Investigation into the Presence of *Helicobacter* in the Equine Stomach by Urease Testing and Polymerase Chain Reaction and Further Investigation into the Application of the ^{13}C-Urea Blood Test to the Horse

by

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INVESTIGATION INTO THE PRESENCE OF *HELICOBACTER* IN THE EQUINE STOMACH BY UREASE TESTING AND POLYMERASE CHAIN REACTION AND FURTHER INVESTIGATION INTO THE APPLICATION OF THE ^{13}C-UREA BLOOD TEST TO THE HORSE

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(ABSTRACT)

Equine gastric glandular mucosal ulceration can have a prevalence of 58%, yet its etiology is poorly understood. In man *Helicobacter pylori* is the most common cause of gastritis and peptic ulcer disease. *Helicobacter* is uniquely able to colonize the stomach, via the action of cytoplasmic urease. Different *Helicobacter* species have been isolated from many mammals but none has yet been cultured from the horse. Three tests used to identify human *Helicobacter* infection were applied to the horse. Test 1: PCR amplification of *Helicobacter* specific DNA, n=12. Test 2: the Pyloritek™ rapid urease test (RUT), n=15. Test 3: the ^{13}C-urea blood test, n=8. Gastroscopy and antral biopsy was performed in all horses.

All horses demonstrated the presence of *Helicobacter* specific gene material by PCR. Biopsy specimens from 7/15 horses were urease positive by RUT. Significant ^{13}C enrichment of the body CO_2 pool was found in all horses after intragastric administration ^{13}C-urea ($p<0.05$). As *Helicobacter* is currently the only known gastric urease positive microorganism, the demonstration of this activity in horses positive by PCR strongly supports the presence of an equine gastric *Helicobacter* species.

Variations of ^{13}C-urea blood test were further examined and a single protocol was found to be most applicable. As the horse is a hind gut fermenter, the effect of cecal urease on the test was examined by laparoscopic intracecal administration of ^{13}C-urea. Significant cecal urease activity was demonstrated however the timing of peak ^{13}C enrichment may limit any effect on the gastric test to 90 minutes onwards.

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LIST OF ABBREVIATIONS

HCl = Hydrochloric acid

NSAID = Non-steroidal anti-inflammatory drug

UreI = Urea channel I specific to gastric dwelling *Helicobacter*

ECL = Enterochromaffin like cells

HIST = Histamine

5-HT = Serotonin

cAMP = Cyclic adenosine monophosphate

ATP = Adenosine triphosphate

Ach = Acetylcholine

PG = Prostaglandin

MW = Molecular weight

CCK = Cholecystokinin

NO = Nitric oxide

NOS = Nitric oxide synthase

cGMP = Cyclic guanosine monophosphate

VFA = Volatile fatty acids

COX = Cyclooxygenase

TNF = Tumor necrosis factor

EGUS = Equine gastric ulcer syndrome

$t_{1/2}$ = Time for one half life

t_{max} = Time to maximum serum concentration

ureI = 16S gene encoding for UreI

mRNA = Messanger RNA

LPS = Lipopolysaccharide

IL = Interleukin

NF-κB = Nuclear Factor κB

IκB = Inhibitory factor κB

MAPK = Mitogen activated protein kinase

PCR = polymerase chain reaction

RUT = Rapid urease test

PDB = PeeDee Belemnite limestone

Pyl = Pyloritek™

ΔC = Absolute variation in ^{13}C enrichment from the international standard, PDB

ΔOB = Difference in ^{13}C enrichment of a sample at given time, when compared to baseline

INTRODUCTION

Equine gastric ulceration has long been identified at post mortem, where it was often described as an "incidental, inconsequential finding".[1] The equine stomach is comprised of both squamous and glandular mucosa, the junction of which is the *margo plicatus*, an area analogous to the human gastro-esophageal junction. Lesions of the squamous mucosa most closely resemble the esophagitis and esophageal erosion seen in man and as such reflect peptic (HCl and pepsin) injury to the poorly protected squamous mucosa.[2,3] In the horse squamous lesions are more commonly reported than glandular lesions and can have a prevalence as high as 90-100% in Thoroughbred racehorses in active training.[4-9] A variety of factors have been shown to influence the development of these lesions including feeding practices, stall confinement, exercise, stress and non-steroidal anti-inflammatory drug (NSAID) administration.[9-17]

Historically endoscopic equipment limitations have prevented a thorough examination of the glandular mucosa of adult horses and as such lesions have been less frequently described. Recently the prevalence of glandular mucosal lesions was reported as 58%, with the pyloric antrum most commonly affected.[18] Interestingly in this study the prevalence of squamous lesions was also 58% although there was no association between the presence or absence of pathology in each mucosa in individual animals. A second study has suggested that glandular lesions of the body can easily be missed by poor endoscopic technique, raising he possibility that the incidence of pathology in this part of the stomach may be greater than previously thought.[19]

Glandular lesions are thought to result from impaired mucosal defense mechanisms rather than primary peptic injury. This is supported by the observation that the feed deprivation models used to create squamous injury do not produce glandular lesions.[10,11] A similar observation was made when assessing the effect of exercise on squamous ulceration where only a few glandular lesions were found.[7] Glandular lesions have been successfully induced in the horse using excessive administration of NSAIDs however no other causes of glandular pathology have yet been determined.[20]

In man gastric ulceration occurs in the antrum, pre-pyloric region and less commonly the body of the stomach.[21] The majority of cases of gastritis and peptic ulcer disease are associated with *Helicobacter pylori* infection, the remainder being associated with NSAID administration or gastric adenocarcinoma, which itself is associated with chronic *Helicobacter* infection.[22-25] Gastric dwelling *Helicobacters* are acid tolerant neutralophiles that are uniquely able to colonize the gastric mucosa due to the ability to regulate intracytoplasmic pH via the action of the enzyme *urease*. Urea passage into the bacterial cell is facilitated by a proton activated channel, UreI which is vital for gastric colonization.[26,27] *Helicobacter pylori* is thought to infect half the world's human population, although most people are asymptomatic, and a variety of other *Helicobacter* species have been described in the domestic species and been associated with gastric pathology.[25,28,29]

Traditionally *Helicobacter* was not thought to be present in the equine stomach, although why such a *carte blanche* assumption was made is unclear.[30] Whilst it is true that *Helicobacter* has never been cultured from the equine stomach, an increasing amount of recent circumstantial evidence suggests that it is present.[31-34] In this study we demonstrate the presence of significant urease activity in the stomachs of clinically normal horses, positive by PCR for *Helicobacter* gene material, using the ^{13}C-urea blood test and the Pyloritek™ rapid urease test strip. Further investigation is then made into the application of the ^{13}C-urea test to the horse, including assessment of the effect of hind gut microbial activity on this test. In all studies the null hypothesis (H_0) was that urease activity is not present in the equine stomach.

PREVALENCE

The first scientific report of equine gastric ulceration was in 1986, detailing the examination of 195 racehorses at post mortem in Hong Kong.[35] Ulceration of the squamous mucosa was found in 66-80% horse's euthanized during training and in 52% of animals who had been retired for one or more months prior to euthanasia. The first report of a method for the ante mortem diagnosis of gastric ulceration was also made in 1986.[36] This technique used a 2m, 9.5mm outer diameter human colonoscope and permitted examination to the level of the pyloric antrum in small horses, but only to the cardia in larger animals. 187 horses were examined of which 87 had clinical signs of gastrointestinal disease. Lesions were most commonly reported in the squamous mucosa of the lesser curvature. In general the prevalence of lesions was much greater in symptomatic horses than asymptomatic ones, and in horses in training compared to those not in training. In general lesions of the squamous mucosa were more commonly reported than glandular lesions, reflecting the limitations of the endoscopic equipment available at that time.

The prevalence of gastric ulceration varies with the breed, use and level of training of the horse. In most reports of gastric ulceration in adult horses, Thoroughbreds have been the predominant breed in the study population. In Thoroughbred racehorses the incidence of gastric ulceration has been reported as high as 100% in animals that had raced, and 93% in horses in training.[4] Overall the prevalence in this population varies between 55 to 100%.[4-9,35] Within this group a slightly higher percentage of gastric ulceration has been reported in geldings (94%), than colts (78%) or fillies (82%).[9] The severity of ulceration is greatest in animals in training and lesions tend to worsen during a training period, particularly at the start. When training is stopped improvement in gastric lesions can occur in some horses.[37] In Standardbred racehorses in training, gastroscopy has shown a similar prevalence of ulceration of 87%, with the risk of ulceration increasing with age in castrated males and decreasing in females and sexually intact males.[38] A separate study of Standardbreds out of training showed an overall prevalence of ulceration of 44%.[39] In show horses who are in active training and have competed in

the 30 days prior to endoscopic examination a gastric ulceration prevalence of 58% is reported.[6] The lowest incidence occurs in elite western performance horses where the rate of gastric ulceration (40%) is similar to that seen in horses not in training or use (36%).[5,40]

All the preceding figures of the prevalence of equine gastric ulceration have included observations of both the squamous and glandular mucosa. Most reports have concentrated on the squamous mucosa either by the necessity of using a 2m endoscope or by choice. Recently endoscopic findings from the equine gastric antrum and pylorus were reported.[18] Erosion or ulceration of the glandular mucosa was seen in the antrum or pylorus in 58% of horses and in the body of the stomach in 8% of horses. The population of this study population was varied, including horses in race training, show horses, event horses and pleasure horses. The prevalence of lesions in the squamous mucosa reflected this and was identical to that of the glandular mucosa (58%) however no association was found between the presence of lesions in the squamous and glandular mucosa; or in the severity of lesions at each site. Hence it is not possible to infer either the presence or severity of lesions of the glandular mucosa based on the appearance of the squamous mucosa. This highlights the need for suitable equipment and a thorough examination of the entire stomach. Further evidence for the need for a through examination was shown by a recent comparison of endoscopic findings with subsequent necropsy and histology.[19] Endoscopy was judged to be a good method of identifying squamous ulceration as all horses that had ulcers in this portion of the stomach endoscopically had them at necropsy. Conversely ulceration of the glandular mucosa was frequently missed endoscopically. The glandular lesions found at necropsy were frequently less than 5mm diameter and may have been missed because of the presence of feed material, gastric juice or inadequate insufflation. Lesions in the glandular body of the stomach were most frequently missed. At necropsy a total of 5/23 horses had glandular lesions here, representing a prevalence of almost 22%.[19] This raises the possibility that the previously described endoscopic prevalence of lesions at this site (8%)[18] may be falsely low. The mean ulcer grade ±SD at this site (0.3±0.7) is therefore also likely to be low.[18] As most of

the lesions reported at necropsy were small (<5mm)[19] the correct glandular ulcer grade in each case would most likely be 1-2, and the mean grade closer to 1.

EQUINE GASTRODUODENAL ANATOMY AND PHYSIOLOGY

Mucosal Anatomy of the Equine Stomach

The equine stomach is single chambered.[41] The mucosal lining is comprised of four histologically distinct regions (fig. 1). The orad one-half of the stomach is lined with squamous mucosa that is similar to the esophageal lining. The squamous epithelium of normal horses is of varying thickness (309-1154μm).[42] This stratified squamous epithelium (fig. 3) is histologically without glandular structures or mucosal protective mechanisms, and by Ussing chamber determination has no active transport mechanisms. The teleological explanation for this functionally inert lining is unknown. This area may allow some fermentative carbohydrate digestion by gastric bacteria to occur as it is normally not exposed to acid. Acetic, butyric and propionic acids have been measured in the stomachs of horses fed both grain and hay diets.[17,43] Histological examination of normal squamous mucosa shows an absence of inflammatory cells within the epithelium and lamina propria.[42]

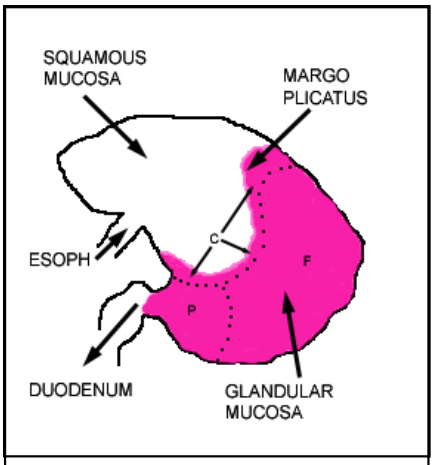


Fig 1. Anatomic Regions of the Equine Stomach. (C – Cardia, F – Fundus, P – Pylorus)

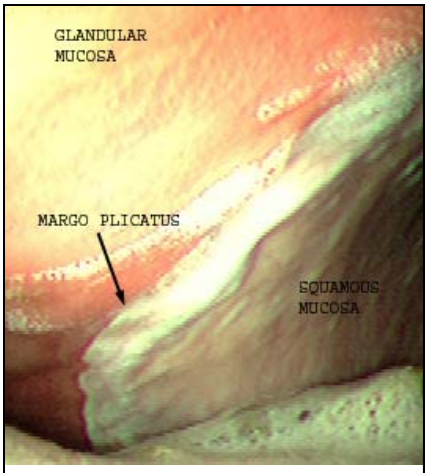


Fig 2. The Lesser Curvature of the Stomach

The squamous epithelium ends abruptly at the *margo plicatus*, a visibly defined junction with the glandular mucosa that lines the aborad half of the stomach (fig. 2).[41]

Glandular mucosa is a compound columnar glandular (fig. 4) that contains frequent invaginations known as gastric pits, the base of which (isthmus) continues into the opening of one or more gastric glands. The luminal surface of the stomach contains cells that produce thick, tenacious mucus. Mucus is also produced by neck mucus cells, found in the neck of the gastric glands.

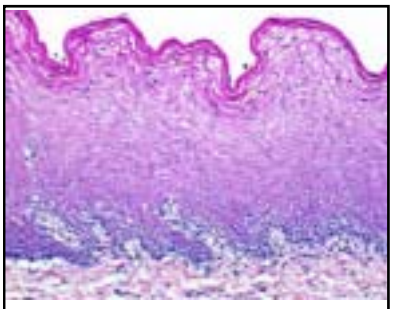


Fig 3. Equine Gastric Squamous Mucosa (H&E Stain)

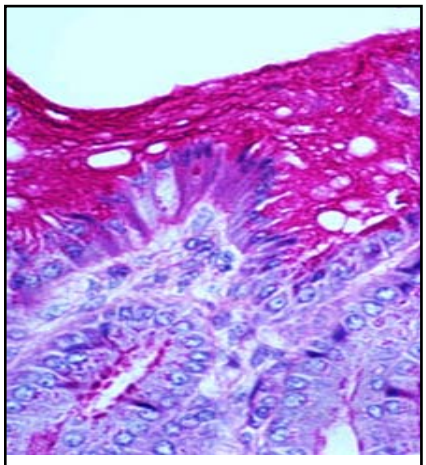


Fig 4. Equine Gastric Glandular Mucosa (H&E Stain).

Both images courtesy of Dr. Mike Murray

The glandular mucosa is divided into three regions with characteristic cell types:

1. **Cardiac Gland Region**.[41,44,45] This narrow strip of mucosa is immediately adjacent to the *margo plicatus* The role of this region in the horse is unknown. In other species a variety of functions have been described. The primary secretion is believed to be sodium bicarbonate, although the importance of this to gastric function is unknown. Large numbers of somatostatin immunoreactive cells have also been shown in this region of the equine stomach. These cells may allow the cardiac gland region to function as an intragastric pH sampling site that modulates G-cell gastrin release and so is involved in the endogenous control of gastric acid secretion.

2. **Fundic Gland Region** (Parietal mucosa).[41,46] This is the largest part of the glandular mucosa and it occupies the ventral stomach along the greater curvature and up the sides of the stomach to the cardiac gland region. The fundic region is the site of the

parietal cells, zymogen (or chief) cells and enterochromaffin like cells (ECL) that make up the "gastric glands". Parietal cells are found throughout the straight, tubular gastric glands but are most numerous in the middle portion. They secrete hydrochloric acid under the stimulation of histamine (HIST), which is produced in a paracrine fashion by the ECL cells. ECL cells also modulate gastric mucosal blood flow via the action of serotonin (5-HT). Amongst the parietal cells are mucus neck cells which secrete thin mucus. The mucus neck cells are the only cells of the stomach lining capable of division and they appear to be the progenitor cells for the gastric mucosa. Gastric glands also contain cells that secrete bicarbonate directly onto the mucosal surface. These combine to form the mucus curtain which is important in maintaining mucosal defense against the corrosive actions of HCl, pepsin and the volatile fatty acids produced by gastric digestion. The zymogen cells secrete pepsinogen into the gastric lumen. D-cells that secrete somatostatin are also present.

3. **Pyloric Gland Region**.[41,46,47] This mucosa lines the gastric antrum and the pyloric outflow (fig. 5) and is distinct from the fundus as the gastric glands are of branched appearance. The majority of cells within these parietal mucosal glands secrete mucus directly into the gastric lumen, producing the mucus curtain. The gastric glands of this region are branched. Gastrin producing G-cells, somatostatin producing D-cells and 5-HT producing ECL cells are scattered amongst the parietal mucus cells. The ECL cells are over twice as numerous as in the fundus. D-cells and G-cells have a close anatomical and physiological relationship within the mucosa and are involved in endogenous modulation of gastric pH.

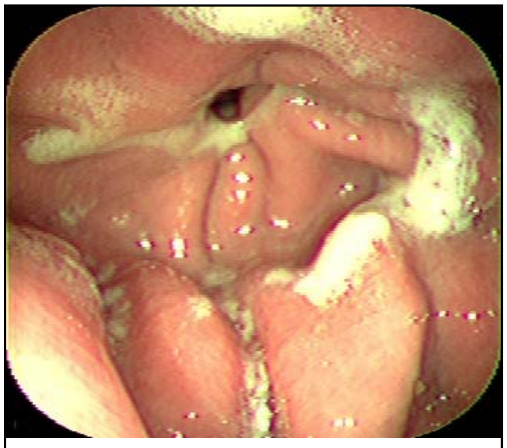


Fig 5. The Equine Pylorus and Rugal Folds of the Glandular Mucosa

The gastric juice is a combination of parotid, gastric, biliary and pancreatic secretions.[41] Basal gastric fluid secretion in the horse is 5.5ml/kg/h, which increases to

11.4ml/kg/h under maximal stimulation. Combined parotid, biliary and pancreatic secretion is ~30L/d in 100kg adult ponies. Gastric juice contains a variety of substances including the cations Na^{+}, K^{+}, Mg^{2+} and H^{+}, the anions Cl^{-}, HPO_4^{2-}, and SO_4^{2-}, pepsins, lipase, mucus and intrinsic factor. Surface mucus cells also secrete HCO_3^{-} however this is trapped in the mucus gel. For further description gastric juice can be divided into parietal and non parietal secretions.

PARIETAL SECRETIONS

Hydrochloric acid[48-51]

Hydrochloric acid kills many ingested bacteria, activates pepsin, aids protein digestion and stimulates the flow of pancreatic juice and bile. The horse is a continuous, variable secretor of gastric acid. The parietal glands secrete an isotonic solution of HCl with a pH <1 into the gastric lumen. Each parietal cell has numerous apical cannaliculi that extend down into the cytoplasm and increase the surface area available for acid extrusion. The method of secretion is different for H^+ and Cl^- (fig 6).

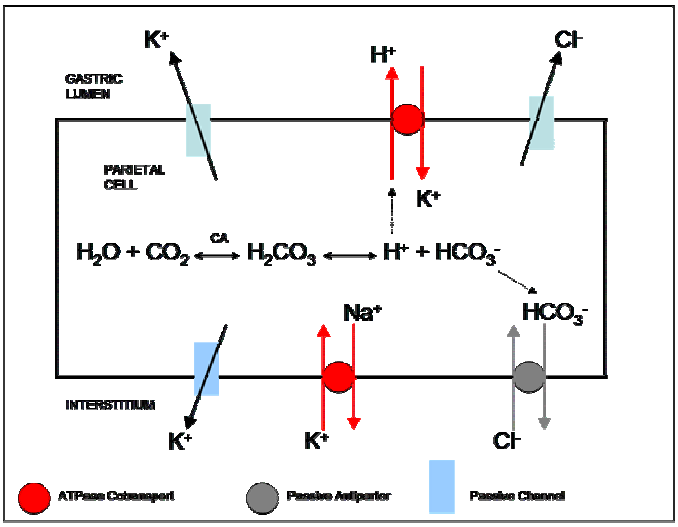


Fig 6. Cellular Transport Mechanisms of the Gastric Parietal Cell

Hydrogen ions are actively secreted by a H^+/K^+ ATPase "proton pump". In the resting cell this pump is contained within coiled cytoplasmic tubulovesicular structures. When the parietal cell is activated increased cAMP and $[Ca^{2+}]_{ic}$ drive the movement of the tubulovesicles to the apical membrane. Here they fuse with the membrane and produce microvilli that project into the cannaliculi of the gland. Hydrogen ions for secretion come from the dissociation of intracellular carbonic acid into H^+ and HCO_3^-. Carbonic anhydrase catalyses the formation of carbonic acid from water and carbon dioxide in the cytosol. The H^+ concentration gradient opposing the proton pump is great and so the process is energy intensive. Outwardly rectifying potassium channels are present on the apical membrane. In the resting state these are inhibited by ATP. Activation of ATPase removes this inhibition and opens the channels allowing potassium to leave the cell down the concentration gradient established by the proton pump. Outwardly rectifying apical

chloride channels open and Cl^- leaves the cell down the strong electrical gradient established by the H^+ transport. Parietal cell secretion is a pure form of HCl that contains 150mEq H^+ and 150mEq Cl^- per L. A bicarbonate ion is left within the cell for each H^+ that is secreted. To prevent accumulation the bicarbonate ion is extruded across the basolateral membrane by a HCO_3^-/Cl^- antiporter. This efflux of HCO_3^- into the blood peaks after a meal as the postprandial alkaline tide.

Secretion of HCl by the parietal cell is stimulated by HIST via H_2 receptors, acetylcholine (ACh) via M_3 muscarinic receptors and gastrin via gastrin receptors. H_2 receptors have dual signaling pathways. One group is G-protein coupled to adenylyl cyclase and exert their effects by increasing cAMP, the second activates phospholipase C and increases $[Ca^{2+}]_{ic}$. Gastrin and ACh also act by this system. Cyclic AMP and Ca^{2+} act via protein kinases to increase H^+ transport. The main pathway by which gastrin stimulates acid secretion is via ECL cells. These are the predominant endocrine cells in the glandular mucosa and act to stimulate acid secretion in a paracrine fashion as gastrin and potentially ACh stimulate the release of HIST.

Gastric acid secretion is inhibited by somatostatin and prostaglandin (PG) E. Somatostatin, produced by D-cells in the fundus and antrum, inhibits the release of gastrin from G-cells, HIST from ECL cells and the secretion of HCl from parietal cells. Somatostatin functions in an autoregulatory fashion as decreased luminal pH stimulates release and increased pH inhibits it. Prostaglandin E_1 and E_2 are inhibitory G-protein coupled and so antagonize HIST stimulation of adenylyl cyclase and any subsequent increase in cAMP.

In the horse a gradient of acidity exists from the squamous mucosa of the fundus (pH5.46±1.82), to the squamous mucosa at the margo plicatus (pH 4.12±1.62), the glandular mucosa of the pylorus (pH3.09±1.9), with the gastric juice being most acidic (2.72±1.86).[52] In another study feed deprivation was shown to increase the acidity of the gastric juice from a median pH 3.1 to 1.55.[53]

Pepsins[48-51]

The chief cells store pepsinogens, the proenzymes of the pepsins, in cytoplasmic zymogen granules. Secretion is stimulated by gastrin and possibly neuropeptide YY (enterogastrone). In man two pepsinogens are secreted: pepsinogen I (found only in the acid secreting regions) and pepsinogen II (found in both acid and non acid secreting regions). Maximal acid secretion correlates with pepsinogen I levels. At pH <4.0 the pepsinogens become active pepsins that hydrolyze the bonds between aromatic amino acids, breaking down proteins into peptones. Their activity terminates in the duodenum and proximal jejunum where pH is raised to approximately 6.5. The effect the large amounts of duodenal reflux commonly seen in the horse have on pepsin activity is unknown.

