeneration is seen more severely in the acute grass sickness than the chronic grass sickness (Gilmour, 1973, Pogson et al., 1992). Sometimes in subacute and often in chronic cases there is evidence that some of the unmyelinated fibres in the coeliac – mesenteric ganglia show some axonal budding, which may point to a conclusion why some chronic cases are able to recover. (Cottrell et al., 1999)

These features can be seen under the light microscope after a variety of staining's, as shown in a 2019 article, looking at the best ways of rapid diagnosis using histological examination for horses that are potentially suffering from EGS (Piccinelli et al., 2019). The experiment evaluated the diagnostic accuracy of Cranial Cervical Ganglion (CCG) scrapings of horses suspected of suffering from EGS, and used May Grunwald Giemsa (MMG), haematoxylin and eosin (HE) and cresyl fast violet (CFV) staining methods, to compare which produced the highest confidence in diagnosis of the disease. The typical histological changes were obvious in all three types of stains, but MMG appeared to show more morphological differences between the EGS and the control cases (Piccinelli et al., 2019).
Below are some examples of the histological results from this experiment, clearly showing the morphological changes associated with EGS, but which are not typical features seen in botulism (Figure 2) (Piccinelli et al., 2019)

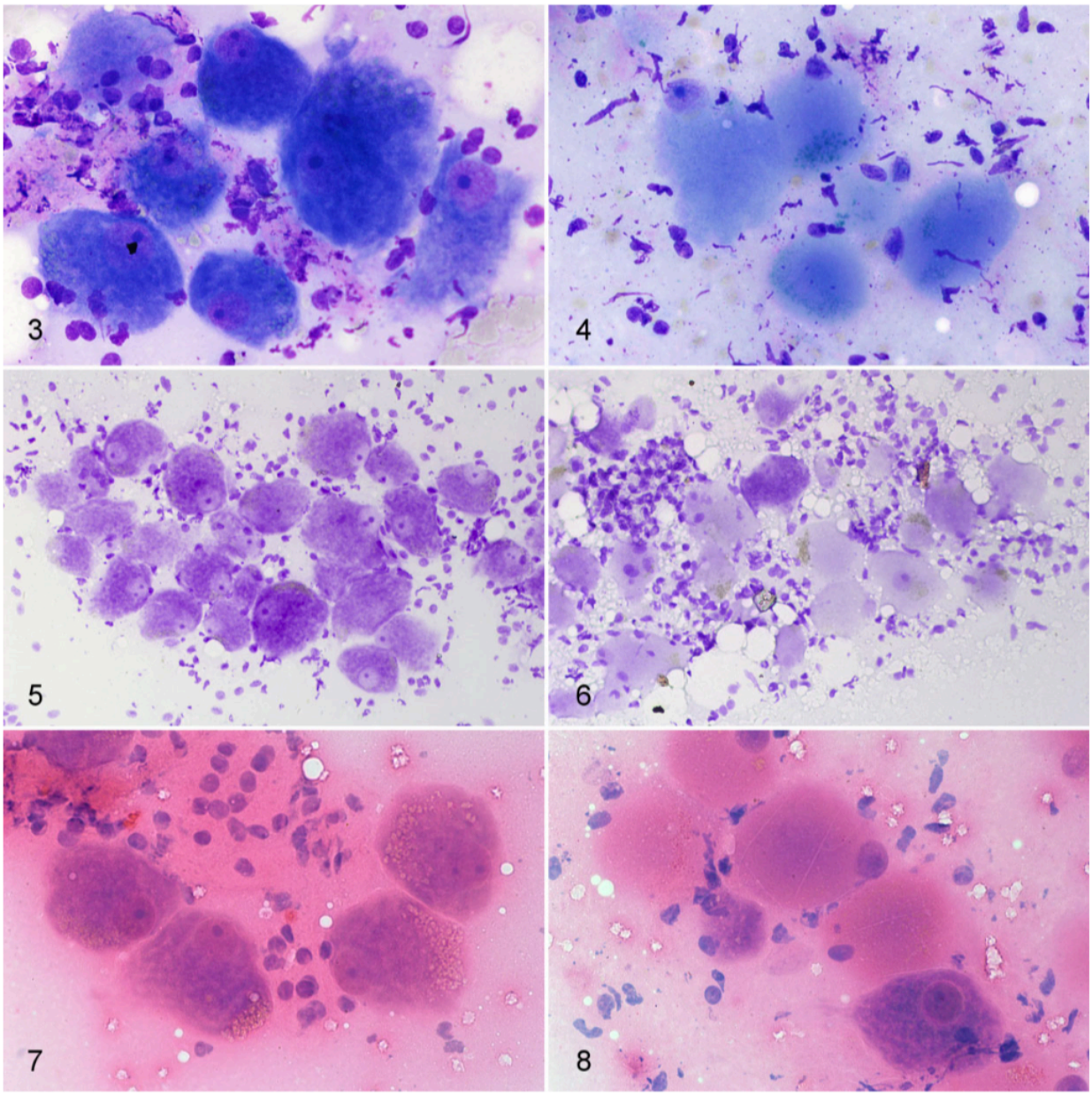

Figure 2: figures 3,5,7 were control horses and 4,6,8 were EGS cases. 3 & 4 are stained with MGG, 5 & 6 with CFV and 7 & 8 with HE. In the EGS slides we can see the chromatolytic neurons are swollen with lack of Nissl Substance, and the nuclei are pyknotic and eccentric. (Piccinelli et al., 2019).

In 2015 an experiment was carried out to examine the direct of effects that the botulism toxins have on body in cases of EGS, compared with equine botulism cases.

Botulism toxins mechanism of action involves the cleavage of Soluble N-ethylmaleimide sensitive fusion attachment receptor or 'SNARE' proteins, each toxin cleaving a different

alpha helix bundle, for example BoNT/C cleaves syntaxin and BoNT/D cleaves Synaptobrevin, each of these providing 1 alpha helix bundle to the SNARE protein. The last, which provides 2 alpha helixes to the SNARE protein is Synaptosomal- associated 25 or 'SNAP-25", which is cleaved by BoNT/A (Barr et al., 2005; Fasshauer et al., 1998).

The investigation aimed to look further at the evidence that Botulism neurotoxins may have a role in EGS, by examining 3 aspects. 1) histology examination of the Cranial cervical ganglion (CCG) and ileal neurons to determine if botulism causes autonomic and enteral neuronal degeneration, similarly to EGS, 2) immunohistochemistry to look at the expression of SNARE proteins of Cranial cervical ganglion and enteric neurons of EGS, botulism and control cases, 3) looking at the concentration of SNARE proteins in extracts of cranial cervical ganglions from EGS and controls (McGorum et al., 2015).

When looking under a light microscope we could see the standard chromatolysis, neuronal swelling, vacuolisation and eccentric, pyknotic nuclei of the EGS horses, but this was not apparent in the Botulism cases and the controls. This was seen in both the CCG and the enteric neurons, which suggests that EGS is unlikely to be caused by the typical BoNT that cause flaccid neuro-paralysis in classic botulism (McGorum et al., 2015).

When looking at the immunohistochemistry for the different SNARE proteins for the CCG and enteric neurons, seen again was some differences between the EGS, the Botulism, and control horses (Figure 3). The CCG were labelled for all three SNARE proteins (syn, syb, SNAP-25) and the ileum sections were labelled with just Syn SNARE protein. In the EGS cases, the CCG had an increased intensity of labelling on all three proteins when compared with the botulism and control horses, who had indistinguishable features with many neurons being either unlabelled or faintly labelled (McGorum et al., 2015).
There was an increased expression of SNARE proteins in the EGS horses neuronal perikarya of the CCG and the enteric neurons but not with those of botulism, suggesting again that it is unlikely that the BoNT's are responsible for the typical findings in EGS (McGorum et al., 2015).

The results from this study suggest no link between the BoNT's that cause the typical flaccid neuro-paralysis that comes with botulism, and EGS. A hypothesis that could be feasible, is

that the BoNT have a different mechanism of action when produced in vivo, through the toxico – infection (McGorum et al., 2015).

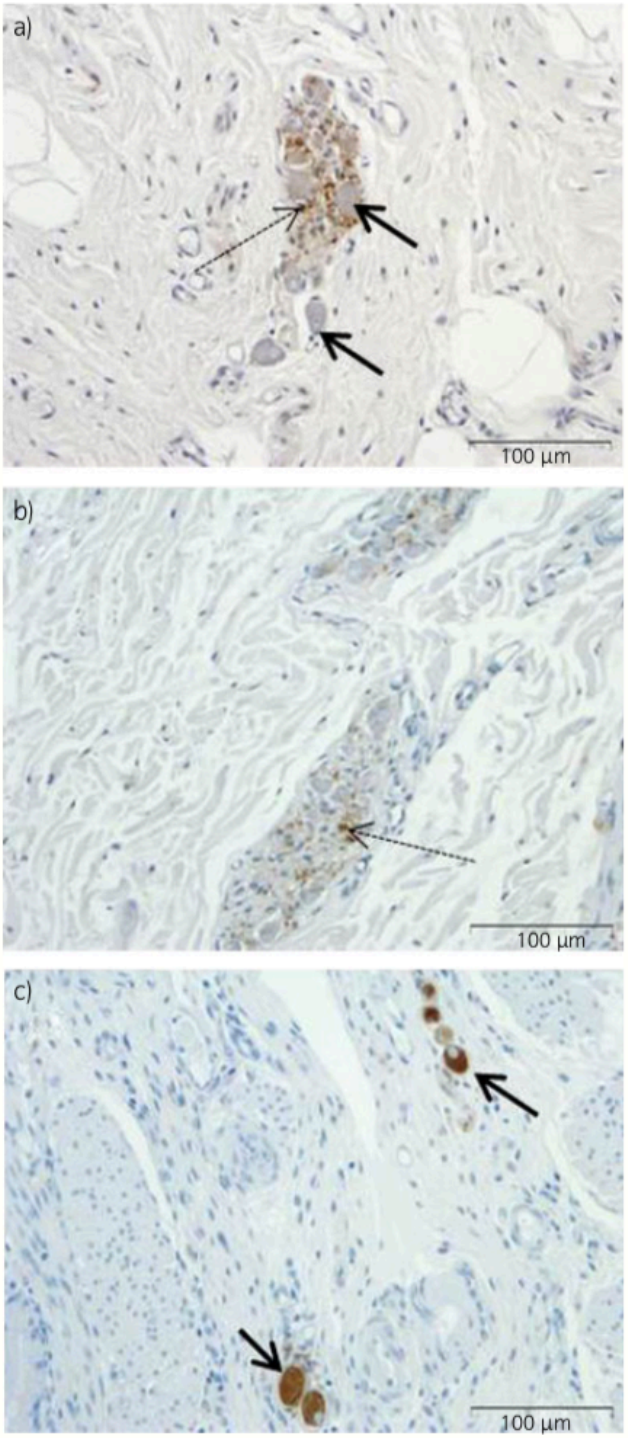

Figure 3: Immunohistochemical localisation of synaptobrevin (Syb) in submucosal plexus neurons from (a) control, (b) botulism and (c) equine grass sickness horses. Synaptobrevin immunoreactivity is confined mainly to axons in botulism and control horses, while neuronal perikarya from equine grass sickness horses have increased Syb immunoreactivity. Neuronal perikarya are labelled with short solid arrows, while long broken arrows label axons (McGorum et al., 2015).

5.3. Faecal and ileal sampling, faecal and urine biomarkers

The first piece of evidence that may suggest *C. Botulinum* as a cause for EGS, was discovered by Tocher in 1919, where he first isolated a bacterium that resembled *Bacillus botulinum* (now *C. botulinum*) from the gastrointestinal tract of a number of horses with EGS (Tocher et al., 1923).

This finding was revisited in 1999 when an investigation was carried out to try to isolate the first exotoxin produced by *C. Botulinum* type C. This toxin, BoNT/C or the classic neurotoxin, is a protease that cleaves syntaxin in the presynaptic membrane, and in turn prevents the release of acetylcholine resulting in the flaccid paralysis, although in EGS it is thought to be a localised toxin production, affecting mainly the enteric nerves, unlike classic botulism where clinical signs can be seen more generalised (Poxton et al., 1999).

The investigation took faecal samples from 45 EGS horses and 77 controls, and samples from the ileal content of 30 EGS horses and 28 controls. Results (Figure 4) concluded that there is a strong connection between EGS and *C. botulinum* with the bacteria being isolated in a significantly higher percentage of horses with EGS than the controls in both the faecal and ileal samples (Hunter et al., 1999).

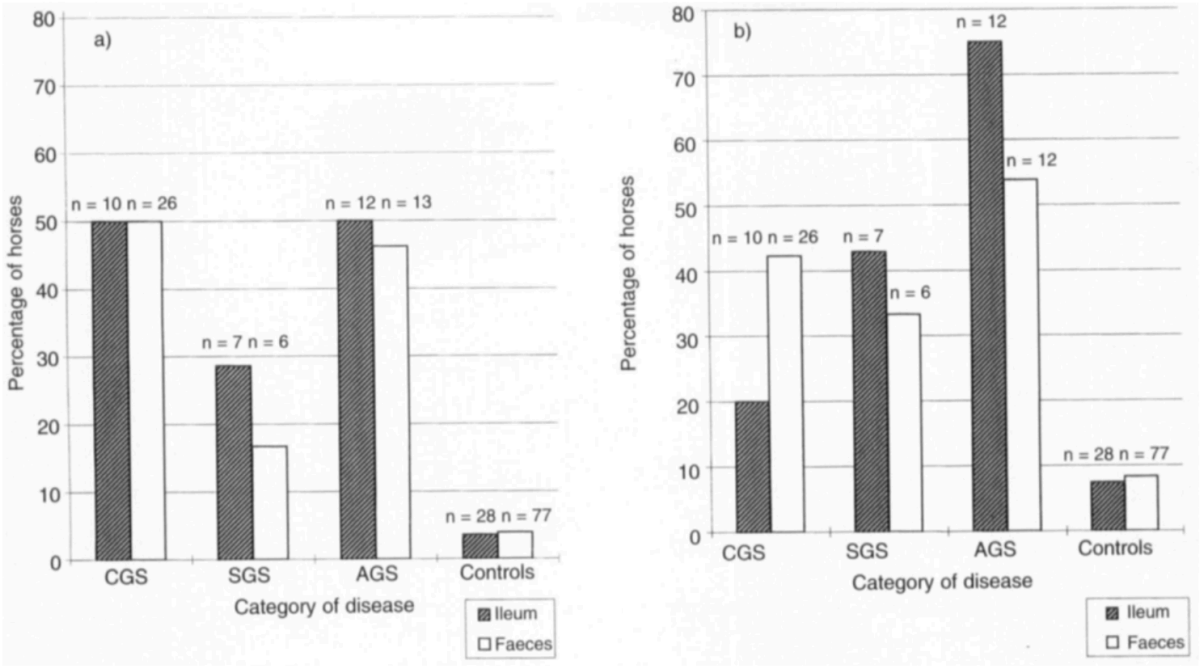

Figure 4: Results from the 1999 study examining the isolation of C. botulinum from faecal and ileal contents in horses with EGS and Controls. a) directly before enrichment, b) after enrichment (Hunter et al., 1999).