Mucus[48-51,54-62]

Gastric mucus is a complex mixture of glycoprotein's, water, electrolytes, antibodies and lipids that forms a hydrophobic flexible gel that covers the gastric epithelium in a continuous layer 100-400µm thick. Mucus is secreted by neck and surface mucus cells and is predominantly composed of glycoprotein units called mucins. Each mucin is a very large biopolymer consisting of a central core peptide with radially arranged oligosaccharides. Each monomer has a MW~$2x10^6$ and at typical gastric concentrations will form extended polymers that interact in 5% aqueous solution to form a gel. Most of the gel consists of water (~95%) that binds to the oligosaccharides forming a substantial barrier to diffusion through the interstices of the gel. Gastric mucus gels are able to significantly retard H^+ diffusion when compared to aqueous diffusion. The formation of gastric mucin gel is pH dependent; at pH 2 a firm viscid gel occurs whereas a loose solution forms at pH 7. Light scattering studies have shown that as pH is lowered gastric mucin changes from a random coil to an extended configuration in which cryptic hydrophobic domains in the coiled peptide are gradually exposed to the bulk solution. These domains form hydrophobic bonds between adjacent monomers resulting in formation of a viscous gel. Further hydrophobicity is provided by mucus associated

phospholipids which form disulfide bridges, enhancing acid resistance. Trefoil peptides interact with the mucin glycoprotein further strengthening the gel forming and protective properties of the mucus layer.

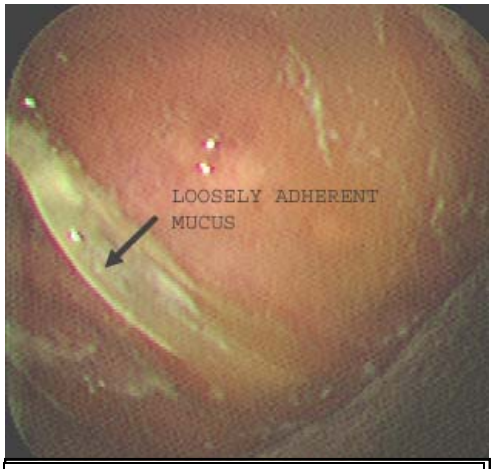


Fig 7. Loosely Adherent Mucus in the Equine Gastric Antrum

Three mucus types are present within the stomach. Soluble mucus is found in the lumen of the stomach and two layers of mucus adhere to the gastric mucosa. The most luminal of the two mucosal layers has been designated the loosely adherent layer as it can easily be removed by suction or cotton tip swab (fig. 7). In vitro removal of the loose layer does not alter pH at the epithelial surface. This layer is thought to lubricate food particles and to bind bacteria. The inner layer is thinner and firmly and continuously attached to the mucosa. This layer is considered the unstirred layer. The physiologic properties and importance of these layers is not currently known.

Multiple pathways for the stimulation of gastric mucus production exist.[63] Secretion of high molecular weight glycoproteins from rabbit fundic and antral explants has been shown in response to acetylcholine (Ach), histamine, and PGE_2. Intracellular Ca^{2+} appears to act as a secondary messenger in these pathways as removal of extracellular Ca^{2+} or administration of a calmodulin antagonist will abolish secretion.

Cholecystokinin (CCK) and the gastrin family of peptides (gastrin, pentagastrin, tetragastrin) are able to stimulate mucus production and the gastrins can act via both gastrin and CCK receptors.[64] Increased mucus production in response to nitric oxide (NO) stimulation has been shown in rat gastric mucosa and was associated with increased intracellular levels of guanosine 3',5'-cyclic monophosphate (cGMP).[65] Calcium dependent constitutive NO synthase (NOS) activity has been found in gastric mucous-epithelial cells suggesting an effector role for NO in mediation of gastric mucus secretion.[66] NO may also be implicated in the cholinergic activation of gastric mucus secretion.[67] Thinning of the firmly adherent mucus layer has been observed after

experimental administration of indomethicin, resulting in direct physical trauma of the gastric epithelium by solid food particles and confirming the importance of prostaglandins in mucus production.[68]

Bicarbonate

Two bicarbonate systems exist. Physiologically the most important component of juxtamucosal neutralization is the blood borne bicarbonate system.[61] For each H^+ produced by the parietal cell and secreted into the gastric lumen, one HCO_3^- is formed and is transported into the blood. The gastric microvasculature is set up to carry this from the parietal cells to the surface cells.[69,70] Some diffusion through the interstitium also occurs.[61] Bicarbonate enters the surface epithelium across the basolateral membrane in co-transport with sodium. Transport across the luminal membrane is dependent on luminal Cl^- and occurs by way of a Cl^-/HCO_3^- co-transporter (fig 8).[71,72] This is in contrast to the parietal cell where basolateral Cl^-/HCO_3^- exchange occurs.

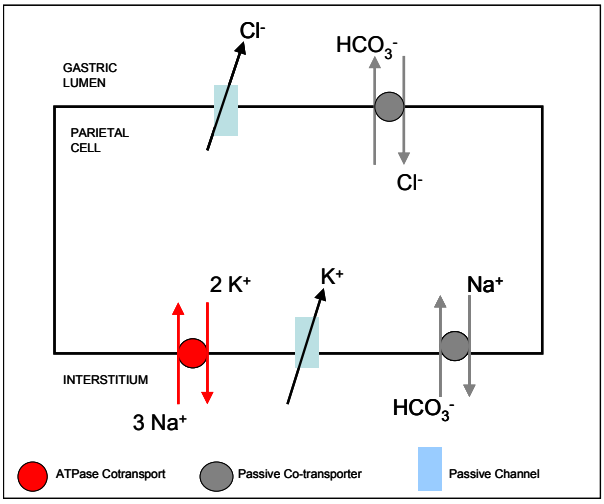


Fig 8. Proposed Mechanism of Secretion of Blood Borne Bicarbonate by Gastric Glandular Epithelial Cells

Physiologically less important is PG-stimulated secretion.[61] Prostaglandin E_2 stimulates bicarbonate secretion by the glandular mucosa of the fundus and experimental inhibition of PG synthesis with indomethicin will reduce this.[73] Prostaglandin stimulated release appears to play only a minor role in juxtamucosal neutralization and pathologically is important only when acid secretion is inhibited and the loosely adherent mucus layer is removed.[61]

Acid Transport Through the Mucus Layer

Acidified spots are observed in the mucus layer above the outlets of the gastric crypts suggesting that acid is transported across the mucus layer only at restricted sites. In

vitro injection of HCl under pressure into mucin produces "viscous fingers".[74] In vivo studies have shown high intraglandular pressures during periods of acid secretion.[62] This pressure may be generated by the muscle fibers which surround the gastric pits.[75,76] Pressure can only be generated against a resistance and this is thought to be generated by constriction of the luminal opening simultaneously as the gland is squeezed. This behavior of acid when injected into a viscous gel is totally different to the free mixing that occurs after injection into a liquid. Maintenance of mucus viscosity by bicarbonate secretion is crucial to acid tunneling as this ceases at pH <4, when viscosity becomes excessive.[74] Thus HCl and pepsinogen, secreted under pressure, form and then pass through slender, thread like channels 5-7μm wide within the surface mucus layer, enabling secreted acid to reach the gastric lumen without disrupting the mucus pH gradient[60] Complete blockade of acid secretion by omeprazole stops formation of these acid channels. Congo red absorbance experiments have shown that the loosely adherent mucus and the mucus within the acid channels consist of the same mucins.[60] This suggests that mucus from neck cells is pushed out of the gastric crypts into the acid channels and then onto the adherent mucus layer to form the loosely adherent layer.

NON PARIETAL SECRETIONS

The first evidence for a large nonparietal fluid component of equine gastric juice was shown by pentagastrin stimulation of gastric secretion.[48] Horses' only increase mean acid concentration, at maximal output, to 60mmol/l, which is less than the 90-100mmol/l seen in other monogastrics. It also results in a steady sodium concentration whilst acid concentration rises, as opposed to the expected fall. This suggested that pentagastrin induced nonparietal as well as parietal secretion in the horse.

Gastroscopic examination of fasted horses supports the presence of a large nonparietal fluid component, which appears to originate from the duodenum (fig. 9).[18] During gastroscopy the gastric juice is quite yellow in color indicating the presence of bile, which can often be seen refluxing through the pyloric sphincter. The pyloric sphincter itself is frequently open. Examination of the interior of the proximal duodenum often demonstrates a large stream of pancreatic juice from the diverticulum duodeni, into which the pancreatic duct empties. This finding allied to the effect of pentagastrin strongly suggests that the nonparietal fluid is of pancreatic origin.

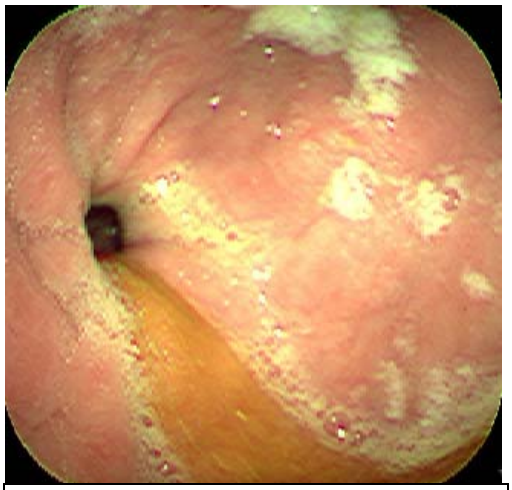


Fig 9. Duodenal Reflux Through the Partially Open Pylorus of a Horse

This watery fluid is rich in sodium and its reflux into the stomach results in periods of spontaneous alkalinization where pH 6.0-7.5 occurs for up to 5 minutes.[53,77,78] This duodenogastric reflux occurs when action potential activity in the antrum ceases, causing pyloric relaxation, coincident with a phase III duodenal contractile event.[79]

MUCOSAL BARRIER FUNCTION

Squamous Mucosa

The squamous mucosa relies mainly upon limited exposure to gastric acid as surface barrier function is limited.[3] The average pH in the dorsal portion of adult horses is 5.5, reducing to 4.1 adjacent to the margo plicatus.[5] Mucopolysaccharides and intercellular tight junctions in the superficial epithelium are the only defense against H^+ entry. Minimal buffering capacity if offered by the intercellular secretion of HCO_3^- and by intracellular buffers.[80-82] Penetration of H^+ deeper into the stratum spinosum leads to cellular death as H^+ is concentrated intracellulary.[82] Adequate mucosal blood flow is required for removal of volatile fatty acids produced by intragastric fermentation of carbohydrate.[83] Interestingly inhibition of cyclo-oxygenase, but not lipoxygenase enhances resistance of esophageal mucosa to acid-pepsin injury suggesting that leukotrienes not prostaglandins are important in squamous epithelial protection.[84]

Glandular Mucosa

The glandular mucosa is covered by a continuous 200-300μm thick sheet of viscoelastic mucus. The mucus provides as a physical protection against ingested particles and digestive enzymes as well as acting as a barrier against luminal acid.[85,86] The mucus contains hydrophobic surface active phospholipids and so is able to repel aqueous HCl.[87] This mucus layer also traps bicarbonate secreted by surface mucus cells and gastric glands creating a pH gradient from the gastric lumen to the surface epithelial cells in the order of magnitude of 100,000.[88] This represents an epithelial pH of 6.5 with a luminal pH of 1.5.

Adequate blood flow is vital for the removal of H^+ and pepsins from the mucosa, and the supply of oxygen and nutrients required for epithelial cellular restitution. This involves migration of cells from the gastric pits to the surface to repair mucosa lost due to irritant injury. Blood flow is regulated by NO, and by PGE_1 and PGE_2.[89-91] PGE_2 analogues can also inhibit gastric acid secretion, increase mucosal blood flow and

promote mucus/bicarbonate secretion at pharmacological doses.[92-94] Some of the resistance of the glandular mucosa to autodigestion is provided by the presence of acid resistant trefoil peptides. Gene knockout mice without these peptides have histologically abnormal mucosa and have a high incidence of mucosal neoplasia.[95,96]

MECHANISMS OF MUCOSAL INJURY

Squamous Mucosa

The predominant mechanism for injury to equine gastric squamous epithelium is acid injury.[10] The squamous mucosa is susceptible to acid injury due to its lack of mucosal protective factors. Continuous monitoring of gastric pH via indwelling pH electrode showed that gastric pH could fall to <2.0 during periods of food deprivation, whereas grazing at pasture or feeding Timothy grass ad libitum often resulted in gastric pH> 6.0.[53] This acid neutralization most likely reflects absorption of acid by ingested hay, buffering by salivary bicarbonate and an overall dilutional effect.[3] Cells deep to the squamous mucosa actually transport H^+ intracellularly.[81] Penetration of acid to this region leads to cellular death and increased ulcer severity.

Experimental periods of prolonged high gastric acidity (pH<2.0) can be produced by alternating 24h periods of feed deprivation and free choice hay and consistently result in squamous epithelial erosion. After 96h of cumulative feed deprivation squamous ulceration was found.[10,11]

Feeding practices will affect acid production. A grain or pelleted diet was associated with significantly higher post prandial serum gastrin concentration, which should lead to increased acid production.[12] Changing pasture fed horses to stall confinement and free choice hay will result in squamous lesions in 7 days.[10] Exercise and training influence gastric injury. The prevalence of gastric lesions in racehorses in training is 70-90%.[1,13,35] Recent work has shown that exercise increases intra-abdominal pressure producing gastric compression. This pushes acidic gastric juice past the margo plicatus up into the squamous lined dorsal half of the stomach.[14] The result was a rapid fall in the pH of the proximal stomach to <4 at the onset of exercise, where it remained until the training session finished. The prolonged exercise routine of the racehorse will obviously result in increased duration of acid exposure. An increase in serum gastrin concentration has also been shown to occur in exercising horses.[15]

Although it has not been proven it is logical to assume that this results in increased acid production by the glandular mucosa and hence may lead to acid damage.

Pepsin and possibly bile acids are involved in the etiology of erosive esophagitis in man and an interaction between HCl, pepsin and bile acids has been proposed.[97,98] As previously described, bile reflux is a normal finding in the equine stomach. Animal models have shown that a combination of HCl and pepsin will induce esophagitis and that in an acidic environment bile acids will augment this damage.[98,99]

The volatile fatty acids acetic, butyric, propionic and valeric acid can be produced during carbohydrate fermentation by gastric bacteria.[100] Volatile fatty acids have been measured in the stomach contents of horses fed both hay and grain diets.[17,101] At pH $\leq$4.0 VFAs are lipid soluble and will penetrate squamous epithelial cells resulting in acidification of cellular contents, inhibition of sodium transport and cell swelling. They may also increase cellular permeability to H^+. In vitro experiments using squamous epithelium in an Ussing chamber have shown that VFAs decreased the mucosal barrier function of the squamous epithelium by affecting tight junction integrity.

Glandular Mucosa

Lesions of the glandular mucosa are thought to result from impaired mucosal defenses rather than being primary acid/pepsin insults. Decreased mucosal blood flow results in reduced H^+ removal from the interstitium, reduced maintenance of epithelial tight junctions and impaired cellular restitution. Failure of the hydrophobic mucus barrier can occur with reduced mucus production and/or reduced bicarbonate secretion.[102] The etiologic difference in glandular ulceration is supported by the observation that the feed deprivation model of prolonged, increased gastric acidity consistently failed to induce glandular ulceration.[10,11] In the race training model of ulceration few glandular lesions were observed.[4]

Reduced mucosal NO synthesis is associated with worsened ulceration and poor ulcer healing in several animal models, whereas increasing mucosal NO attenuates stress and chemical induced ulcers and enhances healing. There is a direct correlation between mucosal blood flow and augmentation/inhibition of mucosal NO.[103] The experimental use

of compounds that increase NO synthesis, such as glyceryl trinitrate, promotes mucosal blood flow, mucosal protection and ulcer healing.[104]

Non steroidal anti-inflammatory drugs exert their damaging action on the gastric mucosa in two ways. Firstly, a major systemic mechanism involving inhibition of cyclo-oxygenase (COX) and secondly, a local COX –independent mechanism. Almost all mammalian cells contain COX which is the first enzyme in the pathway converting arachidonic acid to prostaglandins (PG) such as PGE_2, PGD_2, PGI_2, PGJ_2 and TXA_2.[105] Gene knock out studies have identified the function of each prostanoid.[106-108] Physiologically within the gastrointestinal system, prostaglandin E_2 increases gastric blood flow and inhibits gastric acid secretion, PGD_2 inhibits gastric acid secretion and can be converted to PGJ_2 which stimulates tissue healing and induces NOS.
At least two distinct isoforms of COX exist: constitutive COX-1 which is present in almost all cell types, though can be induced; and inducible COX-2 which is expressed in cells such as the *macula densa* but is not normally present in the gastrointestinal mucosa.[109] Cyclo-oxygenase-2 produces excessive prostanoid as it contains a special large channel that allows arachidonic acid to remain longer and in closer vicinity to the active center of the enzyme.[110] This channel is not present within COX-1.

In man most conventional NSAIDs predominantly inhibit COX-1 with little effect on COX-2.[111] The commonly used NSAIDs in the horse include phenylbutazone, flunixin meglumine and carprofen. In vitro, phenylbutazone and carprofen only inhibit COX-2 1.6 fold greater than COX-1, whereas flunixin meglumine preferentially inhibits COX-1.[112] Expression of COX-1 in gastric mucosa occurs within 1-2 hours of mucosal irritation and acts to increase production of the mucus/HCO_3^- layer, increase gastric blood flow, increase epithelial cell migration, restitution and proliferation.[113] Expression of COX-2 is excessive in gastric mucosa exposed to stress, luminal irritation or ischemia/reperfusion injury.[114] Experimentally, mucosal damage is not caused by selective inhibition of either COX-1 or COX-2, but relies upon inhibition of both enzymes.[115] This effect is believed to result from the inhibition of COX-1 up-regulating the expression of COX-2.[116,117]

The second mechanism of action of NSAIDs is a local COX-independent mechanism. The presence of the drug in the stomach breaks down the gastric mucosal

barrier, penetrating the mucus layer to reach the epithelial cells and diffusing into mucosal cells in the acidic gastric lumen by non-ionic diffusion.[118] During this diffusion the cells lose their hydrophobicity and the ability to repel HCl. The NSAIDs accumulate within the cells and are trapped as they dissociate within the cytoplasm. This results in an uncoupling of oxidative phosphorylation, altered enzyme activity and suppression of the production and expression of heat shock proteins that are vital to the maintenance of cellular integrity.[104] The damaged surface epithelium swells up and forms a "mucoid cap" that allows the penetration of H^+ into the mucosa with resultant release of inflammatory mediators such as leukotriene B_4 and HIST. The microvascular endothelium is damaged causing increased permeability and decreased mucosal blood flow.[119,120]

A third mechanism of action of NSAIDs is the release of tumor necrosis factor (TNF)-α from mucosal cells which up-regulates the expression of vascular adhesion molecules and activates neutrophils. Increased adherence of neutrophils to gastric mucosal vascular endothelium reduces mucosal perfusion and promotes further localized release of tissue destructive mediators.[121] TNF-α also stimulates nuclear factor κB (NF-κB) leading to protection of the P53-DNA repair mechanism and enhancement of the apoptotic pathways mediated via caspases. The induction of mucosal cell apoptosis is accompanied by a fall in prostaglandin biosynthesis due to NSAID inhibition of COX. This results in microcirculation disturbance, augmentation of neutrophil activity and interaction of activated neutrophils with damaged endothelium. The result is the development of "white thrombi" and capillary obstruction.[122] NSAIDs also impair ulcer healing by interfering with growth factor function, by reducing epithelial cell proliferation at the ulcer margin and angiogenesis in the ulcer bed and by slowing the maturation of granulation tissue.[123,124]

The use of NSAIDs is a well described cause of both squamous and glandular ulceration in the horse, and their use can also increase the severity of lesions observed.[3,20,125-127] However several epidemiologic studies have failed to show their administration to be a risk factor for EGUS in competition horses.[4,6,9,38] In two of these studies the entire glandular mucosa was not observed, either due to the use of a 2m endoscope[4] or the chosen endoscopic technique[9]. In the third, the period of starvation

prior to examination was short (6-8h) and the authors admit that this prevented a thorough examination of the glandular mucosa.[6] All of these factors will limit examination of the glandular mucosa of the pylorus and the body, and it is likely that the prevalence of glandular lesions was underestimated.[19]

Stress ulceration of the glandular mucosa is described in man and equine neonates and is thought to result from impaired mucosal blood flow.[16,128] This has been demonstrated in laboratory animal where stress models such as cold restraint and electric shock alter mucosal blood flow resulting in glandular lesions.[129,130] Impairment of mucosal blood flow during hemorrhagic shock is thought to cause gastric injury through neutrophils dependent mechanisms as the extent of ulceration is reduced in neutrophils depleted rats.[104] NSAIDs also appear to directly impair the diffusion capacity of the mucus gel, although the mechanism of this effect is unknown.[131]

Reperfusion is an exacerbation factor in reduced mucosal blood flow of any etiology. A cascade of events occurs after oxygen is delivered to the previously ischemic tissue that results in an increased local inflammatory response. Production of oxygen free radicals in the cytoplasm of ischemic cells damages the cell membrane, reducing the barrier to HCl. Replenishment of cellular oxygen causes reactivation of neutrophils, which undergo a respiratory burst. This can result in endothelial cell damage and further alteration to blood flow.

Helicobacter pylori is considered to be the predominant cause of glandular erosions and ulcers in man.[132] This acid tolerant gastric neutralophile directly injures gastric mucosa and is able to recruit a local inflammatory response. The cytopathic effects of *Helicobacter* are discussed later.

Duodenal Mucosa

Most duodenal disease occurs in foals and with much less frequency than gastric lesions and appears to be associated primarily with enteritis rather than peptic injury.[133] This contrasts sharply with man where duodenal ulceration can have a higher incidence than gastric disease and the overall incidence of disease increases with age.[134] In man *H. pylori* is considered the predominant cause of duodenal disease.

THE PATHOLOGICAL RESPONSE OF THE GASTRIC MUCOSA

The earliest gross change seen in both types of mucosa is hyperemia, which microscopically is seen as capillary congestion within the epithelium and lamina propria.[42] The response of each mucosal type to further assault then varies.

Squamous Mucosa

Prolonged peptic injury to the squamous mucosa results in sloughing of the superficial epithelial layers.[10,11] If this extends to the basal epithelial cells a histological diagnosis of erosion is made. Extension into the lamina propria classifies the lesion as an ulcer. The progression in the severity of epithelial denudation is associated with duration of acid exposure. It is interesting to note that many lesions that endoscopically are characterized as ulcers, histologically have intact epithelial layers and so are in fact erosions.[42] An explanation for this discrepancy may be the reliance on the surrounding tissue to give an estimate of lesion depth and hence severity. The total thickness of epithelium adjacent to experimentally induced lesions is significantly greater than normal epithelium, and this effect increase with lesion severity.[42] The increase in epithelial thickness is attributable to increases in both the keratinized and non-keratinized layers. Variation in lesion type is seen between study populations. A recent histopathologic study of squamous ulcers retrieved from yearlings at necropsy showed 65% of cases to have deep lesions involving the mucosa, submucosa and extending into the tunica muscularis.[19] Twenty percent had lesions of the mucosa and submucosa and 15% had superficial lesions of the mucosa alone. Histopathologically erosion and ulceration is characterized by infiltration of neutrophils, fibrocytes and vascular endothelial cells.[42] The magnitude of the cellular response is greater in ulcers and the density of inflammatory cells is greatest within the lamina propria. Epithelial proliferation, accompanied by vascular proliferation in the lamina propria and extension of capillaries from the lamina propria into the epithelium is also seen. These changes have been associated with increased epidermal growth factor receptor expression in epithelial basal

cells.[135] Progression of an ulcer through the muscularis mucosa is rare, except in foals where this layer is substantially thinner and a pronounced fibrinopurulent response results.

Glandular Mucosa

This is a compound columnar epithelium and so superficial injury will affect superficial epithelial cells, superficial mucus cells and mucus neck cells. Deeper injury will include damage to parietal and chief cells and is often accompanied by a neutrophilic infiltrate. With mild to moderate lesions damage is often localized to individual or groups of gastric glands, whilst neighboring glands appear normal. More neutrophils and lymphocytes are seen in areas associated with mucosal defects than without.[33] With severe ulceration damage extends into the submucosa and produces a more severe inflammatory response.

DIAGNOSIS OF EGUS

As previously stated EGUS is a common problem in horses and foals.[136] Diagnosis can be based on history and clinical signs and should be confirmed by endoscopic examination. The clinical signs described for EGUS are various and often vague (see table 1).[1,5,137,138] In Thoroughbred racehorses in training EGUS is associated with below expectation performance, poor hair coat, poor appetite and colic.[138] Ulcers are identified with an increased incidence in horses reported by the owner to have suitable clinical signs (88-92%) than those which are asymptomatic (37-52%).[5,8,40] The severity of gastric ulceration may also be associated with the severity of clinical signs, although this relationship is not constant.[5,8]

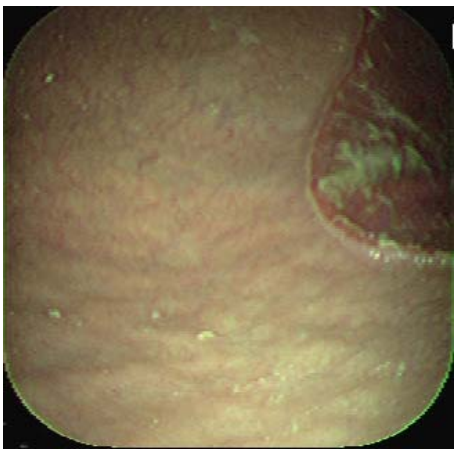
ADULT HORSES	***FOALS***
Acute colic	*Diarrhea*
Recurrent colic	*Abdominal pain*
Excessive recumbency	*Restlessness*
Poor bodily condition	*Rolling*
Partial anorexia	*Lying in dorsal recumbency*
Poor appetite	*Excessive salivation*
Poor performance/training	*Bruxism*
Attitude changes	*Intermittent nursing*
Stretching to urinate	*Poor appetite*
Inadequate energy	
Chronic diarrhea	

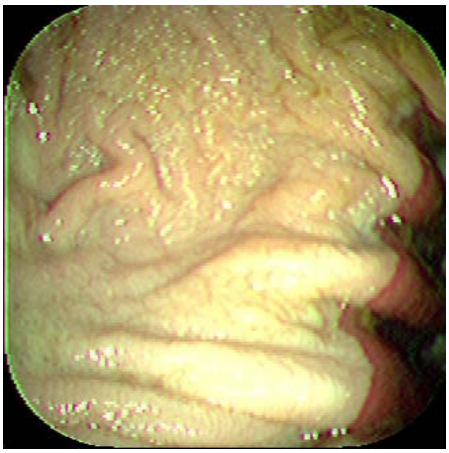
Table 1. Clinical signs associated with EGUS

Several grading systems have been developed to evaluate the gastric ulcer number and severity in individuals.[1,139-141] A simple and straightforward system was proposed in an EGUS consensus statement in 1999.[1] This system can be applied to both the squamous and glandular mucosa, can be used clinically and as a research tool and is similar to grading systems accepted in other body systems. The EGUS system is an extension of the practitioner's simplified scoring system,[142] which when compared to subsequent post mortem gross and histopathologic examination showed no significant difference between endoscopic score and necropsy examination.[142]

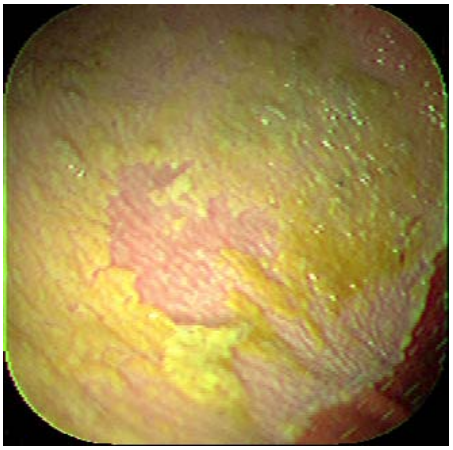
ENDOSCOPIC APPEARANCE OF LESIONS

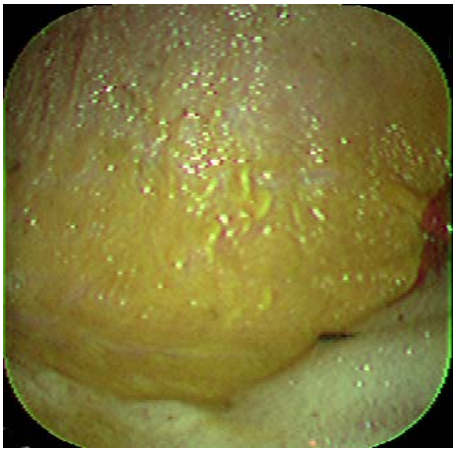
FigureS 10-19. egus grade – squamous mucosa[1]



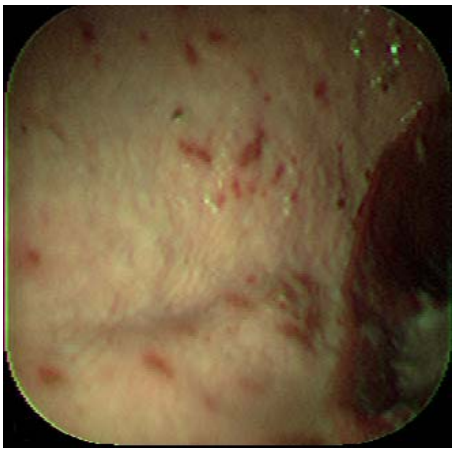
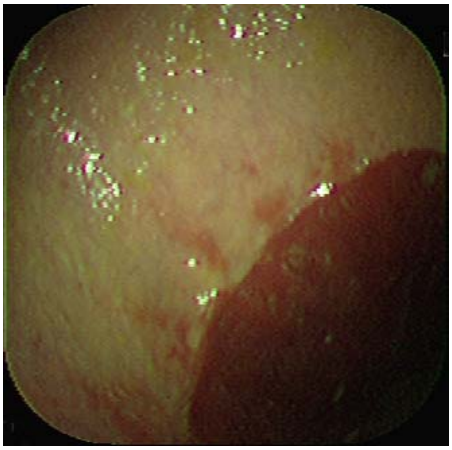


Grade 0 – The epithelium is intact and there is no appearance of hyperemia or hyperkeratosis

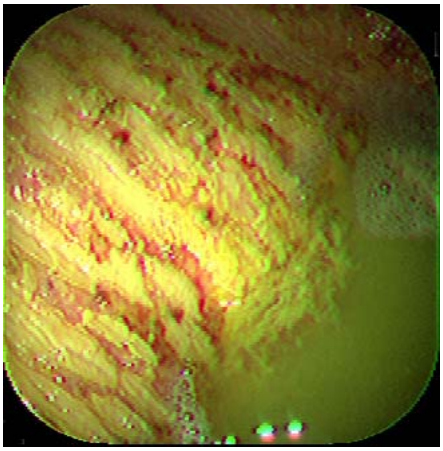
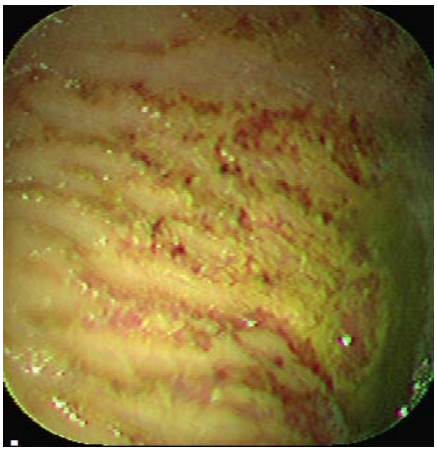




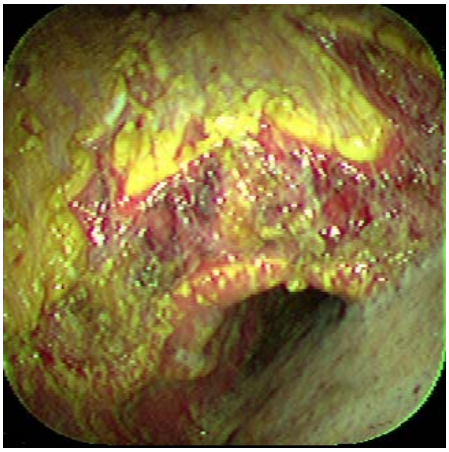
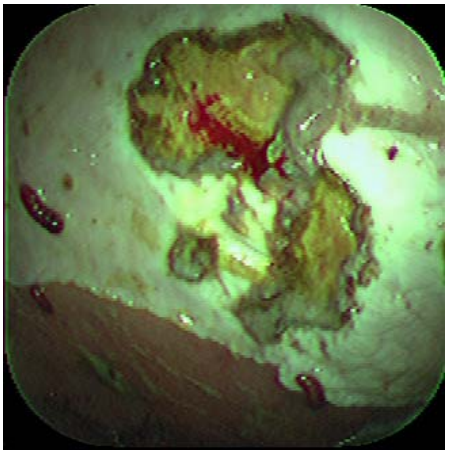
Grade 1 – The mucosa is intact, but there are areas of reddening or hyperkeratosis

Grade 2 - Small, single or multifocal lesions

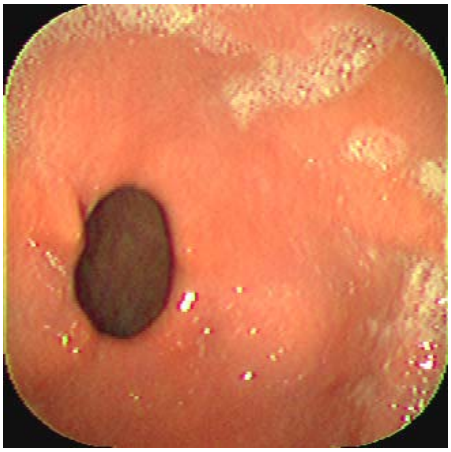



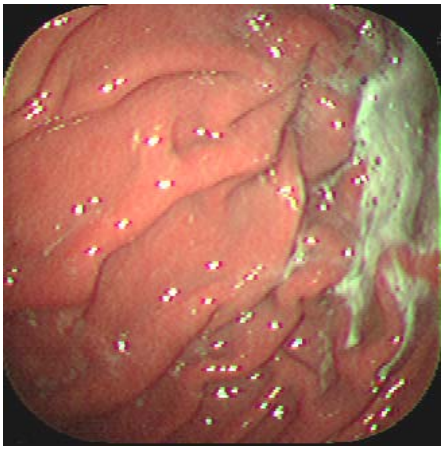
Grade 3 - Large, single or multifocal lesions or extensive superficial lesions

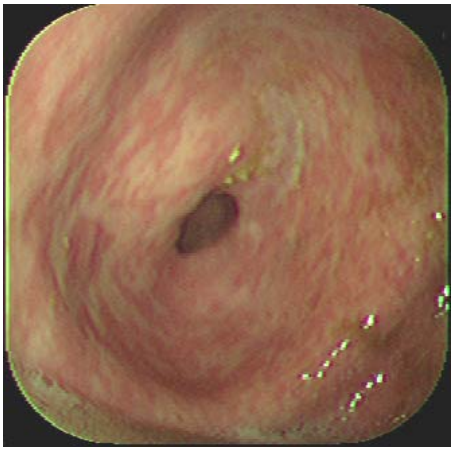
Grade 4 – Extensive lesions with areas of apparent deep ulceration

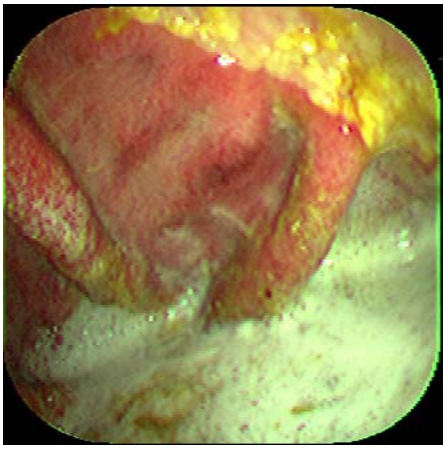
Figures 20-29. egus grade – glandular mucosa[1]



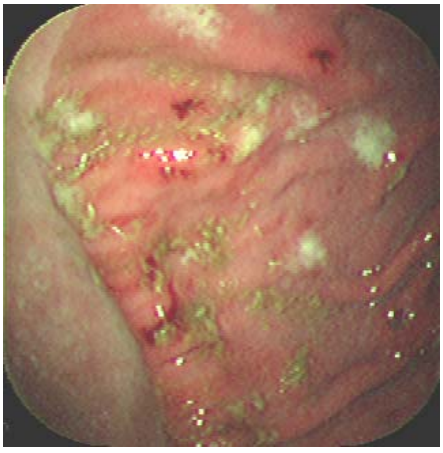
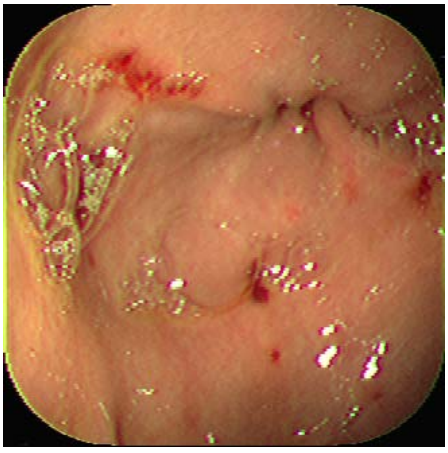


Grade 0 – The epithelium is intact and there is no appearance of hyperemia

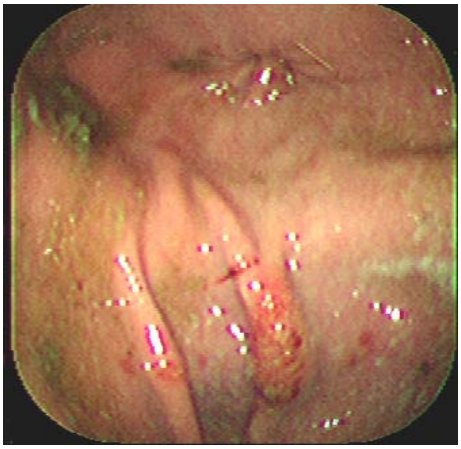
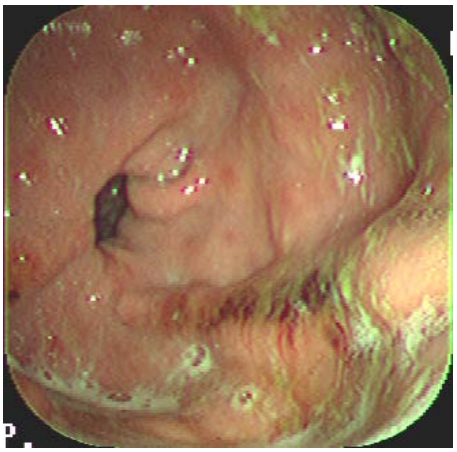




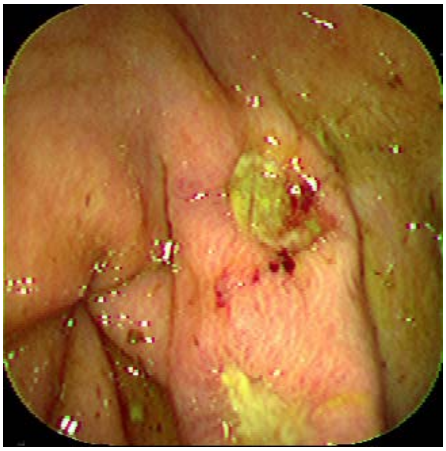
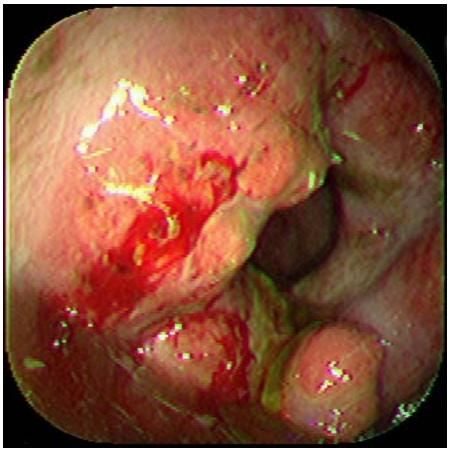
Grade 1 – The mucosa is intact, but there are areas of hyperemia

Grade 2 - Small, single or multifocal lesions

Grade 3 - Large, single or multifocal lesions or extensive superficial lesions

Grade 4 - Extensive lesions with areas of apparent deep ulceration

EQUINE GASTRIC EMPTYING

A variety of methods have been used to assess gastric emptying in the horse, most recently radioscintigraphy, acetaminophen and ^{13}C-octanoic acid has been used.[143,144] Using time data and a suitable model gastric emptying half life ($t_{1/2}$) can then be calculated. The mean ± SD solid phase gastric emptying $t_{1/2}$, using the ^{13}C-octanoic acid breath test, is 2.52±0.35 to 3.79±1.53h.[143,145] A $t_{1/2}$ value of 1.56±1.08h is obtained when using scintigraphic detection of a radiolabeled meal.[143] The difference in values reflects the time taken for absorption and metabolism of ^{13}C-octanoic acid.[143] The use of a "correction factor" is recommended to account for this, in man this has a value of 1.10h,[146] in the horse a value of 1.03-1.71h has been proposed.[143]

Using an acetaminophen solution, which is rapidly absorbed from the proximal small intestine fluid phase gastric emptying has been investigated.[144,147] Using this method the time to peak plasma acetaminophen concentration is 31±4.4mins. This value is very similar to that obtained using a radiolabeled marker and scintigraphic examination, where a fluid phase gastric emptying $t_{1/2}$ of 31.33mins is reported.[148]

The effects of various sedatives and tranquilizers on equine gastric motility have been investigated. For example the α-2 agonist detomidine (0.03mg/kg IV) slows the mean $t_{1/2}$ of solid phase gastric emptying from 3.14±0.81h to 5.28±0.77h.[149] The effect of this particular agent on fluid phase emptying has not been reported, whereas this has been reported for xylazine. Using an acetaminophen marker xylazine increases time to maximum serum level (T_{max}) from 31±4.4mins to 85±9.8mins.[147] Xylazine has also been shown to increase solid phase emptying $t_{1/2}$ to 3.71±0.48h.[149] Using these figures it is reasonable to expect detomidine to increase fluid phase T_{max} to approximately 120mins (5.28/3.71 x 85.9).

The synthetic muscarinic cholinergic agent bethanechol increases gastric contractile activity in the horse.[150] Using scintigraphic detection of radiolabeled marker it reduces mean solid phase gastric empting $t_{1/2}$ to 30.09±10.01mins and fluid phase gastric emptying $t_{1/2}$ to 17.74mins.[148]

GASTRIC *HELICOBACTER*

The stomach was long thought to be a sterile environment due to the "gastric bactericidal barrier"[151] until a novel bacterium was cultured from the gastric mucosa in 1982, marking a turning point in the understanding of human gastric disease.[152] This report described a spiral or curved bacilli in histologic sections from 58 of 100 consecutive biopsy specimens of human gastric mucosa, 11 of which were culture positive for a gram negative, microaerophilic bacterium. Initially this bacterium was thought to be part of the genus *Campylobacter* and was named *Campylobacter pyloridis*, although this was later corrected to *C.pylori*. Subsequent 16S rRNA sequence analysis showed sufficient distance between existing *Campylobacters* and the novel bacterium to warrant renaming as *Helicobacter pylori*, the first member of the new genus *Helicobacter*.[153,154] In man there is overwhelming evidence that *Helicobacter* is capable of inducing gastritis, peptic ulcer disease, gastric adenocarcinoma and gastric non-Hodgkin's lymphoma.[22-24] The World Health Organization has classified *H.pylori* as a class 1 (definite) carcinogen.[155] *H.pylori* is thought to infect approximately half the world's human population, however most people infected with *H.pylori* are asymptomatic.[25,28]

Gastric Helicobacter species

To date nine *Helicobacter* species have been cultured from the stomach of humans and other land animals (table 2), all are capable of hydrolyzing urea.[29] These can be further classified into Lockard types 1, 2 and 3: type 1 has a fusiform to slightly spiral morphology with tapered ends; type 2 is spiral and has sparsely distributed periplasmic fibers and can appear singly or in groups of two to four; and type 3 is more tightly coiled and lacks periplasmic fibers. In general the morphology of gastric *Helicobacter* species isolated from animals other than cats and dogs can sometimes be distinctive and sometimes resemble *H.pylori*.[29] Phylogenetic analysis of current gastric, enteric and hepatobiliary *Helicobacter* species, based on 16s rRNA similarity has been performed

and is shown in figure 30.[29] As can be seen phylogenetic variation between gastric species is minimal.

TAXON	NATURAL HOST
H.acinonychis	Cheetah
H.bizzeroni	Dog
Candidatus *Helicobacter bovis*	Cattle
H.felis	Cat
H.heilmannii	Human, non human primate, pigs
Candidatus Helicobacter suis	Pig
H.mustelae	Ferret
H.nemestrinae	Macaque
H.pylori	Human, monkey
H.salomonis	Dog
H.suncus	Shrew

Table 2. Human and land animal gastric *Helicobacter* taxa.[29]

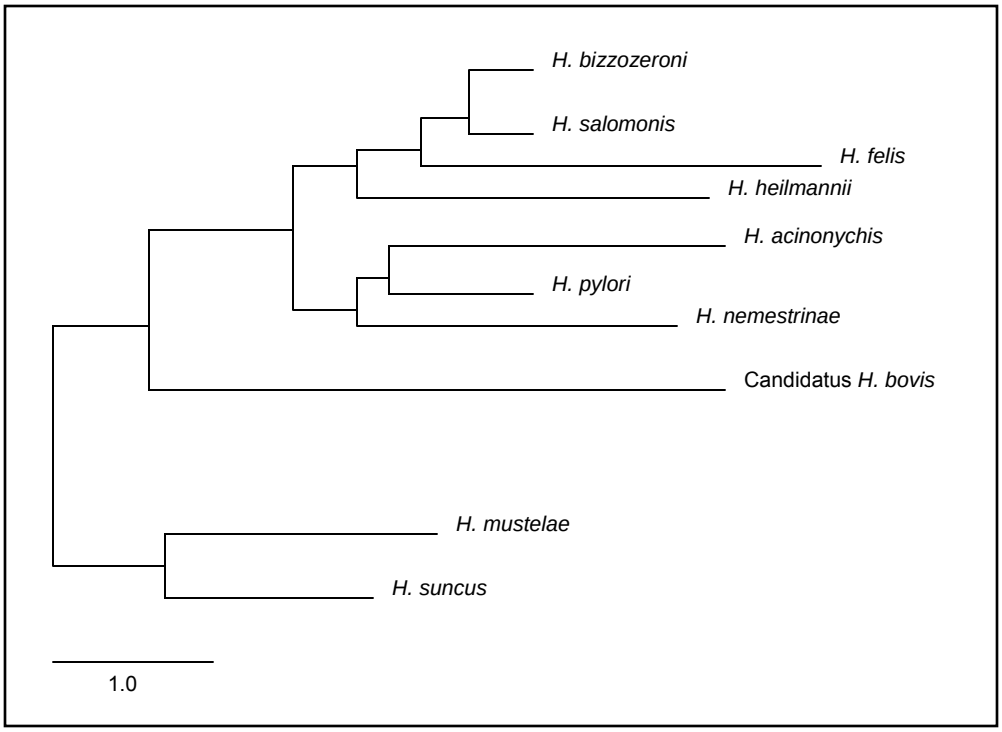


Fig 30. Phylogenetic divergence tree for gastric *Helicobacter* species, based upon 16*s rRNA* sequencing. Asterisk denotes gastric *Helicobacter* species. Scale bar represents 1% difference in nucleotide sequence between species. (Adapted from Solnick J, Schauer D. Emergence of diverse *Helicobacter* species in the pathogenesis of gastric and enterohepatic diseases. *Clin Microbiol Rev* 2001;14:59-97)[29]

ADAPTATION BY *HELICOBACTER* TO THE GASTRIC ENVIRONMENT

Helicobacter is unique in its ability to colonize the stomach.[29,156] In man infection is acquired during childhood and will persist throughout life in the absence of eradication therapy. Other bacteria, such as the neutralophile *Yersinia enterocolitica*, are able to transit the stomach but not colonize it. Recently a urease positive coccoid organism has been cultured from the stomach of people in Korea.[29] Biochemical analysis of the urease from this organism showed it to be in relative abundance and to have a subunit composition similar to *Helicobacter* urease.[157,158] Preliminary taxonomic and 16S rRNA classification has shown the organism to be essential identical to *Staphylococcus saprophyticus* and to have <1% difference to *S.xylosus* and *S.cohnii*.[29] The final identification, prevalence and clinical significance of this organism are currently unknown.