In 2010 another investigation was carried out using similar materials and methods but examined the prevalence of *C. perfringens* instead. The examination used a similar number of EGS horses and control horses, where the control horses were further divided into those that had grazed on high/low risk pastures and a colic group. Results proved to be very similar to the previous investigation which examined the isolation of *C. botulinum* from faeces of horses suffering from EGS. *C. perfringens* was detected in the faeces in every group, but at 41% of EGS, 1% of controls on low risk pastures, 6% of controls on high risk pastures, and 6% of the colic controls. It was detected also in the ileal content in 50% of the EGS horses and 8% of the controls. This evidence may suggest a wider spectrum of Clostridia growth as a cause for EGS (Waggett et al., 2010).

These results could be put down to the fact that the overgrowth of *C. perfringens* was a secondary problem that arose due to the GI dys-motility caused by the EGS, but what is interesting in these results, is that in the colic group of control horses, who most likely also had a GI dys-motility, only 6% had *C. perfringens* isolated from their faeces. As a dys-motility occours often in both EGS and colic cases, these results suggest the presence of *C. perfringens* in the faeces was not due to the dys-motility, but more likely related specifically to EGS and its cause (Waggett et al., 2010).

Although the bacteria in both experiments was isolated in a significantly higher percentage of the EGS horses than the controls, the percentages were not high enough to confirm a *C. botulinum* infection is the cause for EGS. In either experiment, before enrichment no more than 50% of the EGS horses had the bacteria isolated from their faeces. This indicative was not enough to suggest *Clostridia* faecal isolation is pathognomic to EGS. Despite these points not being entirely conclusive to the theory, it does suggest a strong probability that *C. botulinum* plays a role in the pathogenesis of EGS.

In 2018 a study was carried out into examining the faecal microbiota, the urine and the plasma of 40 horses, 19 with EGS, 15 Co grazing horses or matched controls, and 6 hospitalised horses - where EGS was considered before another diagnosis was confirmed. This allowed identification of any microbiota factors that would be specific to EGS, and differentiated from other gastrointestinal diseases (Leng et al., 2018).

From 31 faecal samples bacterial DNA was isolated, amplified using polymerase chain reaction (PCR) and grouped according to their operational taxonomical unit (OUT) at a level of 96% similarity. The bacterial community diversity was examined and showed that the EGS group on a whole had the lowest bacterial community diversity, and the co-grazing horses or matched control horses had the highest. This evidence shows that EGS has an impact on the bacterial diversity of the microbiota of the colon, a factor which has been evident in humans who are infected with *Clostridium difficile*. Reduced diversity of the microbiota can cause a decrease in the competitive nature of the gut, allowing potential harmful bacteria such as *C. botulinum* to grow (Leng et al., 2018).

Further investigation led to examination of groups of bacteria based on taxonomic units. With 20 out of the 82 OTU's being used as potential bacterial biomarkers for EGS, they were grouped into 5 classes: 1. Clostridia, 2. Gammaproteobacteria, 3. Fusobacteria, 4. Bacterioida and 5. Deltaproteobacteria. The results obtained from this data provided no evidence to suggest that *C. botulinum* is the causative agent of EGS, with the analysis showing a lower relative abundance of the bacterial family *Clostridia*, in which *C. botulinum* resides, in the EGS group compared with the Co-grazing or MC (matched controls) group (Leng et al., 2018).

In relation to these results, the metabolic profiles captured from the urine showed a low abundance of hippurate and 4-cresol in the EGS group compared with the co-grazing group. Both these are known gut microbial-host co-metabolites. Hippurate is metabolised from benzoate by the horse, after it has been absorbed from the gut, this in turn is metabolised from benzyl alcohol by the gut bacteria, before absorption (Leng et al., 2018). A lower abundance of hippurate in the urine is consistent with the evidence of a low bacterial diversity of the gut in horses suffering from EGS, and with the pathological evidence of gastrointestinal damage.

4- cresol is metabolised in the gut from tyrosine, and the Clostridium genus, in particular C. *difficile,* are known to be precursors of Cresol as concluded in a study published in 2011 (Dawson et al., 2011). The low abundance of 4-cresol in the urine of EGS horses compared with the co-grazers suggests no evidence of the involvement of *C. botulinum* in the cause for EGS, and it is consistence with the evidence from the faecal biomarkers proving the low abundance of this genus in the faeces and therefore gut microbiota (Leng et al., 2018).

The evidence found in this trial greatly opposes the *C. botulinum* as a cause for EGS theory, although it does not preclude its partaking in the cause. Further investigation would have to be carried out to examine if a trigger factor could lead to the neurotoxin production.

5.4. Soil examination

A novel finding in a study based on prevalence of grass sickness of affected premises showed that the occurrence of EGS is associated more with different soil types of the grazing pastures. The highest prevalence being shown on premises located on loam and sandy soils (Figure 5).

This supports the theory of toxico-infection form of botulism, as *C. botulinum* is a soil-borne bacteria, and that the risk of disease increases when there is increased exposure of horses to soil during grazing. Any form of soil disturbance may increase the risk of grass contamination, thereby bringing the bacterium into contact with grazing horses more frequently. The loam and sand soils are easily turned over, acidic and have good drainage, allowing for soil inhabitants such as earthworms to more easily move through the soil and burrow, disrupting the soil and therefore bringing the bacteria to the surface, for greater access in grazing (Newton et al., 2004).

This evidence coincides with research on faecal removal methods and prevalence of EGS on certain pastures, which concluded a higher incidence rate on pastures where the faeces is removed by mechanical sweepers, as these methods are thought to disrupt the soil in a similar way to the inhabitants (figure 6) (Newton et al., 2004)[1]. In contrast, Hedderson added as a footnote that if the soil remains undisturbed, the mechanical vacuum method to remove faeces may provide some protection against EGS, as this is more similar to the manual method which the study found to be protective against the recurrence of the disease.

[1] **J. Hedderson in 2006 added a footnote to the article Results of an epidemiological study of recurrence of equine grass sickness on affected premises**

Bohnel et al. (2001), investigated 2 cases of EGS on a stud that occurred within 8 months of each other, they investigated among other things, the soil and the grass on the affected pastures. For the first time the evidence here showed growing grass containing free botulinum neurotoxin of types A-E, with 13/19 of the samples taken being positive. *C. botulinum* was also found in the soil, but these did not coincide with the same areas where the toxin was found in the grass. More investigation in this area may need carrying out (Bohnel et al., 2001). These findings also support the toxico-infectious botulism theory as a cause of EGS.

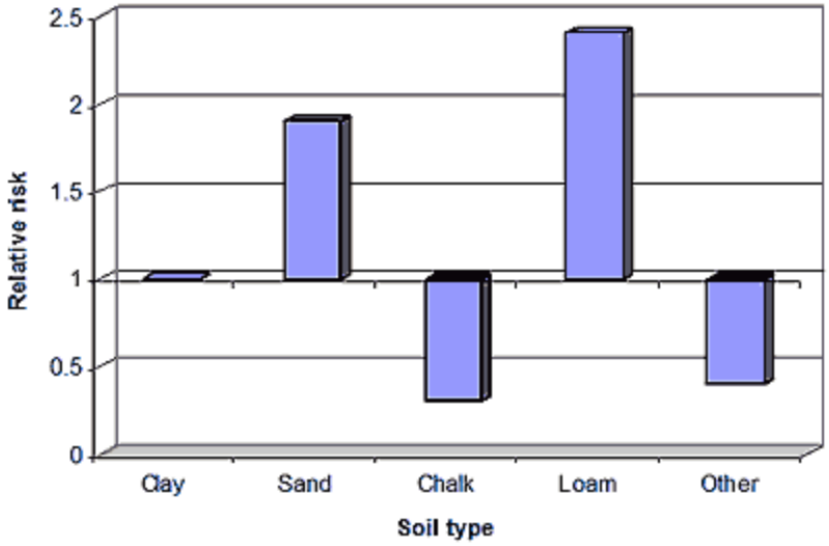

Figure 5: Prevalence of EGS on different soil types (Newton et al., 2004)

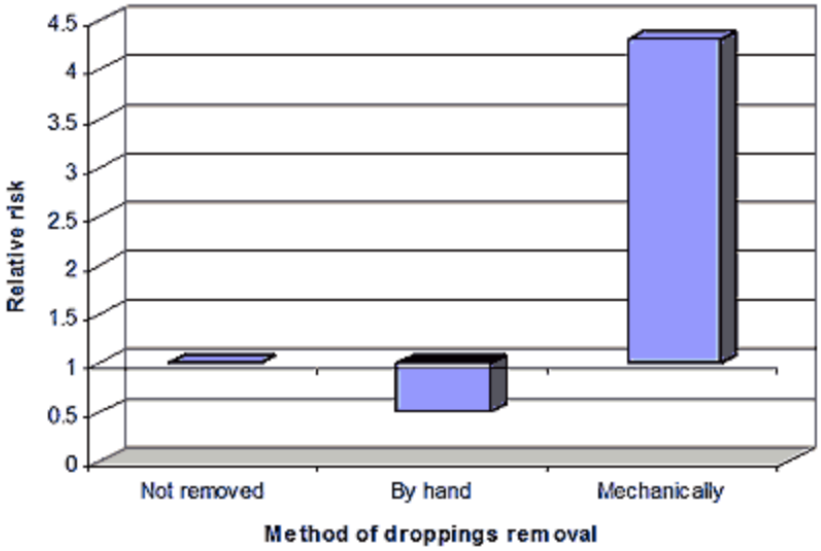

Figure 6: Prevalence of EGS on pastures where the droppings are not removed, removed by hand or removed mechanically. (Newton et al., 2004)

5.5. Antibodies against Clostridium botulinum

After much research, epidemiological data suggests a resistance to EGS could occur with increasing age, as there is a higher prevalence of the disease in horses of 2-7 years of age (Doxey et al., 1991, Wood et al., 1998). The disease is rarely seen in foals, suggesting a maternal immunity. Likewise, evidence suggests development of a resistance after being in contact with a horse who suffered from EGS, with a 10-fold decrease in the prospect of getting the disease (Wood et al., 1998).

Hunter and Poxton (2001), carried out an investigation in vitro to determine if the resistance towards EGS was due to an immune response, in relation to the *C. botulinum* theory. They determined levels of the BoNT/C and the *C. novyi* type A and C antigens in the serum of 80 EGS horses and 142 control horses, which were grouped into 1. Contacts, 2. High risk. 3. Controls. *C. novyi* was used instead of *C. botulinum* due to the risks to humans working on the experiment (Hunter et al., 2001). *C. novyi* is phenotypically similar to *C. botulinum* with their antigens being immunologically cross reactive (Poxton et al., 1984), so was used as a safe and reliable replacement.

Results (Table 4) showed that the EGS horses, had a significantly lower level of IgG's to BoNT/C and surface antigens of *C. novyi* type A than the 3 control groups. In particular, the contact and high risk control groups had much higher IgG levels to both *C. novyi* surface antigens and BoNT/C, suggesting these horses' immune systems have made a systemic immune response to the *C. botulinum* antigens. This may suggest that horses that have been exposed to low toxin level whilst grazing on a high risk pasture may develop a subclinical grass sickness (Hunter et al., 2001).

The results showed that there was no significant difference in the IgG levels between AGS, SGS and CGS cases, for both surface antigens and BoNT/C. These results indicate that the level of IgG do not determine the course of the disease (Hunter et al., 2001).

Specific antibody	Statistics	Antibody level (OD_{405} nm) CGS (n = 31)	SGS (n = 21)	AGS (n = 28)	Controls (n = 36)	Contact (n = 60)	High risk (n = 46)
IgG to surface antigens	Mean	0.75	0.75	0.72	0.80	0.89	0.89
	s.d.	0.31	0.41	0.38	0.36	0.37	0.40
	Range	0.36–1.44	0.20–1.63	0.17–1.94	0.16–1.82	0.31–2.20	0.26–2.06
IgG to BoNT/C	Mean	0.17	0.22	0.18	0.24	0.30	0.36
	s.d.	0.19	0.17	0.15	0.19	0.34	0.30
	Range	0.04–0.62	0.02–0.63	0.03–0.66	0.01–0.94	0.02–2.44	0.07–1.23

Table 4: results of experiment comparing IgG levels to C. novyi type A Surface antigens and BoNT/C from serum of horses suffering from AGS, SGS, CGS, and horses of a high risk group, contact group and control group (Hunter et al., 2001).

Another study in 2004 using similar parameters, also provided strong evidence for the association between *C. botulinum* and EGS, with results showing low IgG levels against *C. botulinum, C. novyi* and *C. botulinum* toxoid in animals suffering from the disease (McCarthy et al., 2004). This experiment followed on from Hunter and Poxtons study which found an invariable statistical association between EGS and *C. botulinum*. Here McCarthy et al., examined multivariable including age of the horses, the diet, the grazing period, faecal egg count, and days since last worming.

Results proved that 4-5 years old is the riskiest age. This may be due to increasing exposure to the agent in horses up until 5 years old, and then a decrease after, or may suggest a relationship between the agent and age related resistance to the disease (McCarthy et al., 2004). The experiment also found that feeding hay or haylage in the diet, rather than silage, may decrease the risk of contracting EGS, which could be related to the known association of botulism caused by *C. botulinum* type B, being associated with feeding silage (Ricketts et al., 1984). Here the results once again strongly supported the *C. botulinum* theory, with added benefits of acknowledging risk factors which could be the base of avoidance strategies (McCarthy et al., 2004).

A similar preliminary study later compared the IgG levels to surface antigens, BoNT/C and BoNT/D, of horses that recovered from CGS and those that were euthanized due to no prospect of recovery. The surviving horses had a significantly higher level of IgG to the

surface antigens and the BoNT/C, but no difference in the BoNT/D (Nunn et al., 2007). The results here in a way contradict the evidence in the 2001 experiment which indicated that the level of IgG's do not determine the course of the disease (Hunter et al., 2001). These results however suggest the level of IgG titres before the infection may be important in the outcome of the disease, as in whether the animal survives or not (Nunn et al., 2007).
These results highly support the *C. botulinum* theory, and suggested potential for a similar immune response from a vaccine, which was trialled in more recent years.

In contrast to the above experiments another trial was carried out in 2007 that examined the quantity of IgA's to *C. botulinum* type C and D in the GI tract, with samples being taken from the duodenum, the jejunum and the ileum from a number of acute grass sickness cases.

Results were somewhat different to previous experiment. They examined IgA's from BoNT/C (Figure 7) and BoNT/D (figure 8), surface antigens of *C. botulinum* type C (figure 9), and *Clostridium tetani* surface antigens (TetSA), as a control variable (figure 10), with results again being expressed at as percentage IgA (Nunn et al., 2007).
The results here showed that there are increased levels of specific IgA in AGS cases against the BoNT/C (figure 7), BoNT/D (figure 8) and surface antigens of *C. botulinum* (figure 9), compared with non- AGS controls, in the gastrointestinal tract, which contradicts the findings of Hunter and Poxton (2001). Here they found evidence of lower systemic IgG in horses suffering from AGS. These findings are more likely to show the current immune response to a recent exposure when compared with systemic IgG, as the IgA antibodies have a shorter half-life, and although little is known about equine gastrointestinal immunity (Nunn et al., 2007), gut and mucosal immunological memory is also short (Pierce et al., 1982). Although levels were higher, we must consider that these IgA levels were not at a protective level, and that the disease still ended in a fatality. There may also be reason to think that the mucosal IgA may have been pre-existing, due to levels being found in the control animals. This may shed some light on length of the disease and production of these antibodies, as we have to remember AGS has a very short duration (Nunn et al., 2007).
This evidence, although contradicting the 2001 study finding low systemic IgG present in AGS cases (Hunter et al., 2001), still can support the *C. botulinum* theory, as it provides optimism that a vaccine may be able to elicit an antibody response in the gastrointestinal tract (Nunn et al., 2007).