The importance of the enzyme urease to *Helicobacter* is shown by the inability of urease negative mutants to colonize the stomach in animal models.[159] Initially it was thought that urease activity was on the surface of the micro-organism and that it acted to create a cloud of ammonia that neutralized the local environment.[160] Subsequent experiments using a green fluorescent cytoplasmic protein marker showed that surface urease is actually the result of cell lysis and not secretion.[161] Another problem with the external urease theory is the pH/activity curve of *Helicobacter* urease: peak activity occurs at pH 7.5, there is virtually no activity below pH4.5 and at pH 4 an irreversible inactivation occurs.[162] The importance of internal urease was discovered when the urease activity of intact *Helicobacter* organisms was measured. At a neutral pH urease activity was low, however a change in pH from 6.5 to 5.5 elicited a 20 fold increase in activity and activity was maintained down to at least 2.5.[162,163] Thus cytoplasmic urease is required for acid resistance. Urease is produced as an apoenzyme and requires insertion of a nickel (Ni^{2+}) cofactor for conversion to active urease.[27] Nickel availability is mediated via the protein NikP, which acts as a transcriptional level control mechanism on

urease activity.[164] The gene cluster for urease consists of seven genes: *ureA* and *ureB* encode the urease structural subunits, and *ureE, -F, -G* and *–H* encode the accessory proteins required for Ni^{2+} insertion into the apoenzyme.[165]

The mere presence of urease is not sufficient to confer acid tolerance; sufficient substrate is also required to saturate the cytoplasmic urease.[166] The permeability of the phospholipid bilayer to simple diffusion of urea is low ($4x10^{-6}cms^{-1}$), even with an external urea concentration of 100mM.[27] In acidic media the Michaelis constant (K_m) of internal urease is equal to that of free urease indicating the presence of an accelerated entry mechanism increasing bacterial permeability to urea.[166] Urea passage occurs through a proton-activated channel, which is coded for by the third gene of the urease gene cluster, *ureI*, hence the channel is often referred to as the UreI channel.[27,166-168] Acid activation of the channel is extremely rapid and allows the bacterium to acutely regulate periplasmic pH.[163] The *ureI* gene is sufficiently distant from the genes encoding urease that its deletion does not affect urease production, but it does abolish the bacteria's ability to increase urease activity in an acidic environment.[27,169,170] Thus the pH dependence of cytoplasmic urease and the ability of gastric *Helicobacter* to react to an acidic environment is determined by Ure-I mediated urea transport.[27] UreI and cytoplasmic urease combine to allow the organism to resist the acute changes in gastric pH associated with digestion and colonize the stomach. *UreI* negative mutants are unable to colonize gastric mucosa, and so the channel is essential for gastric colonization.[163] Ure-I transport is entirely passive (not requiring direct ATP application or reliance on ion gradients) and is driven by cytoplasmic urease maintaining a favorable inward concentration gradient.[27]

Two other, slower, mechanisms of urease regulation have been reported. Firstly the posttranscriptional regulation of urease assembly, which is seen as an increase in levels of different species of messenger RNA (mRNA) transcribed from the urease gene cluster.[171] In an acidic environment, different levels of mRNA and different transcripts, from only the *ureA* and *ureB* components of the apoenzyme to the full sequence of all 7 genes were found. These changes in posttranscriptional regulation of the urease genes act to increase the efficiency of urease assembly. Specific transcripts, such as *ureIE'* are stabilized at acidic pH. Secondly it has also been suggested that increased generation of

active urease from the preexisting apoenzyme could be another posttranscriptional response to acute acid exposure. This is dependent both on nickel as a cofactor and the previously described *ure-* gene products.[164,165] The intrabacterial urease activity of gastric *Helicobacter* is therefore regulated in at least three ways: firstly a rapid response via *UreI* activation, secondly a slower response based on increased enzyme activation and thirdly a chronic mRNA response upregulating synthesis of new urease.

LOCATION OF *HELICOBACTER* IN THE STOMACH

Helicobacter is capable of locomotion within the gastric mucus and is able to counteract peristalsis via the action of multiple (*H.pylori* 5-6) polar flagella. These consist of two structural subunits a 53-kDa FlaA and a 54-kDa FlaB, which are essential to persistent colonization.[172,173] Aflagellate mutants are capable only of transient colonization. The vast majority of *Helicobacter* in colonized hosts are free living within the gastric lumen, but approximately 20% bind to gastric epithelial cells.[174] Adherence is thought to be important to the bacteria as a mechanism of obtaining nutrients and most importantly in resisting shedding of the loosely adherent mucus gel layer during digestion. The binding of gastric *Helicobacter* species is highly specific, for instance when *H.pylori* is found within the proximal duodenum it only overlays areas of gastric metaplasia and not intestinal type cells.[175] In general terms cell adherence is defined either as a "close" attachment between the organism and the epithelial without an intervening space, or as "loose" attachment to microvilli.[174]

In asymptomatic human patients and those with gastritis, *Helicobacter* can be detected at similar rates from all the regions of the stomach.[176] In patients with atrophic gastritis the middle body of the stomach, along the greater curvature and mid antrum have the greatest population. Early transmission electron microscopy studies showed *Helicobacter* organisms arranged in groups close to the intercellular junctions of the gastric epithelium.[177]. Others claimed that this location was artefactual and that *Helicobacter* was mostly located within the mucosa of the gastric pits close to the epithelial surface,[178] however the organisms were never actually observed within, between or underneath the gastric mucosal cells and so the relationship between *Helicobacter* and the pathological changes in adjacent epithelium was obscure at best.[179]

Adherence

Several host and *Helicobacter* ligands involved in adherence have been described:

1. **Haemagglutinin**. 20-kDa haemagglutinin encoded by *hpa* that binds sialic acid components of or erythrocyte membranes.[180-182]

2. **Non-sialated ligands**. *Helicobacter* can bind to ligands of this group including laminin, fibronectin, collagen, heparin sulphate and sulphatide. These are most important in binding to damaged epithelium.[183,184]

3. **BabA**. This family of well conserved ligands share extensive homology within members in the C- and N- terminal domains.[179] BabA binds the blood group antigen Lewis^{b} which is present on gastric epithelial cell membranes and allows *Helicobacter* to move from the mucus gel layer and bind to the epithelium.[185] Several isoforms of BabA exist, BabA2^{+} strains of *H.pylori* are associated with increased risk of gastroduodenal ulceration and gastric adenocarcinoma in man, whereas BabA2^{-} strains usually cause gastritis alone.[186] The presence of BabA2 is associated with the presence of other *Helicobacter* disease related genes such as cagA and is thought to be pivotal to the induction of gastritis.

4. **Lewis**. In vitro pre-incubation of *H.pylori* with anti-Lewis$^{x/y}$ monoclonal antibodies inhibits binding to gastric epithelial cells.[187] *H.pylori* strains that strongly express Lewis$^{x/y}$ are associated with higher colonization densities and a more pronounced neutrophilic infiltration of the gastric mucosa than those which weakly express these antigens.[188]

5. **HopZ**. Inactivation of the gene encoding this protein dramatically decreases the in vitro ability of *Helicobacter* to bind gastric epithelial cells.[189]

6. **AlpA/B**. Inactivation of the genes encoding these also limits binding to epithelial cells.[190] Found in all *H.pylori* strains suggesting a role in colonization.

Helicobacter Lipopolysaccharide

Like all gram negative bacteria *Helicobacter* carries an outer cell wall structure that consists of two components: an inner mucopolysaccharide-peptidoglycan unit and an external lipopolysaccharide (LPS) structure. LPS consists of an O-specific chain, a core polysaccharide and a lipid A moiety.[191-193] The O-antigen of *H.pylori* contains different Lewis antigens, including Lewis^{a,b,x} and ^{y}. Inactivation of Lewis^{x,y} attenuates the colonizing ability of *Helicobacter* in mice.[194,195] These Lewis antigens are also expressed on host astric epithelial cell surfaces and so it has been suggested that their expression by *Helicobacter* may be a form of mimicry that prevents formation of antibodies to these shared epitopes facilitating a persistent infection.[196] This hypothesis is not universally accepted as other studies have failed to show a significant relationship between the Lewis phenotype of the host and invading organism.[197]

Conversely *Helicobacter* expression of Lewis antigens has also been suggested to induce a humoral inflammatory response that enhances gastric inflammation.[198] Infection of mice with *H.pylori* induces production of anti-Lewis^{x} antibodies that have an affinity for host Lewis antibodies on the H^+/K^+ ATPase located in the cannaliculi of parietal cells.[185,199] This role of Lewis antigens is unequivocal as recent data has demonstrated that anti-Lewis$^{x/y}$ antibodies can occur in the sera on uninfected people.[200]

HELICOBACTER INDUCED GASTRIC INFLAMMATION

The inflammatory response induced by *Helicobacter* consists of an active chronic gastritis with varying degrees of epithelial cell degeneration and injury, and a cellular infiltrate comprising neutrophils, B- and T-lymphocytes, plasma cells and macrophages.[201] As previously stated invasion of the gastric mucosa by the organism in vivo is not confirmed and so other mechanisms of induction of inflammation must be postulated. Two possible mechanisms have been put forward: secretion induction and contact-mediated induction; as well as two host immune responses to *Helicobacter*: the humoral response and the T-cell response.

Secretion Induction

Substances secreted from *Helicobacter* may stimulate mucosal inflammation from afar. Urease has been detected within the gastric lamina propria and can stimulate monocyte and neutrophil chemotaxis and activate mononuclear cells.[202] Similarly porins from *Helicobacter* also possess chemotactic abilities.[203] *Helicobacter* cell water extracts are potent promoters of neutrophil-endothelial cell interactions in vitro, increasing leukocyte adherence via CD11a/CD18 and CD11b/CD18 interactions with vascular endothelial ICAM-1.[204] The importance of this effect in vivo is not known.

Contact Mediated Induction

Direct contact of *Helicobacter* with the gastric epithelium stimulates cytokine release. Gastric epithelium from people infected with *H.pylori* demonstrates increased levels of interleukin-1β (IL-1β), IL-2, IL-6, IL-8 and TNF-α.[205-209] This type of interaction has been described in other bacteria such as *Salmonella typhimurium* where bacteria-host cell contact initiates cytokine signaling for polymorphonuclear cell infiltration.[210]

Interleukin-8 appears to be central to *Helicobacter* contact mediation of acute gastritis. IL-8 is a potent neutrophil activating chemokine, the in vivo expression of

which is localized to the gastric epithelial cells and whose levels are directly related to the severity of gastritis.[208,211] In vitro interaction between *Helicobacter* and gastric epithelial cells stimulates IL-8 release.[212]

The rapid increase in IL-8 in response to *Helicobacter* contact is mediated by the action of nuclear factor κB (NF-κB) on the IL-8 gene.[213-215] NF-κB is a transcription factor that is sequestered in the cytoplasm, the activation of which is tightly controlled by a class of inhibitory proteins called IκB's, who mask the nuclear localization signals of NF-κB so preventing its movement into the nucleus.[216] *Helicobacter* contact with the cell leads to phosphorylation of IκB and subsequent ubiquitination and proteosomal degradation. NF-κB is liberated to regulate transcription of the genes for IL-8 and others. This pathway is not reliant on protein synthesis for activation and so is rapid and efficient and is particularly utilized in immune and inflammatory responses where rapid gene activation is required following exposure to bacterial pathogens.

As well as NF-κB, mitogen activated protein kinases (MAPK) have been implicated as mediators of *Helicobacter* induced IL-8 expression. MAPK cascades are signal transduction pathways that target a variety of transcription factors and so participate in a myriad of cellular functions including NF-κB regulation.[217] *Helicobacter* rapidly induces activation of MAPK's in cell culture systems[218], raising the question as to whether *Helicobacter* induction of IL-8 synthesis is dependent on NF-κB activation, MAPK activation or both. Recent work has shown that it is dependent upon activation of both NF-κB and MAPK activation of the transcription factor AP-1, indicating that synergism between AP-1 and NF-κB within the promoter region of the IL-8 gene is required for maximal *Helicobacter* induced IL-8 production.[219]

Helicobacter may also exert a direct stimulatory effect on neutrophils via bacterial production of the 150-kDa neutrophil activating protein, which is a component of the *Helicobacter* cell wall.[220] This protein promotes adhesion of neutrophils to the endothelial cells and in vitro stimulates phagocyte chemotaxis and the generation of reactive oxygen species.[221]

Humoral Responses

Helicobacter colonization induces as pronounced systemic and mucosal humoral response directed at multiple antigens.[222-224] *Helicobacter* is susceptible to in vitro antibody-dependent complement mediated phagocytosis and cytotoxicity however this antibody response does not result in eradication of the *Helicobacter* infection in vivo.[225]

This suggests that the gastric mucus may provide a protective environment which *Helicobacter* exploits as it is relatively inaccessible to antibodies or their effector functions. The excessive and ineffective humoral response may actually contribute to the pathology associated with *Helicobacter* gastritis, monoclonal antibodies directed against *H.pylori* cross react with gastric epithelial cells and can induce gastritis experimentally in man.[226] As previously described *H.pylori* induces the formation of antibodies that recognize the H^+/K^+ ATPase epitope on the luminal surface of acid secreting parietal cells.[226,227] A second auto-antibody reaction may be present as IgM produced by *Helicobacter* colonized gastric mucosal cells also recognizes gastric epithelium.[228] These findings indicate that the distinct histological lesions seen with *Helicobacter* are a combination of both bacterial factors and a mucosal humoral response where an autoimmune reaction against parietal cells leads to gastric atrophy and immunoglobulin mediated destruction of epithelial cells initiates and/or maintains mucosal inflammation and failure of the epithelial barrier.

T-Lymphocyte Mediated Responses

The gastro-intestinal mucosa represents a barrier between the host and the microbial flora. The ability to police this barrier and distinguish between pathogenic bacteria and commensuals is due to T-cell lymphocyte function.[229,230] $CD4^+$ T lymphocytes can be divided into two functional subsets: type 1 (Th1) and type 2 (Th2) T helper cells, which are defined by distinct patterns of cytokine production.[230,231] Th1 cells promote cell mediated inflammatory responses by producing IL-2 and IFN-γ, while Th2 cells induce B-cell activation and differentiation by secreting IL-4, IL-5 and IL-10. The type of immune response that occurs with each particular organism is governed by the

preferential expansion of one T helper cell subset and corresponding down regulation of the other.[230] Most intracellular pathogens stimulate Th1 responses, whilst extracellular pathogens result in a Th2 response. As *Helicobacter* is typified by a non-invasive infection accompanied by an exuberant humoral response it would be expected that Th2 activation would predominate. Paradoxically most T-cell clones isolated from *Helicobacter* infected gastric mucosa produce IFN-γ rather than IL-4 and so reflect a Th1 type response.[232] *Helicobacter* also stimulates in vitro production of IL-12, a cytokine that promotes Th1 differentiation.[233] Thus the hypothesis that an aberrant host response (Th1) to an organism predicted to induce a (Th2) secretory immune response may allow perpetuation of gastric inflammation and *Helicobacter* colonization. This has been supported by a variety of animal models. Strains of mice that develop a Th-1 type response to infection when infected with *H.felis* develop extensive, persistent gastritis, whereas strains that responds to infection with a Th-2 response develop minimal gastritis.[234] Transfer Th2 lymphocytes from infected donor mice to infected recipient mice reduces bacterial colonization, whereas transfer of Th1 cells worsens the gastritis.[235,236]

EPITHELIAL CELL INJURY IN RESPONSE TO *HELICOBACTER*

In addition to the inflammatory response initiated by *Helicobacter* infection several other forms of epithelial cell damage can occur.

Reactive oxygen species (ROS)

In vitro contact of polymorphonuclear cells with *Helicobacter* induces an oxygen burst generating reactive oxygen and nitrogen species that induce oxidative DNA damage and cellular damage from lipid peroxidation and protein oxidation.[237] In vivo, *Helicobacter* increases oxidative DNA damage in man.[238]

Cellular Turnover and Apoptosis

Another consequence of prolonged gastric inflammation may be alterations in cell turnover. Gastric epithelial proliferation rates within *Helicobacter* colonized mucosa are significantly greater than those in uninfected controls and in chronic infection epithelial cell necrosis is reduced, suggesting that other forms of cellular death, such as apoptosis may be induced.[239,240]

Apoptosis is a form of programmed cell death that consists of a series of tightly regulated energy dependent molecular events.[241] Enhanced rates of apoptosis could accelerate the development of gastritis and gastric atrophy, whereas reduced rates could lead to gastric carcinogenesis. Increased levels of gastric epithelial cell apoptosis in *Helicobacter* infected people have been described, however substantial variation does exist within infected groups.[240,242] It is not known whether the differing levels of apoptosis are a result of varying levels of bacterial products or differences in the induced host response. Recently data has suggested that epithelial apoptosis in vivo is likely to be regulated by host mediators such as IFN-γ, a Th-1 lymphocyte derived cytokine that is increased in *Helicobacter* gastritis and which can act synergistically with *Helicobacter* in inducing Fas-Fas ligand (FasL) mediated epithelial apoptosis in vitro.[243,244] FasL is the oligomerizing ligand of Fas and is expressed on many cells including epithelial cells and

activated T-lymphocytes. *Helicobacter* can induce in vitro apoptosis in certain gastric epithelial and T-cell lines by activating Fas.[243,245,246] Overall it appears as though cytokines released by Th-1 lymphocytes in response to *Helicobacter* inflammation of the mucosa may regulate Fas-FasL interaction between T-cells and the gastric epithelium, increasing apoptosis.[232,244]

In addition to host genotypic differences certain bacterial constituents may influence the ability of *Helicobacter* to affect apoptosis. Infection with isolates containing a strain specific gene *cagA* is associated with significantly higher epithelial proliferation rates but lower apoptotic indices than both $cagA^-$ and non infected cases.[247] This is believed to augment the ability of $cagA^+$ strains to induce gastric neoplasia in man.
In vitro urease can also induce apoptosis by binding to class II MHC molecules expressed on the luminal surface of gastric epithelial cells.[248] The helicobacter gene product VacA can induce apoptosis by a separate route of insertion into the mitochondrial membrane, inducing release of cytochrome *c* and activation of cellular caspase-3.[245,249]

IDENTIFICATION OF *HELICOBACTER* STATUS

At present there is no universally accepted "gold standard" for the diagnosis of *Helicobacter* infection and so multiple tests are often required, the choice of which is dependent on the clinical case.[250] The tests available for identification of gastric *Helicobacter* can be divided into those which require endoscopy and biopsy of the gastric epithelium, and those which do not.[251] In human medicine the latter are more commonly used, particularly by general practitioners in primary care.

Invasive Diagnosis

In man diagnosis of *Helicobacter* requires endoscopy and biopsy; endoscopy alone is unsatisfactory.[252] Detection of *Helicobacter* infection is based on histology, culture and/or rapid urease testing (RUT). The use of PCR is also described. There has been suggestion that the performance of all biopsy-based tests can be affected by factors such as sample site, number and dimension of biopsies taken and handling technique. At least three different sites must be sampled (the distal antrum, angularis (lesser curvature) and mid to upper body), or preferably five (two from the antrum within 2-3cm from the pylorus, one form the distal lesser curvature and one from the greater curvature and two from the corpus about 8cm from the cardia).[253,254]

1. Histology. Histology is unique in providing information both about the presence of *Helicobacter* and the presence and type of gastric inflammation. *Helicobacter* can often be readily recognized by its characteristic curved or S shape, and its location within the gastric pits or in the mucus layer overlying the surface. The most commonly used stain is Giemsa which has a sensitivity in man of approximately 90% when compared to culture.[255] The specificity of histology can be increased by using immunohistochemical staining, although clinically this technique is not frequently employed.[256] In order to maximize the results of histology several biopsies must be taken.[254,257] Examination by an expert pathologist is also important in obtaining an accurate diagnosis of the presence of

Helicobacter.[250] The samples are graded according to a defined scale, the modified Sydney scale, that assesses *H.pylori* density, polymorphic neutrophil activity, chronic inflammation, glandular atrophy and intestinal metaplasia.[254] The reader is directed to an excellent reference that describes these graded variables, as well as other non graded variables such as surface epithelial damage, mucus depletion and erosions.[254] *H.pylori* inflammation almost universally demonstrates polymorphic neutrophil activity.[254] Neutrophils are found within the lamina propria, within the epithelium, particularly the glandular neck, and within the lumen of the gastric pits. The density of intra-epithelial neutrophils is correlated with the extent of mucosal damage and the intensity of the *Helicobacter* infection. In man neutrophils are a sensitive indicator of the presence of *H.pylori* and disappear within days of eradication.[258] With chronic inflammation mononuclear effector cells (CD4+, CD8+ T-lymphocytes, B-lymphocytes, plasma cells, monocytes, mast cells) are seen. The normal level of mononuclear cells in human gastric mucosa is 2-5 lymphocytes, plasma cells and macrophages per high power field (hpf).[254] *H.pylori* inflammation typically only has 4-7 lymphocytes per hpf and as such is easily distinguished from lymphocytic gastritis where >25 intraepithelial lymphocytes are found per hpf.[259] Lymphocytic gastritis can be immune mediated, gluten associated or idiopathic.[254]

2. Touch Cytology. Touch cytology is performed by rolling a biopsy specimen onto a glass slide, which after air drying, is then stained using a rapid fixation and staining technique (M+D+Quick, Merz-Dade AG, Dudingen, Switzerland).[260] This stain is a combination of fast green in methanol, and thiazine. The use of touch cytology is advocated for diagnosis of *Helicobacter* species that are difficult to culture *in vitro* such as *H.hominis*.[261] The method has theoretical advantages for recovering organisms from the mucus gel layer that would otherwise be lost during sample handling for histology, although careful and minimal handling when transferring the tissue sample from the biopsy tool to the slide is vital to maximize chances of organism detection.

3. Biopsy Rapid Urease Testing. Given that *Helicobacter* is the only bacteria capable colonizing the stomach, its inherent and abundant urease activity has been used as a surrogate marker for the detection of the organism in gastric biopsies. Some authors have reported RUTs to be "slightly better" than histology in diagnosing *Helicobacter* infection.[250] Several RUTs exist and all are based on the fact that *Helicobacter* urease hydrolyses urea to release CO_2 and NH_3. The release of NH_3 alters the pH of the test medium and this causes a color change in the pH indicator. Some tests use a liquid medium (rapid urease test, Helicocheck™) and others use a solid medium (CLO™ test, Pyloritek™) and some are more rapid than others (1h vs. 24h). The rapid solid medium tests are most commonly used and will be considered further:

a) **CLOtest™** (Delta West Pty LTD., Bentley, Australia). This widely used urea gel test contains urea and a pH-sensitive color indicator in a media gel, yields 90-98% sensitivity and 92-100% specificity for detection of *H.pylori* infection in human gastric mucosal samples.[262,263] The final result of the CLOtest is read at 24 hours.

b) **Pyloritek™** (Serim Research Corp., Elkhart, IN). This test is a membrane test where the biopsy is sandwiched between a substrate pad containing urea and a specialized reagent strip containing the pH-color indicator. The strip contains its own internal positive and negative control areas and is readable in one hour. A sensitivity of 78-92% and a specificity of 100% is quoted fro this test using histology and culture as gold standards.[264] The variation in sensitivity reflects biopsy location, with a combination of antrum and body samples (92%) exceeding body (86%) or antrum (78%) alone.

Given the fact that RUTs only detect urease activity in a small area of the gastric epithelium (the biopsy) they will obviously be most sensitive when bacterial density and urease activity are greatest. This effect is best shown by the reduction in sensitivity of both tests after eradication therapy (clarithromycin/amoxicillin and a proton pump inhibitor)[265] in man: CLOtest™ 60.5%, Pyloritek™ 60.5%.[264] Another effect may be the

down-regulation of UreI and urease caused by the acid suppressive component of eradication therapy. Thus the use of these tests alone is not satisfactory for assessment of low *Helicobacter* loads in patients after eradication therapy.[264] Combination with a second test such as biopsy histology improves sensitivity (71.1%). For optimal post eradication therapy sensitivity a ^{13}C-urea test maybe performed (92.1%).

Recent adaptations to the basic RUT method have included multiscaled rapid urease tests (GIFast™, HpFast™) which are graded to give color reactions dependent on the density of microbial colonization, and the development of a very sensitive chemiluminescent pH indicator that may be suitable for post eradication therapy testing.[266,267]

4. Culture. *Helicobacter* rapidly loses viability when exposed to the environment and so biopsies require careful and rapid handling. Transport should be in Stuart's transport medium at 4^0C, which allows recovery of the organism for up to 48 hours. If the temperature is raised above 15^0C organism viability is poor and recovery rates are reduced even after 6 hours.[268] As most transport devices attain room temperature in 12-24 hours the use of frozen samples stored on dry ice is vital in transport is prolonged.[269] The recovery of organisms from biopsy samples stored at -25^0C in Portagerm™ for 72 hours is reported as excellent and this technique is advised for transport of *Helicobacter* of duration greater than 24 hours.[269] Subsequent culture of *Helicobacter* is then performed using media based upon either Columbia broth or Brain Heart Infusion agar containing blood or blood products, and additives such as starch, charcoal and bovine serum albumin.[270] Selectivity is attained by adding different combinations of antibiotics. Culture of *Helicobacter* from gastric samples is by definition 100% specific for diagnosing *Helicobacter* infection however sensitivity can vary widely between centers.[271] When using concordance between three or more positive tests as an indicator of *Helicobacter* status, culture has been shown to have a sensitivity of 93%. Using Youden's combined estimate of diagnostic validity index, culture shows the best combination of specificity and sensitivity (J value=0.93, ideal value=1). Culture is inexpensive to perform, however it is the slowest and most involved of all the diagnostic modalities as the organism takes a

minimum of 4-5 days to grow on primary isolation and requires .[272] *Helicobacter* has also been cultured from feces and dental plaque, although the clinical relevance of non-gastric isolation to the diagnosis of gastric disease is unknown.[251]

4. Biopsy Polymerase Chain Reaction. Among the bacterial ribosomal molecules the 16S and 23S rRNA units are large enough to contain sufficient phylogenetic material to characterize prokaryotic agents. The use of 16S rRNA gene sequence data is advantageous for identification of many pathogens, although the taxonomic resolution is not sufficient to distinguish between closely related species where the gene sequence homology is 97% or greater.[273] This fact may actually be advantageous when using PCR as a tool to identify new or closely related pathogens in a given and specific environment. The use of PCR in the diagnosis of *Helicobacter* disease is indicated in cases where there are likely to be small numbers of organisms, as the lower limit of detection of *Helicobacter* from clinical specimens by PCR when compared to culture is only 5CFU/ml.[251,274] PCR also has the advantage of being unaffected by the freeze/thaw cycles which are detrimental to bacterial viability.

Given the importance and specificity of urease a great deal of attention has focused on identifying the genes responsible for its function. For instance PCR methods for the detection of both *ureA* and *ureI* have been reported.[163,275] All gastric infective strains of *Helicobacter* have high levels of the urea channel UreI, which is required for urease activation and *UreI* negative mutants are unable to colonize the stomach.[163] As the presence of the urease/ureI system alone appears to be able to confer acid resistance, and as UreI is a protein unique to gastric *Helicobacter*, identification of the encoding 16S rRNA by PCR seems an ideal method for describing the presence of the bacterium.[27,265] As with any PCR procedure false positive results can occur with contamination of the specimens with *H.pylori* gene products either during endoscopy or in the laboratory.[276] To minimize patient side contamination thorough cleaning using protein dissolving solutions and subsequent sterilization of all endoscopy equipment is essential. PCR quality control involves stringent procedures such as separate areas or rooms for sample preparation , PCR reaction and post-PCR analysis and the use of both positive and

negative controls.[277] The thorough evaluation of primers and the nature of the sample site makes false positive results from detection of gene products from other microorganisms extremely unlikely.[278]

Using primers specific for *H.pylori* PCR has a sensitivity of 96% and a specificity of 100% when compared to either culture alone, or a combination of serology and histology as a gold standard for the diagnosis of *Helicobacter* infection in man.[274] PCR has also been described to be as sensitive as culture in detecting *H.pylori* in non conventional specimens.[279] An effect of biopsy location on detection rate is described where specimens obtained from the antrum have slightly better sensitivity (96%) than those obtained from the corpus (84%), in each case the specificity is 100%.[274] In man PCR has also been used to identify a variety of virulence factors such as *cagA* and *vacA* that are significantly associated with clinical disease.[280]

Non Invasive Diagnosis

1. Serological tests. Serology is a widely used and cheap method to detect antibodies to *Helicobacter*. In populations with a low prevalence of *Helicobacter* quantitative serological tests can give results comparable to the urea breath test.[280,281] It is important that serological tests are validated for a particular population as assays evaluated in one country may give different results in other populations with a different prevalence of *Helicobacter* disease.[282] Qualitative antibody tests are also available but are inferior to quantitative tests as they merely indicate exposure to the pathogen and as such are not recommended.[282] Immunoblotting has also been used to detect antibodies to *Helicobacter*, in particular those associated with increased virulence. When detecting antibodies to the virulence factors CagA and VacA in human patients with clinical signs of gastroduodenal disease, very high sensitivity and specificity is reported.[283] In addition to serum and whole blood testing, *Helicobacter* antibodies can also be detected in urine, saliva and feces. These tests are the ultimate in non-invasiveness but also have very low sensitivities and are of limited usefulness in populations with a high *Helicobacter* prevalence.[280]

2. ^{13}C-Urea Testing. Once again this testing modality relies on the intragastric urease action that is unique to *Helicobacter*. A meal of radiolabeled urea is given, which in the presence of gastric urease is hydrolyzed to labelled-CO_2 and NH_4. The labelled-CO_2 is rapidly absorbed across the gastric epithelium and into the blood, from where it is then detectable in both blood and exhaled gases. Either ^{13}C- or ^{14}C- Urea may be used, however as ^{14}C is a radioactive isotope, ^{13}C-labelling is the more accepted method and was first reported in 1987.[284-286] The natural abundance of ^{13}C is in the order of 1.08% of all carbon atoms, however it is not identical in all plant species. Most plants use the Calvin-Benson pathway to form carbohydrates, are known as C3 plants and have a ^{13}C enrichment of 1.08%. A smaller number of plants, such as cane sugar and pineapple produce carbohydrates with a higher ^{13}C content of 1.09% via the Hatch-Slack pathway and are known as C4 plants.[286] Plants enriched with ^{13}C can be fed to animals in order to produce labeled products such as lactose. ^{13}C-enriched amino acids are fed to animals in order to harvest labeled protein substances such as ^{13}C-Urea.

The basis of both the urea breath and blood tests is a urea meal enriched in ^{13}C, typically to a level of 99% ^{13}C, given at a dose of 1-5mg/kg body weight (75-125mg).[287-289] The Ez-HBT system (Metabolic Solutions, Inc., Nashua, NH) uses a 125mg meal that is dissolved in 75ml water to give a 17% solution). In order to slow gastric emptying a proprietary fatty meal is given prior to administering the ^{13}C-urea meal. As previously described, a comparable effect can be obtained in the horse using the α-2 agonist detomidine (0.03mg/kg IV).[149] To limit fluctuations in basal metabolic rate and changes in the size of the body CO_2 pool food is withheld during the test period and the test subject is required to remain seated and still. Exercise depletes the CO_2 pool, resulting in increased CO_2 excretion and falsely elevated results.[290] After exercise the CO_2 pool is then restored to its original size by temporary CO_2 retention. Digestion results in blood alkalization, the "alkaline tide", which increases the body CO_2 pool.[51] This is associated with a negative shift in baseline $^{13}CO_2$ level below natural atomic abundance, and an erroneously low result.[291]

For the breath test expired air is sampled for $^{12}CO_2$:$^{13}CO_2$; with the blood test either serum ^{12}C:^{13}C-bicarbonate levels are measured, or more commonly the $^{13}CO_2$

levels are measured in the gas released from whole blood within a specified vacutainer tube.[287-289] To ensure testing repeatability the use of a single reference laboratory is recommended.[288] Measurement of the ^{13}C:^{12}C ratio of the CO_2 is performed using an isotope mass ratio spectrometer, after separating the CO_2 from the other gas constituents by gas chromatography. These units are usually dedicated to ^{13}C testing.

A baseline sample is always obtained, although in man basal enrichment is typically low on a normal western diet. Blood or breath samples are then taken throughout the test period and are compared to the baseline value. At each time point the increase in ^{13}C enrichment is determined and is called delta over baseline (ΔOB).[286] Thus the absolute baseline ^{13}C enrichment is unimportant. Since changes in CO_2 production will affect the size of the body CO_2 pool and therefore the $^{13}CO_2$:$^{12}CO_2$, a stable metabolic rate and a complete absence of physical exercise are important throughout the test. In man the body CO_2 pool is large, approximately 1mol/70kg.[286] Any $^{13}CO_2$ produced will therefore mix with and pass through this pool, hence being exhaled with a delay of several minutes. The time delay to peak exhalation after administration of a tracer dose of ^{13}C-bocarbonate in man is 7 minutes and represents the time taken for mixing with the body CO_2 pool and subsequent exhalation.[292] Providing the CO_2 pool size is constant (constant production and elimination rates) this lag time is unimportant. Because of the size of the body bicarbonate pool and the consequent dilution of any absorbed $^{13}CO_2$, sufficient labeled substrate and urease activity must be present to produce enough $^{13}CO_2$ per unit time to allow measurements different from the baseline fluctuations.[293] In the presence of gastric urease, the ^{13}C:^{12}C of the body CO_2 pool starts to rise within minutes after ingesting ^{13}C-labeled urea, reflecting the addition of $^{13}CO_2$ derived from the ^{13}C-urea. After a single dose in man the ratio peaks at 50-60 minutes and then declines back to baseline.[287,294,295]

In man the use of the ^{13}C-Urea breath test, with a positive cut off value of ≥6ΔOB, for the diagnosis of *H.pylori* infection has been reported to have a specificity and sensitivity of 100%, with a breath sampling time of 30-50 minutes after labeled meal ingestion.[296] When using a positive result cut off value of >-17Δ ^{13}C per mil, the urea blood test has a sensitivity of 89% and a specificity of 96% when compared to histology

as a gold standard; a sensitivity of 92% and a specificity of 96% when compared to the urea breath test; and a sensitivity of 88% and a specificity of 98% when compared to RUTs.[288,289] Using a cut off ΔOB value of ≥6 the urea blood test has a sensitivity of 90.6% and a specificity of 95.7% when compared to a RUT.[295] This and a second study also demonstrated that gastritis per se did not significantly affect urea absorption.[287,295]

The blood test has become more widely accepted, due largely to its availability as a packaged unit (Ez-HBT), the traditional ease and patient acceptance of venipuncture as a testing method, and the lack of a need for specialist training, equipment or new procedures.[289] The blood test also has the advantage of not being affected by incomplete exhalation or altered respiratory function, most notably in chronic obstructive pulmonary disease where reduced ventilation causes CO_2 retention.[288] A further problem of the breath test is that an insufficient sample volume is only detectable at the testing laboratory, whereas it is immediately obvious when using the blood test.

$^{13}CO_2$ KINETICS IN THE HORSE

There are currently no reports of the use of ^{13}C-urea in the horse and so the data regarding $^{13}CO_2$ kinetics in the horse comes from reports into the use of the ^{13}C-octanoic acid breath test as a marker of gastric emptying.[143,145,149,297] This breath test also relies on detection of $^{13}CO_2$ and alteration of the $^{13}CO_2$:$^{12}CO_2$ ratio. The first of these reports details the basal level of ^{13}C enrichment level in the horse.[297] Four ponies in Great Britain were found to have a basal ^{13}C enrichment of 20.8±5.03ppm (mean±SD).[297] A second report describes the variation in basal ^{13}C enrichment in horses in the southern USA fed two different diets.[143] Basal enrichment of ^{13}C was found to occur with the C4 plant coastal Bermuda grass (*Cynodon dactylon*), but not with a diet of free choice alfalfa hay. Alfalfa is a C3 plant, meaning it uses the Calvin-Benson pathway to produce carbohydrates and as such has a ^{13}C enrichment level of 1.08%, which is equivalent to environmental levels.[286] Basal ^{13}C:^{12}C variation in horses maintained on alfalfa hay has been shown to be very stable at 1.54±6.21ppm excess ^{13}C (mean±SD).[143]

A stable body CO_2 pool is required for the application of ^{13}C test. As previously described, this is can be markedly affected by exercise and digestion. A protocol to limit variation in basal ^{13}C enrichment in the horse has been described.[297] This involves feed withholding and stall confinement for 12 hours prior to and during the study period. During a 13 hour test period following this protocol, ponies had negligible variation in the $^{13}CO_2$:$^{12}CO_2$ ratio.[297] Variation was also negligible after this protocol had been repeated daily for 6 days.

After ingestion of a single dose 250mg of ^{13}C-octanoic acid, breath excretion of $^{13}CO_2$ was detected for 24 hours in ponies, with peak excretion occurring at 100-120 minutes.[297] The pattern of excretion was constant with varying doses of ^{13}C-octanoic acid (125mg, 250mg or 500mg) and is similar to a mathematical model used to describe the human gastric emptying curve.[298] It must of course be noted that these values reflect ingestion of labeled marker given as a solid meal. A rough estimate of the kinetics after a liquid meal can be obtained by comparing solid phase to liquid phase gastric emptying in

the horse. Using a radiolabeled marker and scintigraphic detection the mean $t_{1/2}$ for the solid phase is 93.6mins, and the liquid phase is 31.33mins.[143,148] From this is reasonable to assume that the peak excretion of $^{13}CO_2$ after ingestion of liquid dose of ^{13}C-octanoic acid could occur at 33-40 minutes.

HELICOBACTER AND THE HORSE

Traditionally *Helicobacter* was not thought to be present in the equine stomach.[30] Given the wide host species diversity and the extent of gastric specialization this assumption appears short sighted. The basis of this assumption was the lack of direct visual identification of *Helicobacter* organisms in equine gastric mucosa.[3] The presence of *Helicobacter* specific gene material has been demonstrated by PCR in post mortem samples on two separate occasions. Firstly, from three horses, two of which had squamous mucosal erosions and the third a glandular lesion, and secondly in samples from 29 of 32 animals.[31,33] On this second occasion histology was also performed and this showed a variable degree of lymphocytic/plasmacytic inflammation. Lymphocytes were significantly ($P<0.01$) more numerous than plasma cells and were distributed throughout the mucosa.[33] The mean frequency was greater in the deeper mucosa (44.1±17.5 per hpf) than in the superficial layers (22.0±15 per hpf). There were significantly more polymorphic neutrophils associated with areas of erosion than intact areas ($P<0.05$).

Recently a technique for obtaining a large biopsy of the glandular mucosa from the gastric antrum of live horses has been described.[34] In this description of 10 horses the glandular mucosa appeared normal with mild hyperemia in 2 cases, mild erosions in 2 cases and ulceration in 1 case. Histological examination of the biopsy specimens demonstrated mild to moderate lymphocytic/plasmacytic mucosal inflammation (n=8), mild to moderate mucosal capillary congestion (n=6), mucosal erosion (n=2), *muscularis mucosa* edema (n=2) and proliferation of the of the *muscularis mucosa* (n=1). Neutrophils were only rarely seen in these normal biopsies. The consistent finding of a lymphocytic/plasmacytic infiltration in the equine glandular mucosa does not correlate with the findings of active *Helicobacter* infection seen in other species. The association of neutrophils with the active lesions in the post mortem study certainly may more closely resemble the type of inflammation typical of *Helicobacter* gastritis.

Further evidence for the possibility of an equine *Helicobacter* spp. was shown by a longitudinal sero-epidemiological study using a western blot test for antibodies to

H.pylori.[32] An absence of antibodies was detected in the foals at birth. After colostrum ingestion all foals had band patterns that were identical to their dam. As the foals matured the band patterns developed more variability from the dams, likely reflecting a waning of maternal antibodies and acquisition of innate antibodies due to exposure to *Helicobacter* infection.

MATERIALS AND METHODS

I. PILOT STUDY

SUBJECTS

Two healthy adult Thoroughbred geldings (aged 7 and 16 years, weight 442kg and 451kg) were used from the research population of the Marion duPont Scott Equine Medical Center. The absence of clinical disease was determined by thorough physical examination including thoracic auscultation and rebreathing.

^{13}C-UREA DIAGNOSTIC DRUG COMPONENT

Pharmaceutical grade synthetic ^{13}C-urea (chemical formula: $^{13}CH_4N_2O$) was obtained as a lyophilized white powder which contained greater than or equal to 99% of the carbon molecules in the form of the stable, non-radioactive isotope ^{13}C[a]. Combination with 50ml sterile water produced an oral dose of 500mg ^{13}C-urea as a 1% solution. This clear, colorless solution was prepared within 1 hour of administration and stored in an airtight 60ml syringe. The solution was visually checked for the presence of particulate matter and was discarded if any was found. The solution was administered by nasogastric intubation and the tube was flushed with further 100ml water prior to removal. Unused solution was discarded if not used within 4 hours after reconstitution.

^{13}C-UREA TEST PROTOCOLS

All horses were fasted for 12 hours prior to and throughout the testing protocol. The ^{13}C-urea breath test and the ^{13}C-urea blood test were performed simultaneously on both horses during a single day using a single 500mg dose of ^{13}C-urea. Blood urea nitrogen (BUN) was measured prior to the urea infusion and 30 minutes afterwards.

^{13}C-UREA BREATH TEST

Expired air was collected prior to and 30 minutes after administration of the ^{13}C-urea solution. Expired air was taken directly from the mid cervical trachea using an 18G

[a] Helicosol™, Lyophilization Services of New England, Inc., Manchester, NH

1½in needle placed aseptically using a standard tracheal aspiration technique. Expiration was identified by outward air movement from the nares and a 10ml sample of air was aspirated using a 20ml syringe and immediately transferred into a breath collection tube[a]. The samples were stored at room temperature for a maximum of 36 hours prior to shipping to a reference laboratory (Metabolic Solutions, Inc., Nashua, NH). All samples arrived within 3 days from collection. The tracheal site was palpated daily to check for evidence of subcutaneous emphysema or abscess formation.

^{13}C-UREA BLOOD TEST

Blood was collected prior to and 30 minutes after administration of the ^{13}C-urea solution by standard jugular venipuncture technique. Each sample was aspirated by vacuum into 13 x 75mm, 3ml draw, 45 USP Heparin blood tube[b]. Samples were stored at room temperature for a maximum of 36 hours prior to shipping to a reference laboratory for testing (Metabolic Solutions, Inc., Nashua, NH). All samples arrived within 3 days from collection.

$^{13}CO_2$ DETECTION TECHNIQUE

Samples were analyzed for $^{12}CO_2$:$^{13}CO_2$ ratio using a Europa Scientific 20/20 ABCA Gas Isotope Ratio Mass Spectrometer[c] (GIRMS). The GIRMS separates the CO_2 from other constituents by gas chromatography before measuring the $^{13}CO_2$:$^{12}CO_2$ ratio. Data is reported as delta ^{13}C per mil (parts per thousand difference) (Δ^{13}C). This represents the relative difference between the ratios of $^{13}CO_2$:$^{12}CO_2$ in the sample and the international ^{13}C standard, PeeDee Belemnite Limestone (PDB). PDB is significantly enriched in ^{13}C when compared to physiological samples and so results have a negative value. Sample enrichment occurs in the presence of urease and results in a ^{13}C level closer to that of PDB and so a less negative value. The analysis method has a precision of 0.1 per mil. In horses fed free choice alfalfa the basal variation in $^{13}CO_2$ output is

[a] Labco 139
[b] Becton Dickinson Vacutainer™ # 367672
[c] Europa Scientific Systems, Cincinnati, OH

1.54±6.21 ppm excess ^{13}C ($1.54±6.21x10^{-3}$ per mil).[143] At each time point during the test the increase in blood CO_2 ^{13}C enrichment was determined as Δ over baseline (ΔOB).

For a given time point (x):

$$\Delta OB = \Delta^{13}C_{tx} - \Delta^{13}C_{t0}$$

Fig 31. Calculation of ΔOB

II. PRINCIPAL STUDY

SUBJECTS

Seventeen healthy grass fed horses were obtained from two sources: a commercial dealer and the research herd of the Marion DuPont Scott Equine Medical Center. These horses had a median age of 7 years (mean 8.9 years, range 3-22 years) and a median weight of 451kg (mean 443kg, range 312-522kg). Physical examination of each horse was unremarkable, none had a history of gastrointestinal disease and all were grass fed for at least 1 month prior to commencing the study. No antimicrobials or proton pump inhibitors had been administered for at least 6 weeks prior to the study. All horses were housed in large outdoor pens throughout the study period and fed free choice alfalfa hay. On day 1 gastroscopy was performed on all horses and an antral biopsy was taken. Based upon reagent availability, 15 horses were then randomly selected for Pyloritek™ RUT; and 12 for PCR for *Helicobacter* specific 16*S rRNA*. After a 48 hour period eight horses were then randomly selected to be used for ^{13}C-urea blood test study. Table 3 shows the signalment of each horse and the protocols it completed in the gastric study

HORSE ID	AGE (yr)	BREED	GENDER	BODY WEIGHT (kg)	PROTOCOLS PERFORMED
1	12	QHX	GELDING	442	G, P, R, D+, D-, H
2	6	WP	GELDING	312	G, P, R, D+, D-, H
3	4	TBX	GELDING	465	G, P, R, D+, D-, B
4	5	TBX	GELDING	498	G, P, R, D+, D-, H
5	7	WP	GELDING	334	G, R, P
6	3	PA	GELDING	421	G, R, P
7	7	TBX	GELDING	453	G, P, R, D+, D-, H
8	3	PA	MARE	430	G, P, R, D+, D-, B
9	8	TBX	GELDING	506	G, P, R, D+, D-, B
10	7	TBX	GELDING	467	G, P, R, D+, D-, B
11	12	TB	GELDING	522	G, P, D+
12	22	TB	GELDING	352	G, P, D+
17	8	TB	GELDING	497	G, R, D+
18	12	PA	GELDING	432	G, R, D+
19	7	PA	GELDING	446	G, R, D+
21	12	TB	GELDING	510	G, R, D+
22	16	TBX	GELDING	451	G, R, D+

Table 3. Signalment Data On All Horses Used In The Gastric Study, Showing Group Of Origin And Protocols Performed On Each Horse.

(Breeds: PA –Paint, QHX – Quarter Horse Cross, TB – Thoroughbred, TBX – Thoroughbred Cross, WP –Welsh Pony)

(Protocols Performed: G – gastroscopy, P - PCR, R – Pyloritek™ RUT, D- - standard protocol, D+ - detomidine (0.03mg/kg iv), H – 250mg ^{13}C-urea, B - bethanechol protocol

GASTROSCOPY

Gastroscopy was performed in all horses taking part in the gastric study. All horses were fasted for 12-14 hours to ensure emptying of solid gastric contents. Horses were tranquilized using acepromazine[a] (0.03mg/kg IV), followed 15 minutes later by sedation with xylazine[b] (0.6mg/kg IV). Gastroscopy was performed using a three meter long, 9.5mm diameter, 2.8mm biopsy channel diameter video endoscope[c]. The technique has been described in detail elsewhere.[18] Briefly upon first entering the stomach the squamous mucosa of the greater curvature is seen. Advancing the endoscope around the curvature of the right side of the stomach and then dorsally demonstrates the cardia, lesser curvature, margo plicatus and the glandular mucosa of the body. To demonstrate the antrum and pylorus the endoscope is further advanced ventrolateral to the lesser curvature into the dependent portion of the stomach. The entire squamous and glandular mucosa and proximal duodenum were examined in all cases. The endoscopic appearance of the squamous and glandular mucosa was graded according to the EGUS Council grading system.[1] During gastroscopy biopsy samples were obtained from the glandular mucosa of the gastric antrum of each horse by one of two techniques:

1. A trans-endoscopic 2.6mm fenestrated biopsy cup was passed down the biopsy channel and advanced to grasp the mucosa. The biopsy tool was then sharply retracted with the endoscope insertion tube held in place. At least two tissue samples from the glandular mucosa of the gastric antrum of each horse were obtained. In the horse this technique typically obtains superficial mucosa.
2. A polypectomy snare technique was used, as previously described.[34] Briefly a 3.2m polypectomy snare[d] was attached to the outside of the endoscope insertion tube using zinc oxide tape. The snare was connected to a unipolar electrocautery unit[e] set at 100W cutting power that was grounded to the neck or flank of the horse using a surcingle and electrical contact gel. Passage of the endoscope/polypectomy snare unit

[a] Promace, Fort Dodge Laboratories Inc., Fort Dodge, IO
[b] Rompun, Bayer Corporation, Shawnee Mission, KS
[c] Video Gastroscope EV-P2900L, Pentax, Orangeburg, NY
[d] Wilson-Cook Medical, Winston-Salem, NC
[e] Aspen Excalibur, ConMed Aspen Labs, Englewood, CO

was aided by application of lubricant jelly. Once in the antrum the snare was advanced out of the sheath until it formed a 1cm diameter loop up against the antral wall. Using the transendoscopic fenestrated biopsy cup the center of the tissue was grasped and retracted through the open polypectomy loop. The loop was tightened and the biopsy separated by electrocautery. The biopsy was recovered by removing the entire endoscope/snare unit from the horse.

Gastroscopy was repeated after 24 hours in horses undergoing the polypectomy snare technique. If excessive bleeding was observed afterwards serial monitoring of heart rate and mucus membrane color was performed hourly for 6 hours.

PYLORITEK™ RAPID UREASE TESTING

Gastric urease activity was detected using the PyloriTek™ Rapid Urease Strip Test following the manufacturer's protocol. Briefly, Pyloritek™ is a dry format reagent strip that detects ammonia generated by *Helicobacter* urease-catalyzed hydrolysis of urea. Ammonia causes the pH indicator bromophenol blue to change color and gives a result within one hour. The Pyloritek™ system has three components. Firstly the reagent strip, which has an inbuilt positive and negative control, an area for up to three small gastric biopsies and a patient data section. The strip is made of two pads with a central perforated crease to allow easy folding. The substrate pad contains 3.3% urea and the reaction pad contains 0.1% bromophenol blue behind a semi-permeable membrane. At pH 3.0 - 4.6 this indicator turns yellow to blue. Secondly, an inert hydration reagent which contains 1.8% Tris buffer is required to activate the strip. Thirdly, a plastic reaction chamber is used to hold the folded strip and provide a solid contact between the gastric biopsy and the substrate pad, ensuring that any ammonia gas generated is directed through the membrane to the pH indicator. For the test to be valid the positive control spot must turn an intense blue within 60 minutes and the negative control must remain yellow. The test was counted as positive if an intense purple or dark blue color develops over the biopsy sample. This dark color can cover the all or only a small part of the biopsy specimen. No color change or a pale blue/faint grey haze over the specimen was considered a negative result (See fig 32). Color formed after 60 minutes was disregarded.