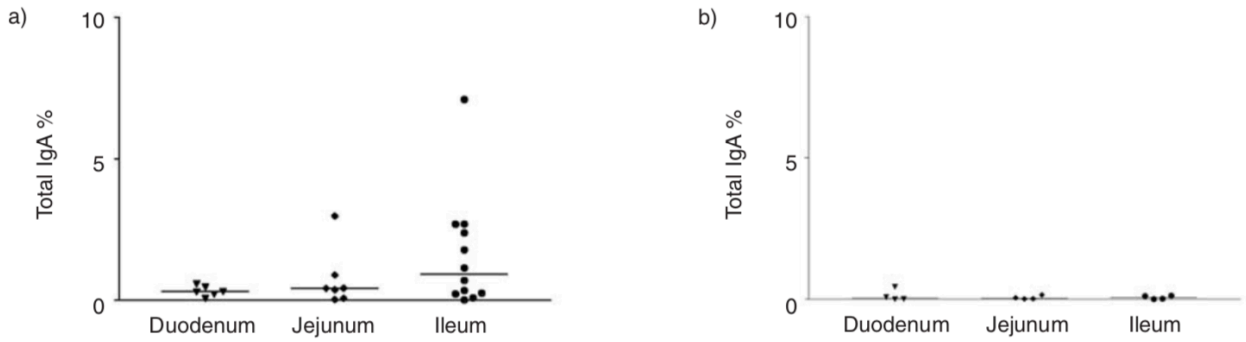

Figure 7: Range of specific IgA found along different areas of the GI tract against BoNT/C in a) AGS cases and b) Non AGS controls. Horizontal lines indicate the median (Nunn et al., 2007).

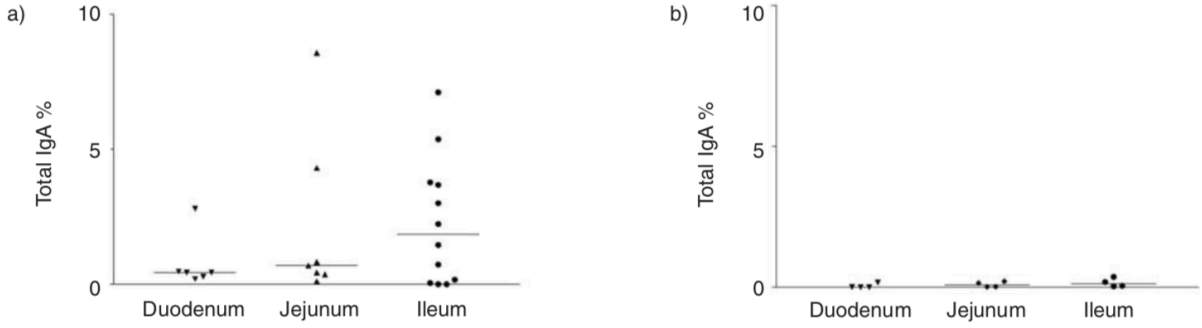

Figure 8: Range of specific IgA found along different areas of the GI tract against BoNT/D in a) AGS cases and b) Non AGS controls. Horizontal lines indicate the median. (Nunn et al., 2007).

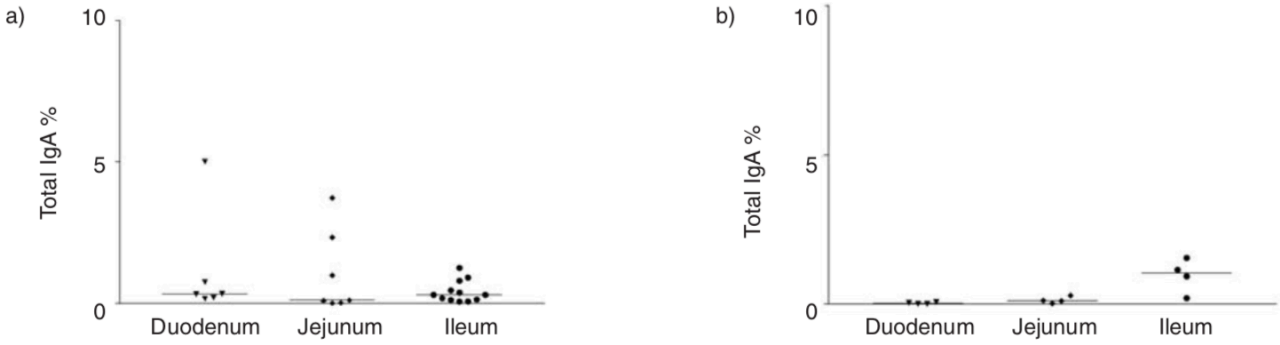

Figure 9: Range of specific IgA found along different areas of the GI tract against Surface antigens of C. botulinum in a) AGS cases and b) Non AGS controls. Horizontal lines indicate the median. (Nunn et al., 2007).

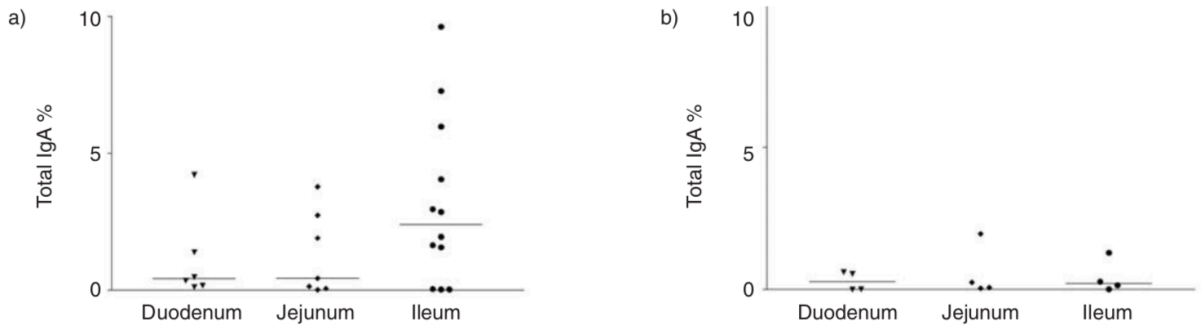

Figure 10: Range of specific IgA found along different areas of the GI tract against Surface antigens of C. tetani in a) AGS cases and b) Non AGS controls. Horizontal lines indicate the median. (Nunn et al., 2007).

5.6. Vaccine trials

Just short of 15 years after the first case of equine grass sickness was reported in 1909 (Tocher et al., 1923), a randomised controlled vaccine field trail was carried out in 1922 and 1923, which used a neutralised toxin of *C. botulinum*, and concluded that there was at reduction in the cases of EGS in those vaccinated animals (Tocher et al., 1923), although this failed to convince Tochers contemporaries (Hunter et al., 1999).