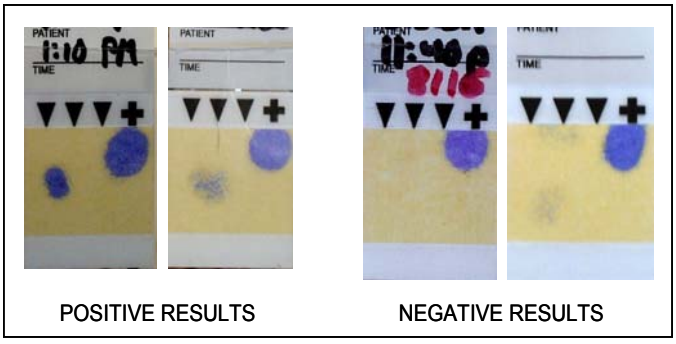


Fig 32. PyloriTek™ Test Interpretation

A single test strip was used for each horse and the horse's identification number and test start time were written on the reagent strip. On each strip 2-3 fenestrated biopsy cup tissue specimens or 2-3 small (1.5mm diameter) sections from the large polypectomy biopsy sample were used. Specimens were transferred directly from the biopsy tool to the

reaction pad to limit loss of urease activity. Following the manufacturer's protocol the upper quadrant opposite the reaction pad was left clear to act as a negative control area. Each test strip was read at 60 minutes.

GASTRIC *HELICOBACTER* SPECIFIC *16S rRNA* POLYMERASE CHAIN REACTION

To identify the presence of 16S rRNA specific to gastric *Helicobacter* PCR was performed using specific primers and a previously validated method.[163] A gastric antral biopsy specimen was obtained from each horse as previously described and frozen in sterile phosphate buffered saline at -70^{0}C. The total genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method.[299] Briefly the biopsies were washed with 10mM Tris-HCl-1mM EDTA (pH 8.0) (TE buffer) and dissolved by the use of 0.5% sodium dodecyl sulfate in the presence of 100μg of proteinase K per ml at 37^{0}C for 1 hour. The solubilized specimens were mixed with NaCl (0.5 M, final concentration), followed by 10 min of incubation at 65^{0}C in the presence of 1% CATB. Protein was removed using a chloroform-isoamyl alcohol (24:1) wash, followed by a second wash with phenol-chloroform-isoamyl alcohol (25:24:1). Genomic DNA was precipitated with ice cold isopropanol and resuspended in TE buffer. A 1μg DNA template was used for each PCR reaction. The upper and lower primer used were: 5/-GCT AGG ACT TGT ATT GTT ATA ATG-3/ and 5/-CCC AGT GTT GGA TAA GAG C-3/, respectively. ExTAQ polymerase (TaKaRa) was used for PCR for 30 cycles at an annealing temperature of 50^{0}C. The PCR products were size separated on a 0.8% agarose gel in the presence of ethidium bromide, and ΦX174DNA/*Hae*III markers were used as molecular weight standards. The positive control was *Helicobacter pylori* strain American Type Culture Collection (ATCC) 43504, gDNA, 1μg. One microgram DNA corresponds to approximately 500-550 *Helicobacter* cells.[300] The negative control contains no template. A standard ladder for the DNA 7500 kit and an Agilent automated Bioanalyzer[a] were used. Data analysis was performed using Agilent Data Review Software[b].

[a] Agilent 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA
[b] Bio-Sizing, Agilent Technologies, Palo Alto, CA

^{13}C-UREA BLOOD TESTING

^{13}C-UREA DIAGNOSTIC DRUG COMPONENT

Pharmaceutical grade synthetic ^{13}C-urea (chemical formula: $^{13}CH_4N_2O$) was obtained as a lyophilized white powder which contained greater than or equal to 99% of the carbon molecules in the form of the stable, non-radioactive isotope ^{13}C. The drug is the diamide of ^{13}C-carbonic acid and is highly soluble in water (1 gram per ml at 25^0C). Combination with 50ml sterile water was used to produce an oral dose of 500mg ^{13}C-urea as a 1% solution. This clear, colorless solution was prepared within 1 hour of administration and stored in an airtight 60ml syringe. The solution was visually checked for the presence of particulate matter and was discarded if any was found.

^{13}C-UREA BLOOD TEST PROTOCOLS

Four separate protocols were followed and horses were assigned randomly in a cross over design. To limit basal fluctuations in VCO_2 and $^{13}CO_2$ production all horses were fasted for 12 hours prior to and throughout the testing period, and exercise was limited by confining each horse to an individual stall 15 minutes prior to commencing the test protocol.[143] A 48 hour washout was allowed between test days, during which free access to water and the alfalfa hay was given. The standard protocol was named the D-, the remaining three protocols were variations upon this:

1. **D- Protocol.** In eight horses, a blood sample was obtained by standard jugular venipuncture to determine baseline ^{13}C enrichment of CO^2 prior to the administration of 500mg of the ^{13}C-urea solution by nasogastric intubation. The nasogastric tube was flushed with 100ml water and 100ml air prior to removal. Blood sampling was repeated at 30 minute intervals for two hours after ^{13}C-urea dosing. Each sample was aspirated by vacuum into 13 x 75mm, 3ml draw, 45 USP Heparin blood tube. The blood samples were stored at room temperature (between 15 and 25^0C) for a maximum of 36 hours prior to shipping to a reference laboratory for testing (Metabolic Solutions, Inc., Nashua, NH). All samples arrived within 3 days from collection.

2. **D+ Protocol.** In eight horses, the standard protocol was followed with the exception of Detomidine[a] (0.03mg/kg IV) being administered five minutes prior to dosing of the ^{13}C-urea solution, in order to delay gastric emptying.[149]
3. **B protocol**. In 4 horses, the standard protocol was followed with the exception of Bethanechol[b] (0.025mg/kg SC) being administered 30 minutes prior to dosing with 500mg ^{13}C-urea solution, in order to increase gastric contractile activity and promote gastric emptying.
4. **H protocol**. In 4 horses, the standard protocol was followed except a half dose of the 1% ^{13}C-urea solution (250mg) was given.

$^{13}CO_2$ DETECTION TECHNIQUE

A sample of gas released from the whole blood was withdrawn from the vacutainer and then analyzed for $^{12}CO_2$:$^{13}CO_2$ ratio using a Europa Scientific 20/20 ABCA Gas Isotope Ratio Mass Spetrometer (GIRMS). The GIRMS separates the CO_2 from other constituents by gas chromatography before measuring the $^{13}CO_2$:$^{12}CO_2$ ratio. Data is reported as delta ^{13}C per mil (parts per thousand difference) (ΔC). This represents the relative difference between the ratios of $^{13}CO_2$:$^{12}CO_2$ in the sample and the international ^{13}C standard, PeeDee Belemnite Limestone (PDB). At each time point during the test the increase in blood CO_2 ^{13}C enrichment is determined as Δ over baseline (ΔOB).

[a] Dormosedan, Animal Health (Pfizer Inc.), Exton, PA
[b] Sigma Chemical Co, St Louis, MO

DATA ANALYSIS TECHNIQUES

Repeated measures analysis of variance was performed using the MIXED procedure of the SAS system[a]. Model adequacy was assessed using standardized residual plots. The means of all time points were compared to time zero (baseline) means using Dunnett's test. A *P* value of ≤0.05 was considered significant.

[a] ver 8.02, SAS Institute Inc., Cary, NC

III. CECAL STUDY

INTRODUCTION

The horse as a hind gut fermenter has substantially different gastrointestinal physiology to the monogastric human. Small intestinal absorption of carbohydrate in the horse is not very efficient and large amounts of starch and sugars reach the hindgut where there is a tremendous capacity for fermentation.[301] The cecum and large intestine form three functionally separate fermentation chambers: the cecum, the ventral colon and the dorsal colon. The horse is also capable of extensive enterohepatic urea recycling as a non-protein nitrogen source and urease activity may be present within all three chambers. As the fluid phase gastric emptying $t_{1/2}$ is approximately 30 minutes the significant ^{13}C enrichment at 90 and 120 minutes in the D- protocol indicated that persistent urease activity was present at these times even though 87.5-93.75% of the solution should have left the stomach. Similarly in the B protocol persistent urease activity was found even though the addition of urease reduces fluid phase $t_{1/2}$ to approximately 17 minutes. In order to investigate the effect that cecal urease would have on the application of the ^{13}C-urea blood test to the horse, laparoscopic guided intracecal administration of ^{13}C-urea was performed. The test dose was administered into the cecal lumen and the protocol was completed following the previously described D+ protocol. The intracecal protocol was termed IC.

SUBJECTS

Eight horses were randomly selected from the research herd of the Marion duPont Scott Equine Medical Center (See table 4). These had a median age of 15years (mean 14.25years, range 8 – 22years) and a median weight of 502.5kg (mean 493.3kg, range 352 - 587kg). All the horses were grass fed for at least 1 month prior to inclusion in the study. The horses were kept in a large outdoor pen for at least 24 hours prior to the study and fed free choice alfalfa hay.

HORSE ID	AGE (yr)	BREED	GENDER	BODY WEIGHT (kg)
12	22	TB	GELDING	352
13	18	TB	GELDING	501
14	19	AR	GELDING	587
15	8	TB	GELDING	566
16	19	TB	GELDING	512
17	8	TB	GELDING	497
18	12	PAINT	GELDING	432
20	8	TB	GELDING	504

Table 4. Signalment Data On All Horses Used In The Cecal Study.

(Breeds: PA –Paint, QHX – Quarter Horse Cross, TB – Thoroughbred, TBX – Thoroughbred Cross, WP –Welsh Pony)

DIAGNOSTIC DRUG COMPONENT

A 500mg dose of ^{13}C-urea was prepared as previously described.

INTRA-CECAL (IC) ^{13}C-UREA BLOOD TEST PROTOCOL

All horses were fasted for 12 hours prior to and throughout the testing protocol. An area of the right paralumbar area, extending from the tuber coxae caudodorsally to the 17th rib cranially and the level of the stifle fold ventrally, was clipped and prepared aseptically in a standard fashion. Detomidine (0.03mg/kg IV) was administered five minutes prior to beginning the surgical procedure. Local anesthesia was provided by subcutaneous mepivacaine HCl[a] in an inverted L-block pattern over the surgical site and by intramuscular mepivacaine HCl at the incision sites. The surgical site was draped in a standard fashion including application of an iodinated bio-occlusive film[b]. A 10mm vertical incision was made through the skin and superficial adipose fascia to create a laparoscopic portal. Straight Metzenbaum scissors were used to dissect through the external oblique abdominal and internal oblique abdominal muscles. Using the blunt tip

[a] Carbocaine, Breon Labs Inc., New York, NY
[b] Steri-drape™, 3M Medical Products Division, St Paul. MO

trocar with a 10mm cannula, the peritoneal cavity was entered by passing through the transverse abdominal muscle, associated fascia and the parietal peritoneum. Intra-peritoneal pressure was allowed to equilibrate to atmospheric pressure to facilitate laparoscopy. Laparoscopy was performed using a 57cm 30^0 rigid video laparoscope system[a]. The axial wall of the body of the cecum was visualized (fig. 33).

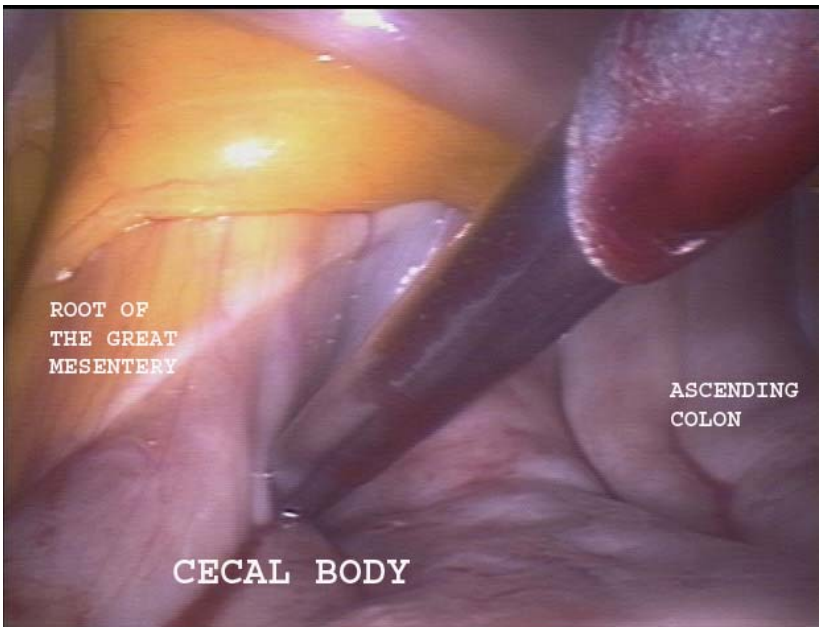


Fig 33. Laparoscopic view of the cecal base from the right paralumbar fossa.

A 5mm incision for an instrument portal was made 4 cm ventral to the camera incision using the previously described technique. A 40cm injection cannula, with a 4cm long 1.2mm diameter needle tip[b] was introduced through an instrument sleeve. The laparoscope was oriented to allow visualization of the instrument and the cecal base. The entire needle tip was purposefully introduced through the dorsomedial wall of the cecal body into the cecal lumen. Twenty ml of sterile saline was then rapidly infused to visually confirm placement and to ensure that leakage was not occurring. The ^{13}C-urea solution was then instilled into the cecum and the needle was flushed with 50ml sterile

[a] Veterinary Video Camera 62230120 and Large Animal Laparoscope 62032BPA, Karl Storz Veterinary Endoscopy, America, Inc., Bethel, CT
[b] Laparoscopic Injection Cannula, Karl Storz Veterinary Endoscopy, America, Inc., Bethel, CT

water prior to removal. The laparoscope was removed and single layer closure of the skin incisions was performed using 3.5Metric polypropylene[a] in a simple cruciate pattern. Blood was collected by standard jugular venipuncture prior to cecal infusion to determine baseline ^{13}C enrichment of CO^2. Sampling was repeated at 30 minute intervals for two hours after ^{13}C-urea infusion. Each sample was aspirated by vacuum into 13 x 75mm, 3ml draw, 45 USP Heparin blood tube. The blood samples were stored at room temperature (between 15 and 25^0C) for a maximum of 36 hours prior to shipping to a reference laboratory for testing (Metabolic Solutions, Inc., Nashua, NH). All samples arrived within 3 days from collection. Food and exercise were withheld throughout the test period to limit basal fluctuations in VCO_2 and $^{13}CO_2$ production.
Serial monitoring of heart rate, temperature, fecal output and demeanor was performed every six hours for 2 days following the surgery.

$^{13}CO_2$ DETECTION TECHNIQUE

Enrichment of $^{13}CO_2$ was performed as previously described.

DATA ANALYSIS TECHNIQUES

Repeated measures analysis of variance was performed using the MIXED procedure of the SAS system. Model adequacy was assessed using standardized residual plots. The means of all time points were compared to time zero (baseline) means using Dunnett's test. A P value of ≤ 0.05 was considered significant.

[a] Prolene™, Ethicon, Somerville, NJ

RESULTS

I: PILOT STUDY

Both horses tolerated the urea infusion well. No complications from nasogastric intubation (epistaxis), tracheal aspiration (subcutaneous emphysema, abscessation) or venous sampling (hematomas) were observed. BUN prior to ^{13}C-urea infusion was 18 mg/dl and post infusion was 17mg/dl.

i. ^{13}C-Urea Breath Test

Horse	*Time (mins)*	*∆C (per mil)*	*∆OB*
A	*0*	*-29.18*	
	30	*-29.14*	*0.04*
B	*0*	*-31.03*	
	30	*-32.19*	*-1.16*

Table 5. Results of pilot ^{13}C-urea breath test

At t_0 (baseline) the mean ± standard deviation ΔC was -30.11 ± 1.31 per mil. The mean ±SD ΔC at 30 minutes post infusion was -30.665 ± 2.16 per mil. Comparison of the mean ΔC at t_0 and t_{30} by paired *t*-test gave a *t* value of 0.933. This corresponds to a two tailed P value of P = 0.522.

ii. ^{13}C-Urea Blood Test

Horse	*Time (mins)*	*∆C (per mil)*	*∆OB*
A	*0*	*-24.57*	
	30	*-25.46*	*-0.89*
B	*0*	*-27.77*	
	30	*-29.05*	*-1.28*

Table 6. Results of pilot ^{13}C-urea breath test

The mean ± SD baseline ΔC was -26.17 ± 2.26 per mil. The mean ± SD 30 min ΔC was -27.26 ± 2.54. Comparison of the mean ΔC at t_0 and t_{30} by paired *t*-test gave a *t* value of 5.564. This corresponds to a two tailed P value of P = 0.113.

Comparison of the mean ΔOB of the breath and blood tests by paired *t*-test did not show statistical difference in results (P=0.21).

II. PRINCIPAL STUDY

a) Gastroscopy

All horses tolerated gastroscopy without complications. The results of EGUS grading are shown in table 7. The incidence of gastric lesions is shown by location and EGUS grade in tables 8 and 9 and figure 34. No lesions greater than grade 2 were observed in any horse. No duodenal abnormalities were observed in any case.

Gastric biopsies were successfully obtained from all horses examined endoscopically. No complications occurred with the fenestrated biopsy cup technique. In 13/15 of the horses undergoing polypectomy snare biopsy no complications occurred and normal granulation tissue was seen on 24 hours follow up examination (fig. 35). Complications occurred in two cases. In the first case persistent bleeding from the biopsy site occurred after retrieval of a large (>1.5cm diameter) sample, despite the use of electrocautery (fig 36). Repeat gastroscopy after 24 hours showed early granulation and no further bleeding. In the second case a large hematoma had formed at the biopsy site (fig 37).

HORSE ID	SQUAMOUS MUCOSA		GLANDULAR MUCOSA	
	DESCRIPTION	GRADE	DESCRIPTION	GRADE
1	Normal	0	Normal	0
2	Normal	0	Mild antral hyperemia dorsal to pylorus	1
3	Normal	0	Mild, diffuse antral hyperemia	1
4	Normal	0	Normal	0
5	Single erosion of the lesser curvature	2	Single area of antral thickening and hyperemia	2
6	Normal	0	Mild, diffuse antral hyperemia	1
7	Normal	0	Normal	0
8	Normal	0	Normal	0
9	Normal	0	Mild, diffuse antral hyperemia	1
10	Normal	0	Normal	0
11	Normal	0	Normal	0
12	Multiple small erosions of the lesser curvature	2	Normal	0
15	Normal	0	Mild hyperemia	1
16	Normal	0	Normal	0
17	Normal	0	Normal	0
18	Multiple small erosions of the lesser curvature	2	Normal	0
19	Mild hyperkeratosis	1	Normal	0
21	Multiple erosions of the lesser curvature	1	Normal	0

Table 7. Results of Gastroscopy on Horses, graded according to EGUS guidelines.

ANATOMIC LOCATION	FREQUENCY OF ABNORMALITY	
	n	%
SQUAMOUS MUCOSA	5/18	27.8
GLANDULAR MUCOSA	6/18	33.3

Table 8. Overall Prevalence Of Gastric Lesions

EGUS GRADE	FREQUENCY OF EGUS SCO			
	SQUAMOUS MUCOSA		GLANDULAR MUCOSA	
	n	%	n	%
0	13/18	72.2	12	66.7
1	2/18	11.1	5	27.8
2	3/18	16.7	1	5.5
3	0/18	0	0	0
4	0/18	0	0	0

Table 9. Prevalence Of Gastric Lesions Of A Particular Grade

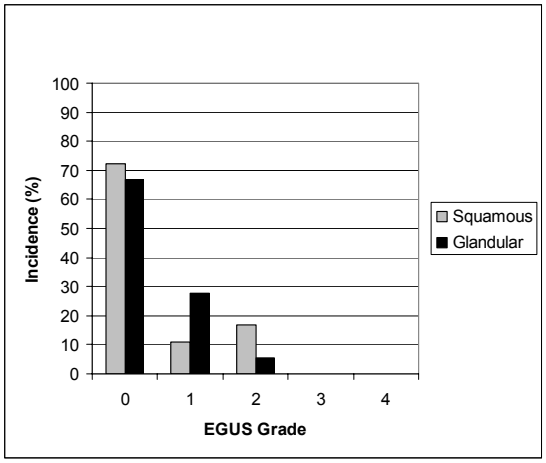


Fig 34. Prevalence of Lesions by EGUS Grade

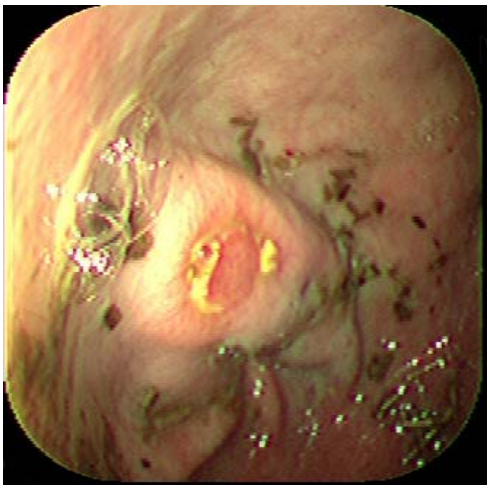


Fig 35. Normal granulation tissue adherent to the biopsy site, seen at 24 hours

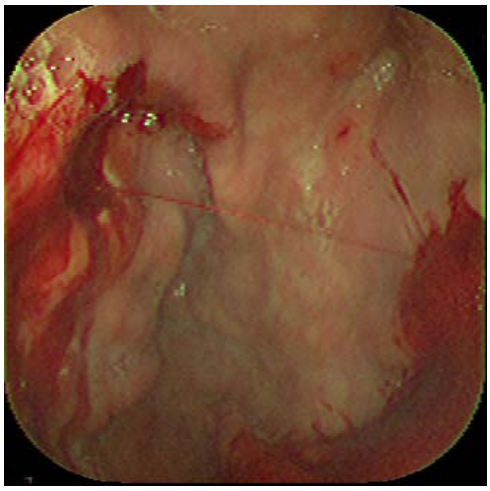


Fig 36. Excessive bleeding from the biopsy site immediately after the biopsy was obtained

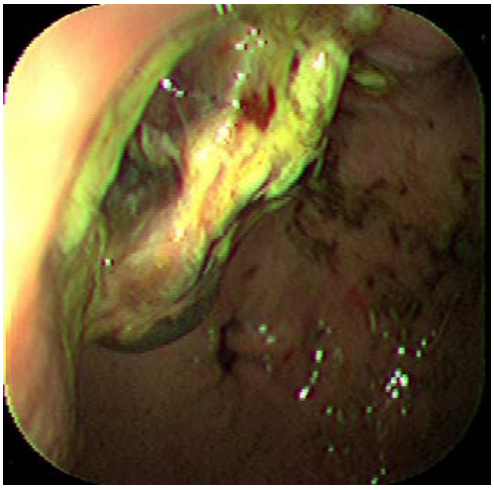


Fig 37. Large hematoma adherent to the biopsy site, seen at 24 hours

PyloriTek™ Rapid Urease Strip Testing

Positive results were obtained in 7/15 horses, negative results in the remaining 8/15 (table 10 and figure 38). All strips exhibited successful negative and positive controls.