After years of investing other potential causes of EGS, the focus more recently has been reviewing the *C. botulinum* hypothesis. After carrying out a feasibility study of a field trial (Ireland et al., 2016), the Animal health trust launched a placebo controlled – field trial for a vaccine that could potential act against EGS, in collaboration with the universities of Edinburgh, Liverpool and Surrey, involving veterinary practices registered with the EGS surveillance scheme in England, Wales and Scotland (Wylie et all., 2011). 119 locations were used, all of which had an EGS risk factor of 2.23 cases per 100 horses prior to the beginning of the investigation. Care was taken to record other influential factors such as the length of time each horse spent on each pasture, and the horses age, which ranged from 3 months to 29 years, at an average age of 8 years old (Ireland et al., 2019).

1029 horses and ponies entered the trial, 541 receiving the *C. botulinum* type C toxoid vaccine and 488 receiving the placebo, through the process of randomisation. The location of these trial horses extended all over the UK. Throughout the trial, observations and monitoring's were carried out primarily by the owners, as well protocols such as blood tests. This is shown in figure 11.

Results of the trial (Table 5) show that 9 cases of EGS occurred over the 4-year vaccine trial, 3 cases from the vaccine group and 6 cases from the placebo. This shows that the risk of EGS was not significantly reduced by the toxin vaccine, providing little evidence that *C. botulinum* toxoid vaccine provides protection against EGS (Ireland et al., 2019). Thus opposing the theory of EGS being linked to *C.* botulinum type C.

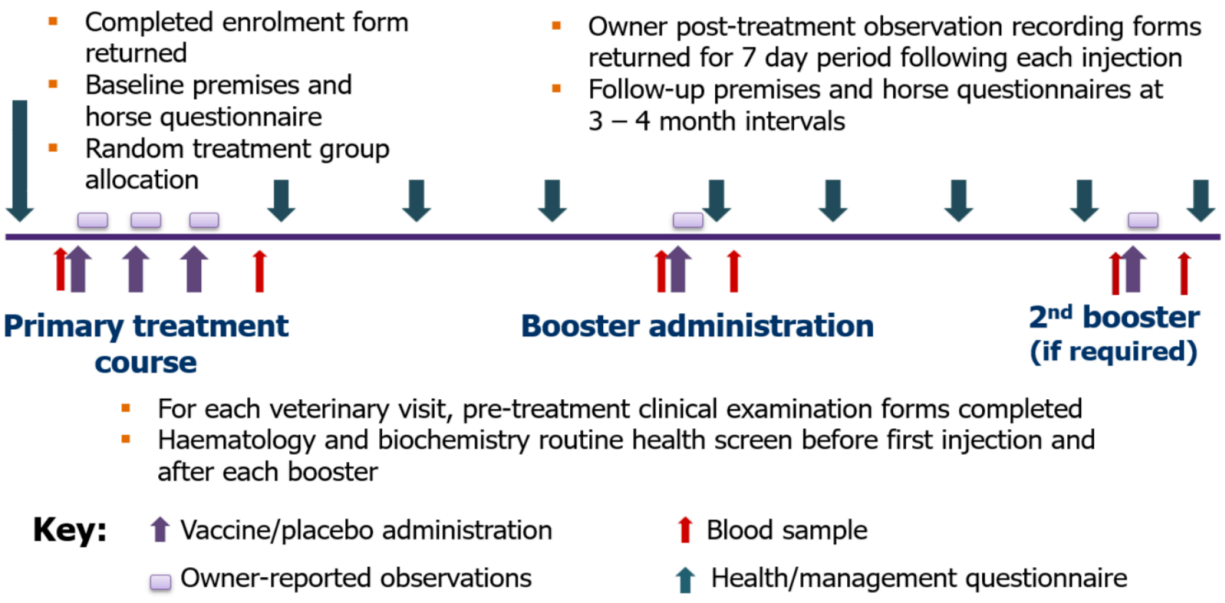

Figure 11: figure showing the protocols schedule of the treatment and monitoring of horses and ponies who took part in the EGS vaccine trial (Ireland et al., 2019).

Year of trial	Treatment group	Total number of horses/ponies enrolled during each year	Number of EGS cases in each year	Prevalence of EGS (percentage of horses/ponies affected)	EGS incidence per 100 horse-years at risk
2014	**Overall**	**604**	**1**	**0.17%**	**0.43**
	Vaccine	319	1	0.31%	0.82
	Placebo	285	0	0%	0
2015	**Overall**	**927**	**2**	**0.22%**	**0.30**
	Vaccine	481	1	0.21%	0.30
	Placebo	446	1	0.22%	0.31
2016	**Overall**	**824**	**2**	**0.24%**	**0.27**
	Vaccine	418	1	0.24%	0.27
	Placebo	406	1	0.25%	0.27
2017	**Overall**	**634**	**4**	**0.63%**	**0.82**
	Vaccine	323	0	0%	0
	Placebo	311	4	1.29%	1.68
Total	**Treatment group**	**Total number of horses/ponies enrolled**	**Number of EGS cases**	**Prevalence of EGS (percentage of horses/ponies affected)**	**EGS incidence per 100 horse-years at risk**
Entire trial period	**Overall**	**1,029**	**9**	**0.87%**	**0.43**
	Vaccine	541	3	0.55%	0.28
	Placebo	488	6	1.23%	0.58

Table 5: Prevalence and incidence of EGS for each year of the trial period, and for the entire trial period overall (Ireland et al., 2019).

Although the results did not associate EGS with botulism there was thought that the results were unlikely to provide evidence due to the low number of cases that occurred over the trial period. The overall incidence of EGS dropped significantly from 2.23/100 horses to 0.43/100 horses, from before to after the trial period.

There may have been speculation about the evidence supporting how effective the botulism vaccine is when being used against Botulism itself. Commercially available vaccines against botulinum toxin for equines are formalin-inactivated toxoids (serotypes B and C/D). The efficiency of these vaccines are considered satisfactory since its development in 1938. An experiment carried out in 2009, used a recombinant Botulism vaccine HcBoNT/C, which was produced in E. coli, and proved its reliability (Stahl et al., 2009). The results showed that recombinant HcBoNT/C and D are able to produce neutralizing antibodies against botulinum neurotoxin types C and D in the horse, and hence fulfil the basic requirement for

the development of a potent and clinically well tolerated vaccine, to protect horses against equine botulism. The evidence from this experiment once again opposes the connection between botulism and equine grass sickness, although you could argue that only 10 horses were given the vaccination in this experiment, and that an increased number of animals in the trial would provide a more reliable conclusion (Stahl et al., 2009).

Although the vaccine trial (Ireland et al., 2019) provided no evidence of a working vaccine, it did reinforce other evidence and our understanding of other risk factors of EGS. Due to regular antibody testing after each vaccination, there was evidence of those horses who had a lower final *C. botulinum* type C antibody titre were at an increased risk of developing EGS regardless of which group they were allocated to, and those who developed the disease had low antibodies before the onset of EGS, thus suggesting evidence towards the horses and ponies individual immune system playing a role in the prevalence of the disease (Ireland et al., 2019).

5.7 Similar diseases in other species and their association with Clostridium botulinum

In 1982 a disease of cats very similar to EGS was investigated by Key and Gaskell (Key and Gaskell, 1982). They stated Feline dys-autonomia is characterised by degeneration of the autonomic nervous system. It is known either as feline dys-autonomia or Key-Gaskell syndrome. There are striking similarities in the clinical and pathological signs in a number of unrelated species including horses, dogs, cats, rabbits and hares (Hahn, 2020). These clinical signs are mainly related to gastrointestinal dys-motility as well as similarities to Horner's syndrome showing miosis of the pupil and ptosis (Hahn et al., 2005).

In 2004 an investigation was carried out on cats in a similar fashion to the experiment on EGS horses. Samples were taken from 8 cats who were clinically infected with Key-Gaskall syndrome, and from 11 control cats. The samples were taken from the faeces and the ileum, as well as their food in this experiment. Results showed that the botulism toxin (BoNT/C) was isolated by ELISA from 4 out of the 8 clinically infected cat's faeces, and after enrichment, 7 out of the 8 cases. The highest concentration was seen in the 3 most clinically

infected cats. The toxin was not detected at all in the controls or in the food samples. This evidence supports the theory of Feline dys-autonomia and EGS being caused by a toxico-infection of botulism (Nunn et al., 2004).

In the same experiment they investigated the IgA antibodies to BoNT/C toxins and *C. botulinum* surface antigens, similarly to the 2007 experiment examining gastrointestinal IgA levels in horses suffering from AGS (Nunn et al., 2007). The results here (figure 12), again showed a significantly higher level of IgA in the affected cats than the controls, to both the toxoid and the surface antigen, from the faeces and the ileum samples, which were taken 14 weeks after the onset of clinical signs. One sample of an affected cat which had lower values of IgA than the others was one where the sample was taken in the 3rd week and not the 14th week after onset of clinical signs. This could suggest a resistance may develop over time (Nunn et al., 2004). Again this evidence supports the toxico- infectious botulism theory as a cause for feline dys-autonomia as well as EGS.

Similar to the horse and the cat, rabbits and hares present with similar clinical signs. An investigation was carried out in 2005 on rabbits to determine if the pathological lesions matched those of horses with EGS, and if this disease of rabbits and hares could be considered a dys-autonomia (Hahn et al., 2005).
A post mortem examination was carried on 3 domestic pet rabbits and 2 wild carcasses, who appeared to have clinical signs that resembled those of horses suffering from EGS. Results showed an impacted large intestine on all carcases. Histological examination showed degeneration of the post ganglionic autonomic neurons, peripheral ganglia and enteric nervous system. The pathological changes included chromatolysis of the autonomic motor neurons of the lower cranial nerves and brainstem, and did not appear in the lower motor neurons of the spinal cord (Hahn et al., 2005). These findings appeared to be very similar to the appearance of dys-autonomias of horses (Gilmour, 1973), and cats (Griffiths et al., 1982). In regards to the botulism theory behind these dys-autonomias, the gut content of the wild rabbits did also test positive to *C. botulinum* (Hahn et al., 2005), which again supports the theory, although further examination may need undertaking due to the low numbers of carcasses investigated.

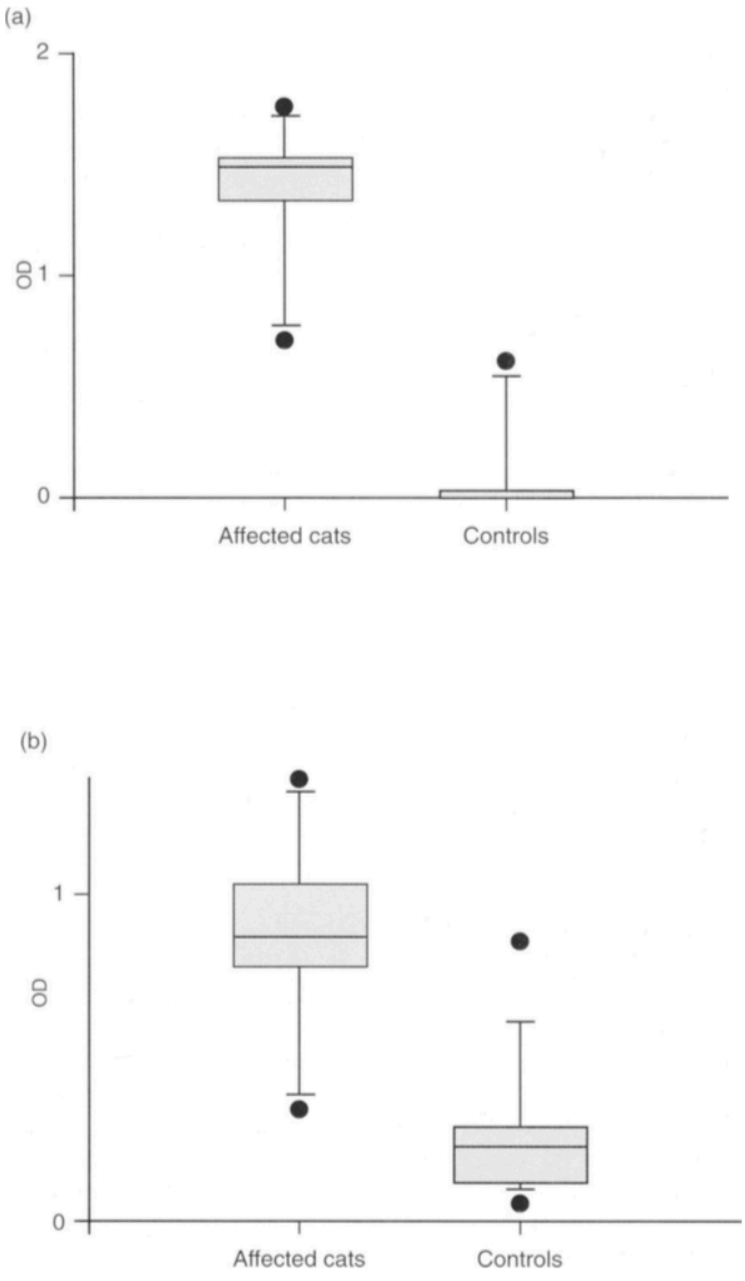

Figure 13: box and whiskers plot of faecal immunoglobulin A expressed in terms of optical density against a) C. botulinum type C toxoid. b) against EDTA-extracted surface antigen of C. botulinum type C (Hahn et al., 2005).

6. Conclusion

The arguments within this work show some correlation between *Clostridium botulinum* and Equine Grass Sickness, but on the other hand there are a few factors which don't show a relation, such as some histological changes and the more recent vaccine trials.

The examination into the IgG and IgA levels against *C. botulinum* of horses suffering from grass sickness proved to be massively supportive to the *C.* botulism hypothesis, with EGS horses showing low levels of antibodies towards BoNT/C and surface antigens, when compared with control horses and horses who were considered high risk, such as those who had been in contact with diseased horses and co-grazers. The high risk group had the highest antibody level. This suggested that horses with high antibody levels against *C. botulinum* have a decreased chance of getting EGS, indicating the bacterium have a role in the pathogenesis of the disease, and that horses are capable of developing a resistance to EGS (Hunter et al., 2001).

This proposed optimism that a vaccine could be developed to protect horses against grass sickness, but after a vaccine trial across the UK, results did not provide any evidence that the toxoid vaccine of *C. botulinum* provided any protection against EGS, (Ireland 2019), which opposed the evidence collected in the antibody trials. I did think that the efficiency of the botulism vaccine may be questionable but the results from a 2009 experiment concluded that the vaccine did produce neutralizing antibodies against *C. botulinum* (Stahl et al., 2009).

Potentially one of the strongest pieces of evidence was first discovered in 1919 by Tocher (Tocher et al., 1923), and was further revisited at the end of the century, where *C. botulinum* was isolated from the faeces and samples of ileal content in a higher concentration in EGS horses, when compared with control horses and colic horses (Hunter et al., 1999). Again similar evidence in 2010 using *C. perfringes* found almost identical percentages of the bacterium, in gut content and faeces of EGS horses when compared with colic and control horses. This evidence may suggest a wider clostridia growth but overall supports the theory of *C. botulinum* playing a role in the pathogenesis of equine grass sickness. What was interesting, was looking at the low levels of Clostridia in the colic cases, which suggested the increased levels of the bacterium in the EGS cases, is not just due to a dys-motility in the gastrointestinal tract, but more specifically related to EGS pathogenicity (Waggett et al., 2010).