RESULT (60 min)	***n/15***	***Prevalence***
Positive	*7*	*47%*
Negative	*8*	*53%*

Table 10. Pyloritek™ Rapid Urease Strip Test Results in 15 horses

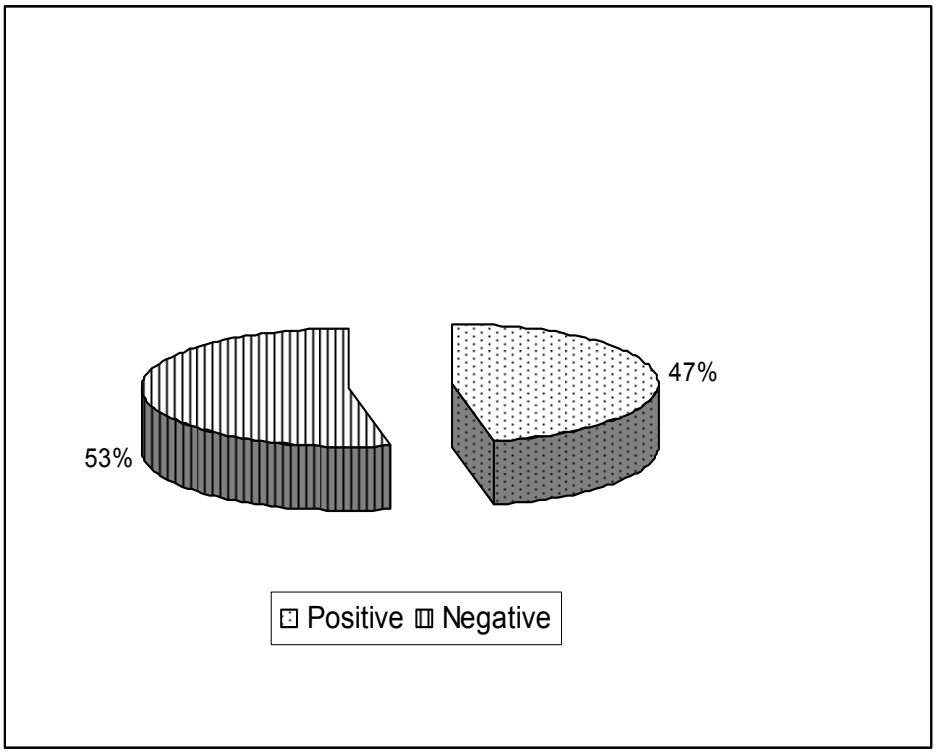


Fig 38. Pyloritek status in 15 horses.

b) *Helicobacter* specific *16s rRNA* Polymerase Chain Reaction

The results are shown in figures 39-42. The positive control is labeled "16s +CTRL" and has a primary band at 1483bp. Table 11 and figures 43 and 44 show the interpretation of the PCR results.

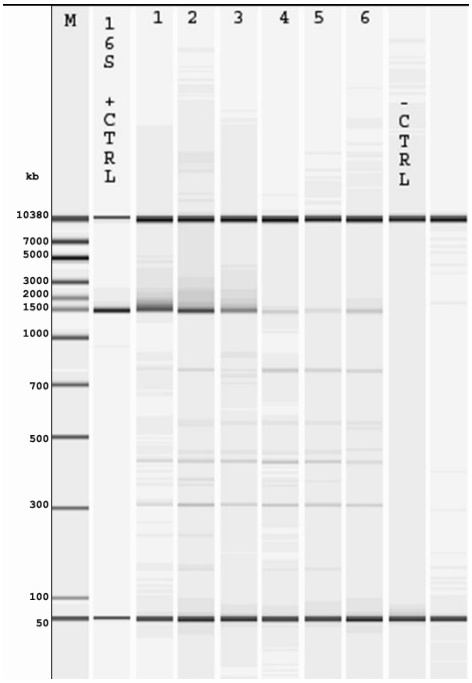


Fig 39. PCR data horses 1-6

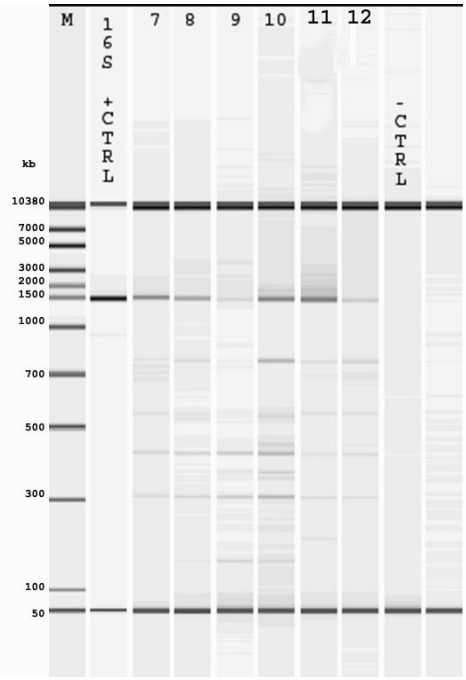


Fig 40. PCR data horses 7-12

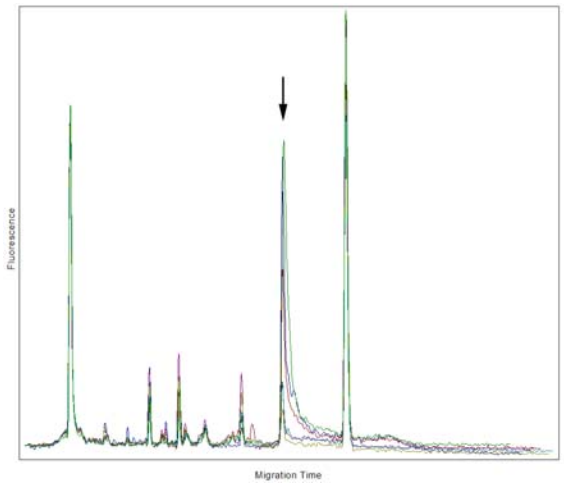


Fig 41. Composite Graph of PCR Fluorescence Horses 1-6 (Arrow indicates +ve control region)

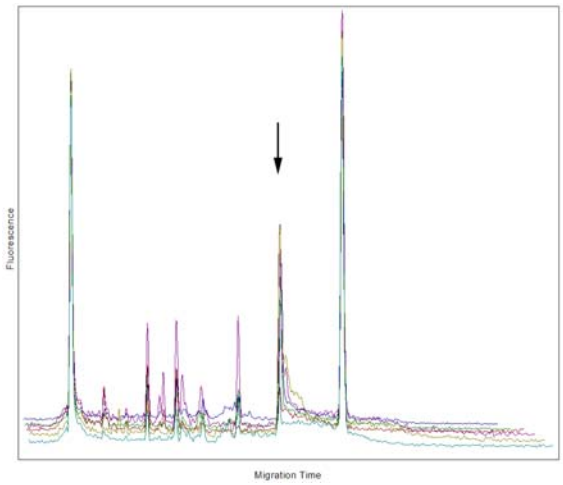


Fig 42. Composite Graph of PCR Fluorescence Horses 7-12 (Arrow indicates +ve control region)

HORSE	RESULT		HORSE	RESULT
1	*Positive*		*7*	*Positive*
2	*Positive*		*8*	*Positive*
3	*Positive*		*9*	*Weak Positive*
4	*Weak Positive*		*10*	*Positive*
5	*Weak Positive*		*11 (London)*	*Positive*
6	*Weak Positive*		*12 (Sunny)*	*Weak Positive*

Table 11. Results of PCR for *Helicobacter* specific gene material

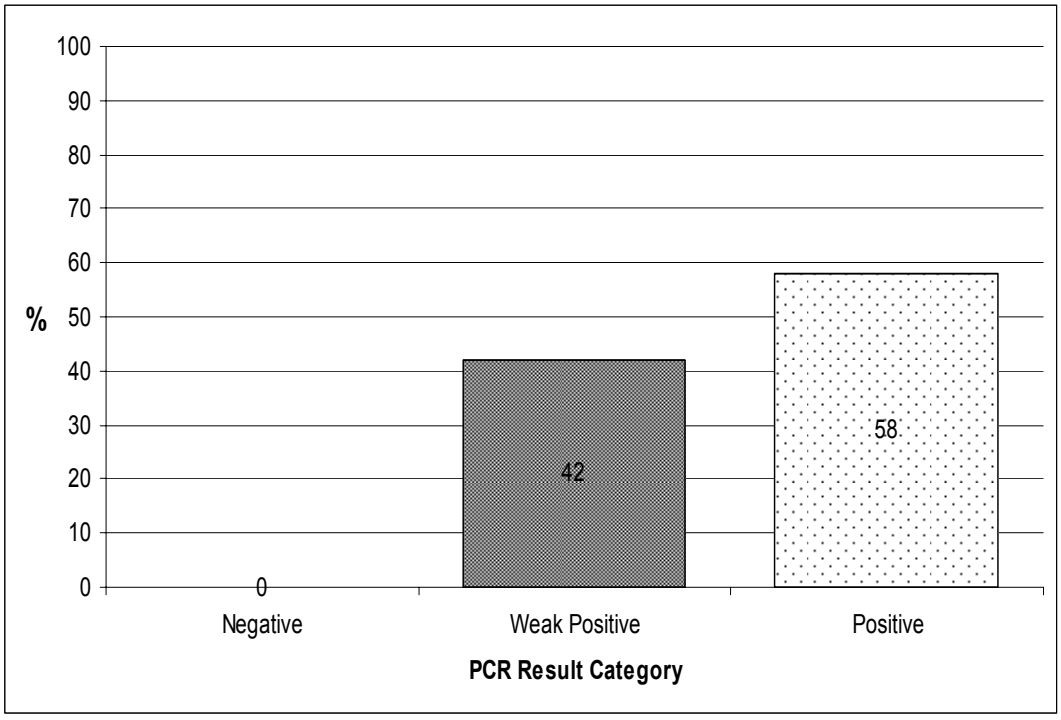


Fig 43. Prevalence of *Helicobacter* Specific PCR status in 12 Horses

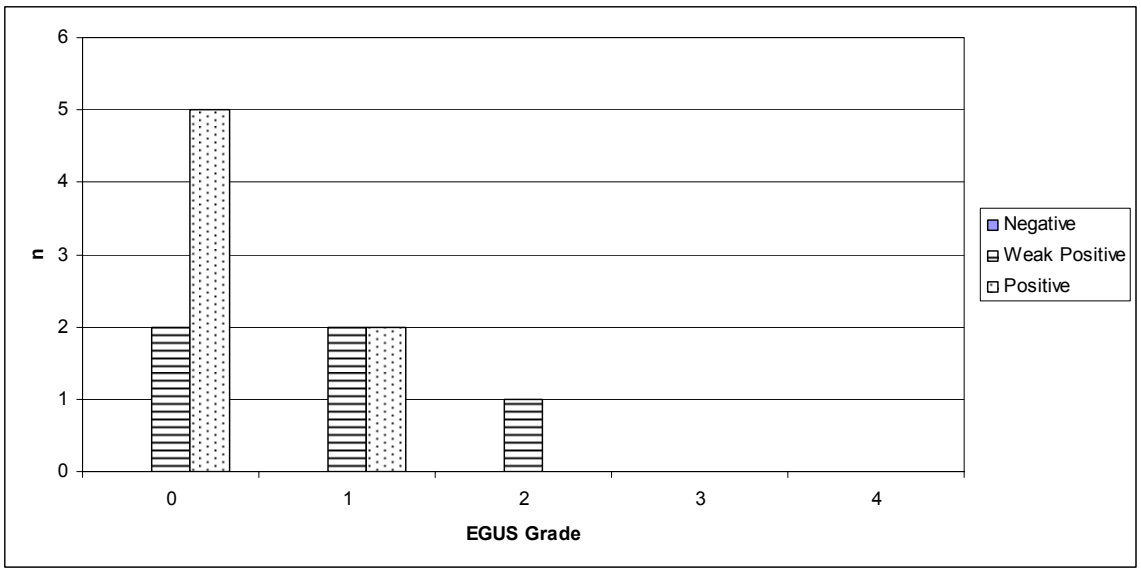


Fig 44. Prevalence of *Helicobacter* Specific PCR status in 12 Horses by EGUS Grade

c)

^{13}C-Urea Blood Testing

All horses tolerated the urea infusions well. No complications from repeated nasogastric intubation (epistaxis) or serial venous sampling (hematomas) were observed. No complications related to administration of the sedative, detomidine, or the intestinal prokinetic, bethanechol, occurred.

Analysis of one sample was not possible due to low tube $^{13}CO_2$ labeled gas content. This error occurs when an improper seal on the tube bung allows gas escape during transport. All other samples were analyzed without complication.

The mean±95%CL values for baseline ^{13}C enrichment was 25.88±1.05 ^{13}C per mil. The mean individual variation in baseline ^{13}C enrichment was 0.31±0.24 per mil.

The results for the various ^{13}C-urea protocols are described below.

i. Detomidine Negative Protocol

Time	n	Geometric mean	Lower 95% CL	Upper 95% CL
0	8	-26.104	-26.8849	-24.7764
30	8	-19.8547	-23.2094	-14.151
60	8	-11.0123	-18.0087	0.8833
90	8	-12.4464	-18.8522	-1.555
120	8	-13.2205	-19.3074	-2.8711

Table 12. Descriptive statistics for ΔC for D- Protocol for 8 Horses

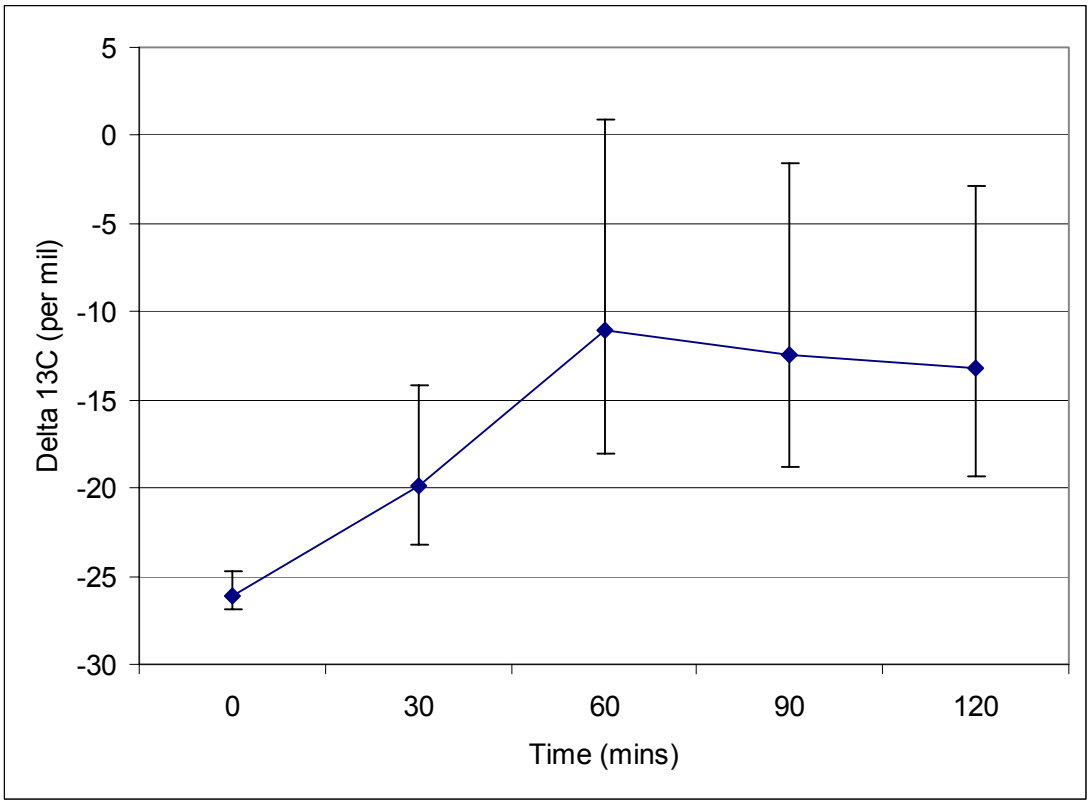


Fig 45. Graph of ΔC geometric mean±95%CL for D- Protocol

Time	n	Geometric mean	Lower 95% CL	Upper 95% CL
30	8	7.0237	-1.7112	15.7587
60	8	21.0000	12.2650	29.7350
90	8	18.5075	9.7725	27.2425
120	8	16.2525	7.5175	24.9875

Table 13. Descriptive statistics for D- protocol ΔOB values for 8 Horses

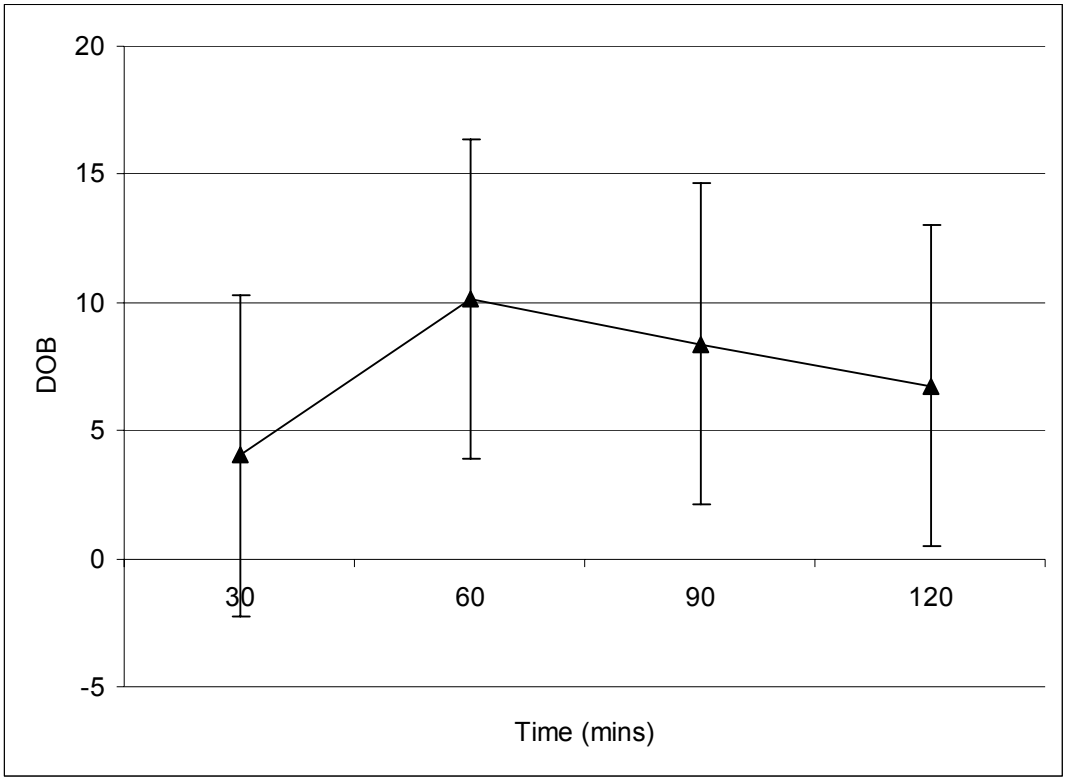


Fig 46. Graph of ΔOB mean±95%CL for D- protocol

Effect	*N*	*Time*	*Time*	*P*	*α*
Time	*8*	*30*	*0*	*<.0001*	*0.05*
Time	*8*	*60*	*0*	*<.0001*	*0.05*
Time	*8*	*90*	*0*	*<.0001*	*0.05*
Time	*8*	*120*	*0*	*<.0001*	*0.05*

Table 14. Comparison of ΔC means by RMANOVA using the Dunnett-Hsu adjustment for the D-protocol.

At all time points P <0.05 indicating that ΔC is different to the baseline value therefore the null hypothesis, that no urease activity is present within the equine stomach, is rejected.

ii. Detomidine Positive Protocol

Time	n	Geometric Mean	Lower 95% CL	Upper 95% CL
0	8	-25.3919	-26.401	-23.7462
30	8	-22.8924	-24.8962	-19.5951
60	8	-21.4502	-23.9842	-17.3172
90	8	-20.2821	-23.268	-15.412
120	8	-19.8633	-23.0113	-14.729

Table 15. Descriptive statistics for ΔC for the D+ protocol for 8 Horses

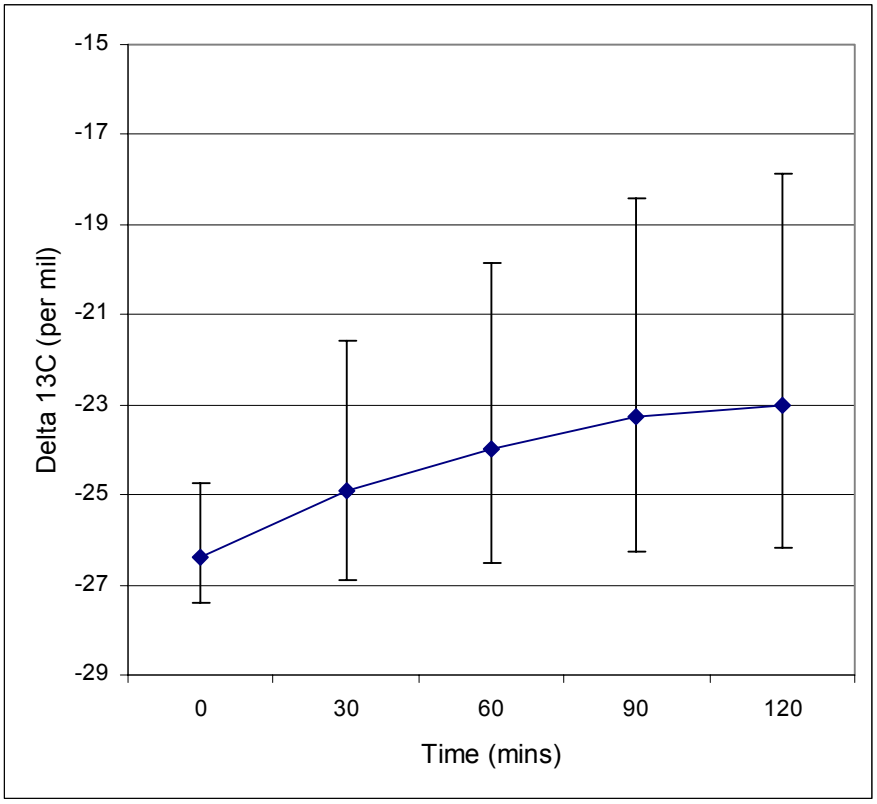


Fig 47. Graph of ΔC geometric mean±95%CL for D+ Protocol

Time	n	Geometric mean	Lower 95% CL	Upper 95% CL
30	8	4.2798	-2.8976	11.4571
60	8	6.4550	-0.4769	13.3869
90	8	8.7512	1.8193	15.6832
120	8	11.2225	4.2906	18.1544

Table 16. Descriptive statistics for ΔOB for the D+ protocol for 8 Horses

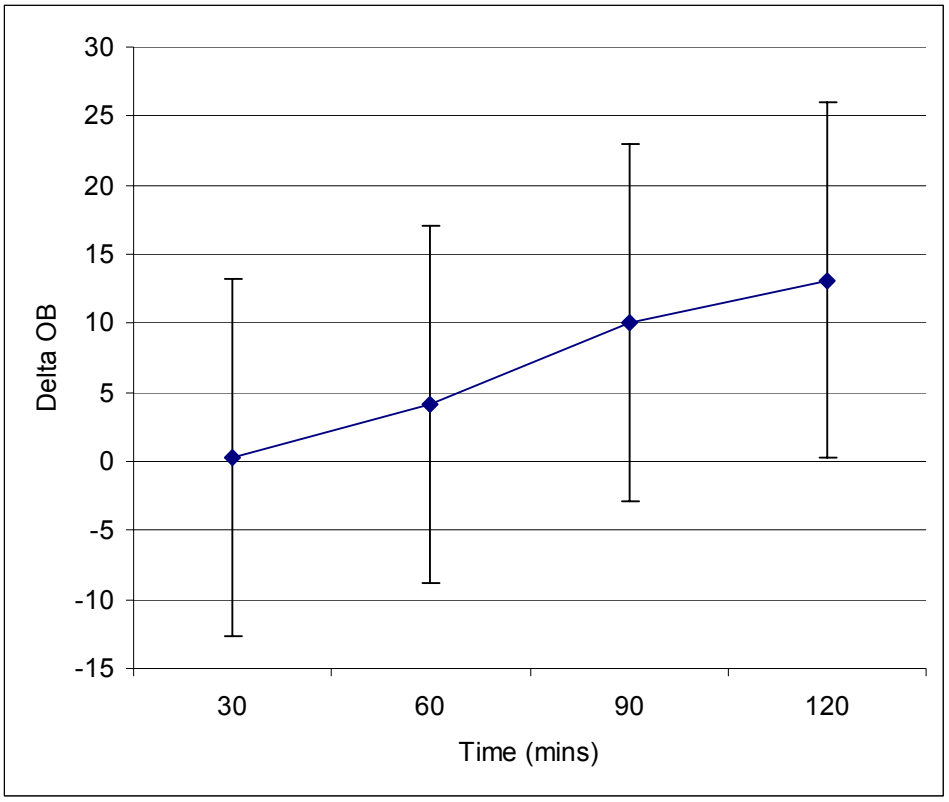


Fig 48. Graph of ΔOB mean±95%CL for D+ Protocol

Effect	N	Time	Time	P	α
Time	8	30	0	0.0015	0.05
Time	8	60	0	0.0007	0.05
Time	8	90	0	0.0006	0.05
Time	8	120	0	0.0009	0.05

Table 17. Comparison of ΔC means by RMANOVA using the Dunnett-Hsu adjustment for the D+ Protocol.