Although *C. botulinum* has been isolated from faeces in Hunters 1999 study (Hunter et al., 1999), and Tochers 1919 study (Tocher et al., 1923), the more recent study in 2018, using faecal biomarkers, found a very low abundance of Clostridia species in EGS horses compared with MC (match control) and co – grazers. This opposes previous findings and contradicts Hunter's findings in 1999 (Hunter et al., 1999), providing no evidence that *C. botulinum* plays a role in equine grass sickness's pathogenesis (Leng et al., 2018).

C. botulinum is a known soil born bacterium, and with examination of the of the soil on affected pastures in 2001 there was evidence of the presence of botulism neurotoxins and the bacterium on the pastures (Bohnel et al., 2001), as well as studies finding that looser and more manipulability of the soil, increased the risk of horses grazing here to contact the disease. Evidence found surrounding soil manipulation and faeces removal showed this further increased the risk of grazing horses contracting equine grass sickness (Newton et al., 2004). These findings, although need further investigation and experimentation, suggest that *C. botulinum* could play a role in the pathogenesis of EGS.

When looking at other animal dys-autonomias such as feline dys-autonomia and rabbit dys-autonomia, we can see more positive evidence that *C. botulinum* plays a role in their pathogenesis. An experiment in 2004 managed to isolate *C. botulinum* from faeces and ileal samples from 7 out of 8 cases after enrichment, and found high levels of IgA antibodies in these samples of the infected cats when compared with the controls (Nunn et al., 2004). Similarities in the histological lesions of rabbit and equine dys-autonomia, were seen in the 2005 experiment. Damage such as chromatolysis was evident in the autonomic ganglions, especially the enteric nervous system, which mirrors histological lesions found in horses who were diseased with EGS. *C. botulinum* was also isolated from the gut of these rabbits (Hahn et al., 2005). These findings fully support the hypothesis that *C. botulinum* plays a key role in EGS pathogenicity.

The clinical signs of EGS have some relation to those of botulism, supporting the theory that *C. botulism* may have a role in grass sickness's pathogenesis, but on the other hand the macroscopic and microscopic pathological lesions show very little correlation between the two diseases, contradicting the theory. The microscopic lesions of the EGS samples show neuronal degeneration of the autonomic nervous system and specifically the enteric nervous

system, where under light microscope we can see chromatolysis of the neurons with a lack of Nissl substance, and the nucleus is pyknotic and eccentric. The botulism and control samples do not show these changes, suggesting *C. botulinum* does not play a role in the pathogenesis of EGS (McGorum et al., 2015). These typical signs now used to diagnose equine grass sickness, with the samples being taken via laparoscopy from the ileum.

In my opinion *C. botulinum* has a very high chance of causing EGS. I believe the theory that is a caused by a toxico-infection (Hunter et al., 1999), where the BoNT is produced in the gastrointestinal tract, is a very valid hypothesis. The examinations involving antibody levels against the surface antigens and the BoT/NT (Hunter et al., 2001), as well as isolation of the bacterium from faeces and GI content (Hunter et al., 1999, Waggett et al., 2010), I believe are very supportive of the *C. botulinum* theory. With regards to vaccine trials, there could have been very promising results, but unfortunately evidence provided did not match the research performed on antibody levels, although you could argue that during the vaccine trials, not enough cases of EGS occurred, so the results are not reliable. You could also argue that there is uncertainty of the efficacy of the vaccination used, and whether it is efficient against an actual botulism infection a horse, as this is something that we do not routinely vaccinate against in the UK. If another vaccine trial was carried out over a longer period of time, with a higher number of horses grazing on the high risk pastures, we may see different results. This could be something to think about for future research.
With regards to faecal samples and isolation of the bacterium from the GI tract, I believe further investigation will need to be carried out with higher number of cases to sample from, to confirm that *C. botulinum* plays a leading role in the pathogenesis of equine grass sickness.

7. Summary

The cause of EGS has been a mystery for over 100 years when it was first described in eastern Scotland in 1904. Many hypotheses have been proposed such as mineral components of the soil etc. The most recognised hypothesis being that *Clostridium botulinum* type C may play an unknown role in EGS pathogenesis, most likely a toxico-infectious type, in which exotoxins are produced within the GI tract. Much evidence supports the theory, but the aetiology has not been confirmed.

The first evidence indicating that *C. botulinum* could play a role in the development of EGS, was in the early 1900's when Tocher isolated the bacterium from the gut of infected horses (Tocher et al., 1923). Further evidence of this was seen in 1999 where again *C. botulinum* was isolated from the faeces and the gut of horses affected with EGS, although evidence investigating faecal biomarkers showed opposing results, where there was significantly lower abundance of the *Clostridia* group in the affected horses compared with controls. *C. botulinum* was also isolated from the faeces and the gut of cats and rabbits suffering from feline and rabbit dys-autonomias. These diseases present clinically in a very similar manor to equine grass sickness.

Experiments looking at soil types showed positive evidence towards the theory. The looser, sandier soils showed a higher prevalence of EGS, which meant *C. botulinum*, a known soil bacterium, was able to reach grazing level more easily. *C. botulinum* was also isolated from a number of infected pastures, as was BoNT, although they did not coincide.

Experiments involving *C. botulinum* antibody levels proved very promising in proving the *C. botulinum* theory. Infected animals had very low levels IgG's against the BoNT/C and surface antigens, whereas healthy co-grazers, and horses grazing on high risk pastures had much higher levels which indicated animals could develop immunity to EGS. Following this a recent vaccine trial was looking promising, but unfortunately due to low levels of EGS cases the results appeared inconclusive, and did not show any evidence that horses could be protected from EGS by a vaccine containing *C. botulinum*.

IgA levels were also taken from the gut, which contradicted the serum IgG levels taken in the previous experiment. Here they were higher in the EGS cases when compared with

controls. These findings are more likely to show the current immune response to a recent exposure when compared with systemic IgG, as the IgA antibodies have a shorter half-life, and although little is known about equine gastrointestinal immunity (Nunn et al., 2007).

It is clear from the work in this text that although there is valid and promising research proving that *C. botulinum* could potentially play a role in EGS pathogenicity, still further experiments need to be undertaken. It seems the case number in each experiment is often not high enough to make a valid conclusion regarding the cause of EGS, and that any future research would benefit from cases taken from a larger number of horses, grazing on known high risk pastures.

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9. Acknowledgements

I would like to say a massive thank you to the Equine Grass Sickness Society in the UK, who provided me with lists of endless articles and papers regarding my topic, as well as putting me in touch with Veterinarians who specialise into the research of Equine Grass Sickness. I would also like to thank them for their encouragement and support, and for taking an interest in my thesis.
I would also like to thank in particular Professor Ian Poxton, who was available to answer any questions I had.

I'd like to take this opportunity to thank the University of Veterinary Medicine Budapest for high level of education I have received, especially all the members of staff who work tirelessly every day. I'd like to say a huge thanks to Peter Lessi, for always being there to answer any questions I had over the years, and for all his hard work.

I would also like to thank my family for all their support over the years, not only financially but the encouragement I was given, and emotional support. Also thank you to my friends, who became like family over the years, for all the help and support we have given each other.

Also I would like to say thanks to Mike Barrott, Daniel Carroll and all the vets of Cinderhill Equine Veterinary Clinic, who have been extremely encouraging and helpful not only during my studies, but prior to beginning university.

Finally, a massive thank you to my thesis supervisor, Dr. Fodor László, who has been nothing but enthusiastic and supportive.

Appendix 4.

I hereby confirm that I am familiar with the content of the thesis entitled "Exploration of the evidence supporting and opposing the role of *Clostridium botulinum* in the pathogenesis of Equine Grass Sickness" written by Rosie Ansell which I deem suitable for submission and defence.

Date: Budapest, 15th November 2020

László Fodor
Department of Microbiology and Infectious Diseases

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