At all time points $P < 0.05$ indicating that ΔC is differs from the baseline value therefore the null hypothesis, that no urease activity is present within the equine stomach, is rejected.

iii. 250mg ^{13}C-urea Test Dose Protocol

Time	n	Geometric mean	Lower 95% CL	Upper 95% CL
0	8	-25.3527	-26.4952	-23.3426
30	8	-21.8063	-24.4794	-17.1036
60	8	-16.4927	-21.4591	-7.7556
90	8	-18.0740	-22.3579	-10.5375
120	8	-19.7148	-23.2906	-13.4241

Table 18. Descriptive statistics for ΔC for H Protocol for 8 Horses

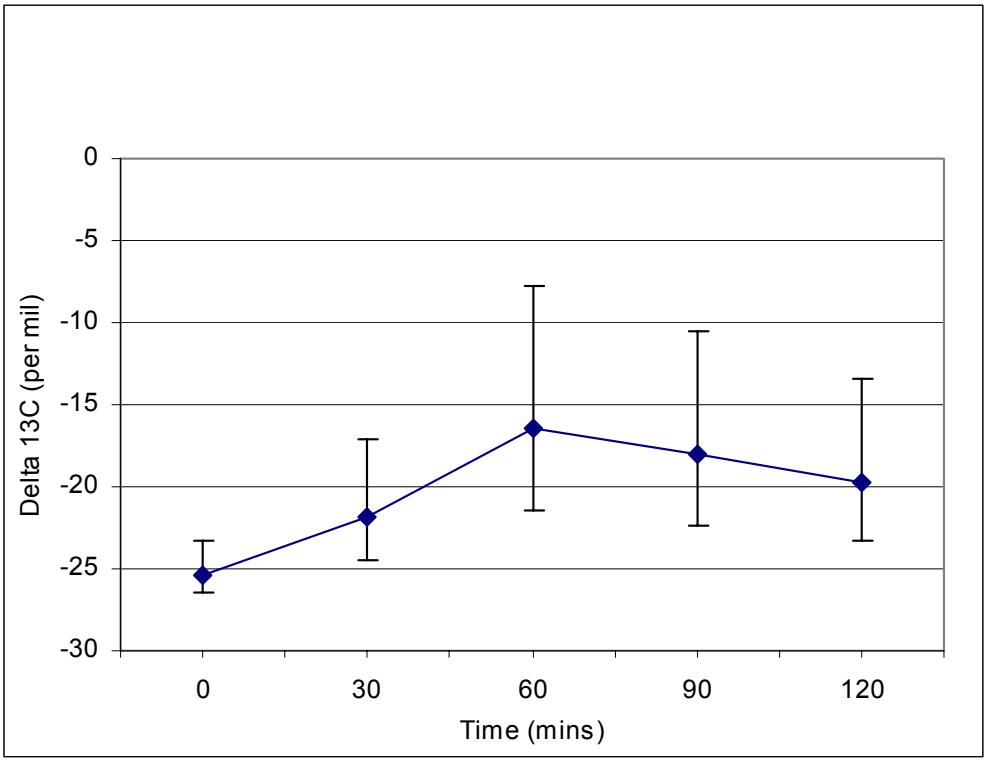


Fig 49. Graph of ΔC geometric mean±95%CL for H Protocol

Time	n	Geometric mean	Lower 95% CL	Upper 95% CL
30	8	4.0450	-2.2211	10.3111
60	8	10.1325	3.8664	16.3986
90	8	8.3850	2.1189	14.6511
120	8	6.7525	0.4864	13.0186

Table 19. Descriptive statistics for H Protocol ΔOB values for 8 Horses

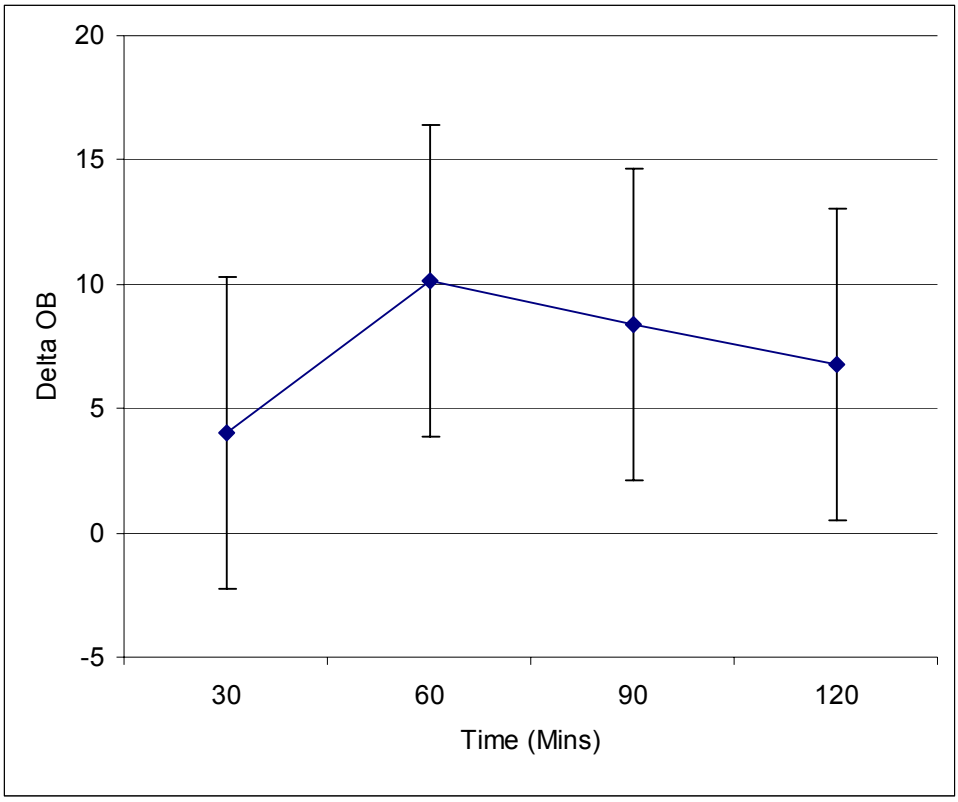


Fig 50. Graph of ΔOB mean±95%CL for H Protocol

Effect	*n*	*Time*	*Time*	*P*	*α*
Time	*8*	*30*	*0*	*0.0051*	*0.05*
Time	*8*	*60*	*0*	*0.0003*	*0.05*
Time	*8*	*90*	*0*	*0.0016*	*0.05*
Time	*8*	*120*	*0*	*0.0079*	*0.05*

Table 20. Comparison of ΔC means by RMANOVA using the Dunnett-Hsu adjustment for the H Protocol.

At all time points $P < 0.05$ indicating that ΔC is different to the baseline value therefore the null hypothesis, that no urease activity is present within the equine stomach, is rejected.

iv. Bethanechol Protocol

Time	n	Geometric mean	Lower 95% CL	Upper 95% CL
0	8	-26.1966	-27.0491	-24.5796
30	8	-23.7946	-25.7827	-20.0241
60	8	-17.3146	-22.3660	-7.7340
90	8	-14.3798	-20.8186	-2.1679
120	8	-13.2048	-20.1991	0.0606

Table 21. Descriptive statistics for B Protocol for 8 Horses

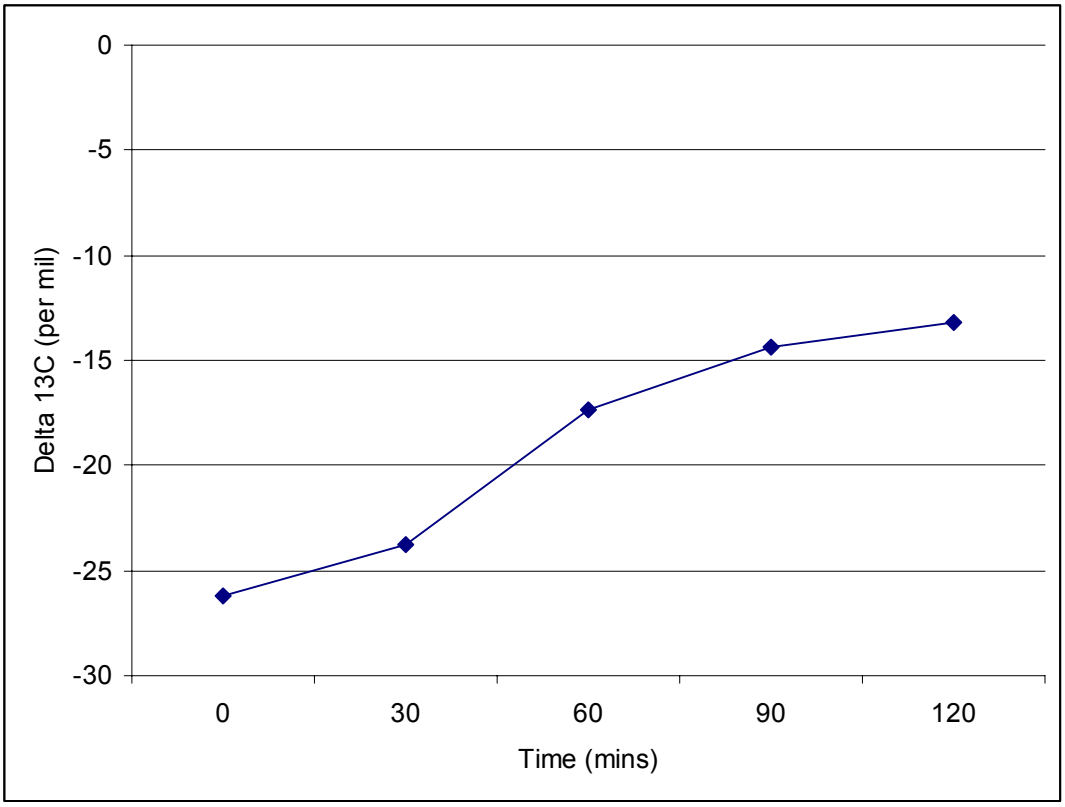


Fig 51. Graph of ΔC geometric mean±95%CL for B Protocol

Time	n	Geometric mean	Lower 95% CL	Upper 95% CL
30	8	1.7175	-5.9520	9.3870
60	8	9.7775	2.1080	17.4470
90	8	13.3025	5.6330	20.9720
120	8	15.1725	7.5030	22.8420

Table 22. Descriptive statistics for B Protocol ΔOB values for 8 Horses

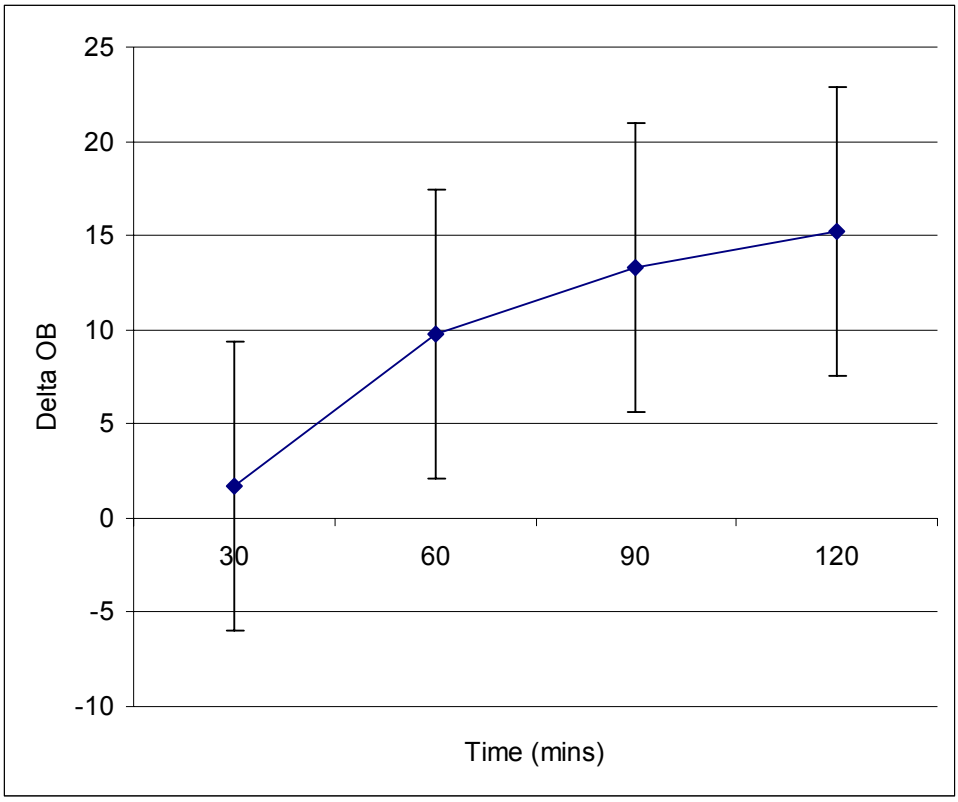


Fig 52. Graph of ΔOB means±95%CL for B Protocol

Effect	*n*	*Time*	*Time*	*P*	*α*
Time	*8*	*30*	*0*	*0.0009*	*0.05*
Time	*8*	*60*	*0*	*<.0001*	*0.05*
Time	*8*	*90*	*0*	*<.0001*	*0.05*
Time	*8*	*120*	*0*	*<.0001*	*0.05*

Table 23. Comparison of ΔC means by RMANOVA using the Dunnett-Hsu adjustment for the B Protocol.

At all time points P <0.05 indicating that ΔC is different to the baseline value therefore the null hypothesis, that no urease activity is present within the equine stomach, is rejected.

v. Principal Study Summary Tables and Figures

PROTOCOL	MEAN PEAK (Δ^{13}C per mil)	MEAN PEAK ΔOB	TIME OF PEAK ENRICHMENT (min)
D-	-11.01	21.00	60
D+	-19.86	11.23	120
H	-16.49	10.13	60
B	-13.20	15.17	120

Table 24. Peak ^{13}C Enrichment of the Various ^{13}C-urea Blood Test Protocols and the Time of Occurrence

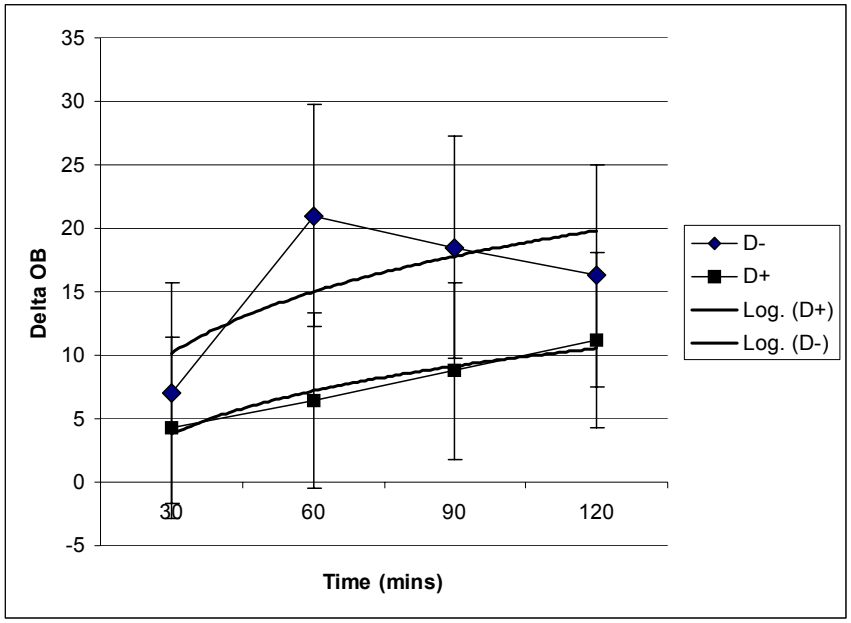


Fig 53. ΔOB Mean±95%CL at each time point for the D- and D+ Protocols, with a Logarithmic Trend Line Applied

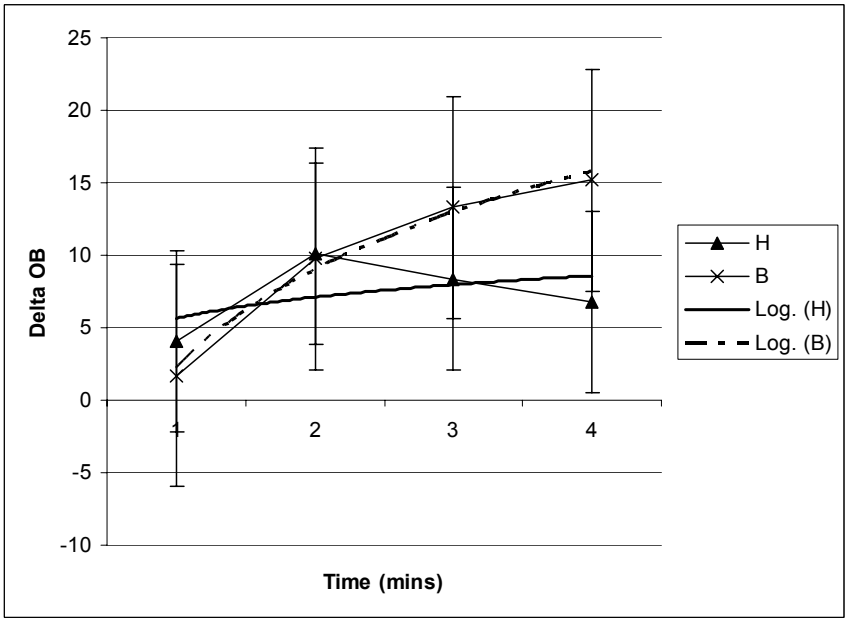


Fig 54. ΔOB Mean±95%CL at each time point for the B and H Protocols, with a Logarithmic Trend Line Applied

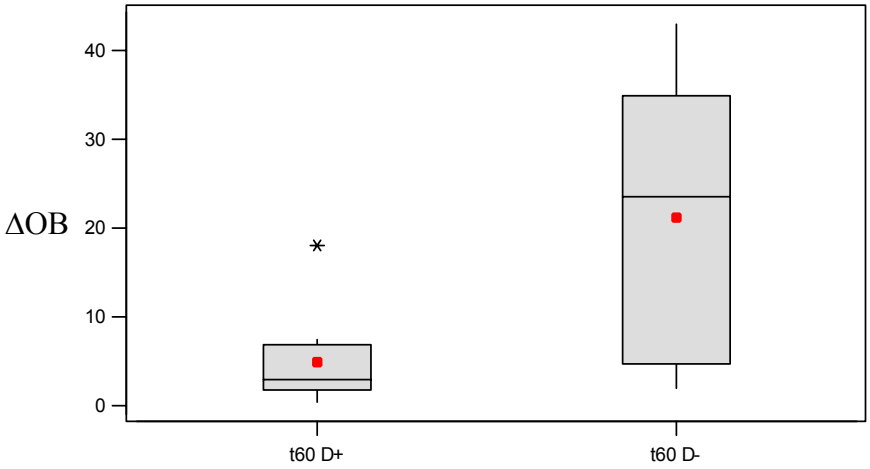


Fig 55. Boxplots of the ΔOB Mens at t60 for the D- and D+ Protocols (* indicates significance *P*≤0.05)

PROTOCOL	TIME (mins)	n ΔOB > 6	SENSITIVITY (%)
D-	60	6/8	75
D+	90	3/8	37.5
H	60*, 90, 120	2/4	50
B	60, 90, 120*	3/4	75

Table 25. The Sensitivity of the Various Protocols of the ^{13}Urea Blood Test with a Positive Cut Off ΔOB Value of >6 When Compared to PCR as a Gold Standard. (asterisk indicates peak enrichment period when multiple time points are shown)

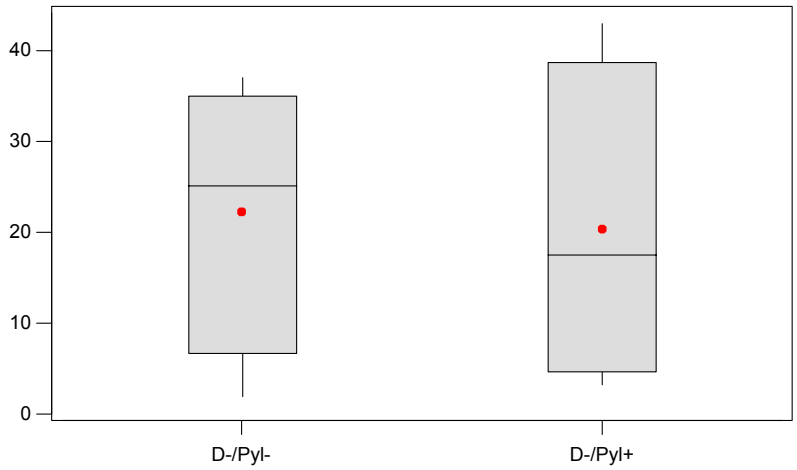


Fig 56. Boxplots of the ΔOB Means in the D- Protocol for Pyloritek Positive (Pyl+) and Negative (Pyl-) Horses

III. INTRA-CECAL (IC) STUDY

The cecum was visualized and the ^{13}C-Urea solution successfully injected into the lumen of the cecal body of each of the 8 horses. The laparoscopic procedure was tolerated in all horses without incident. The surgical incisions healed normally with no complications (dehiscence, abscess formation) and sutures were removed routinely at 10 days. No post operative complications (colic, peritonitis) occurred. Serial monitoring of heart rate and temperature were within published normal limits in all horses. Normal fecal output and demeanor were observed for 2 days following the surgery.

The results of the intracecal protocol are shown below.

Time	n	Geometric Mean	Lower 95% CL	Upper 95% CL
0	8	-29.0976	-29.8934	-27.7296
30	8	-16.9145	-22.8066	-6.7851
60	8	-12.4609	-20.2160	0.8713
90	8	-11.4515	-19.6289	2.6066
120	8	-12.5956	-20.2944	0.6396

Table 26. Descriptive statistics for ΔC for IC Protocol for 8 Horses

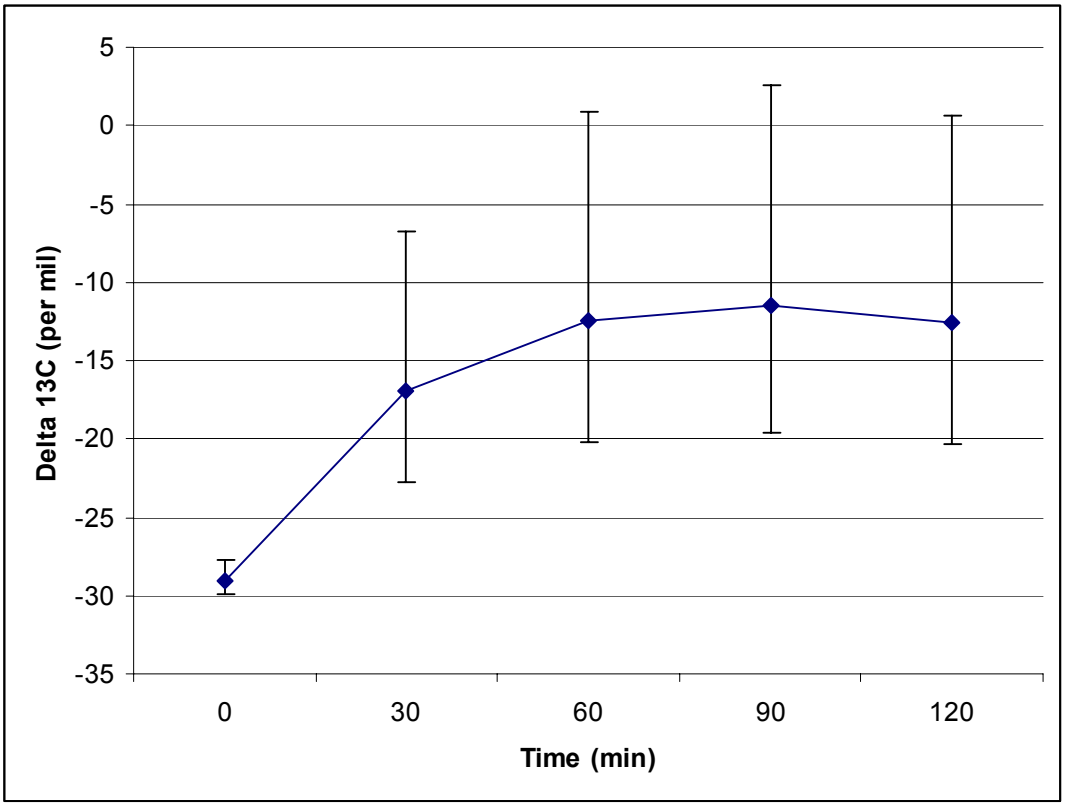


Fig 57. Graph of ΔC geometric mean±95%CL for IC Protocol

Time	n	Geometric mean	Lower 95% CL	Upper 95% CL
30	8	13.6213	7.9983	19.2442
60	8	18.4900	12.8671	24.1129
90	8	19.0363	13.4133	24.6592
120	8	17.8288	12.2058	23.4517

Table 27. Descriptive statistics for ΔOB for IC Protocol for 8 Horses

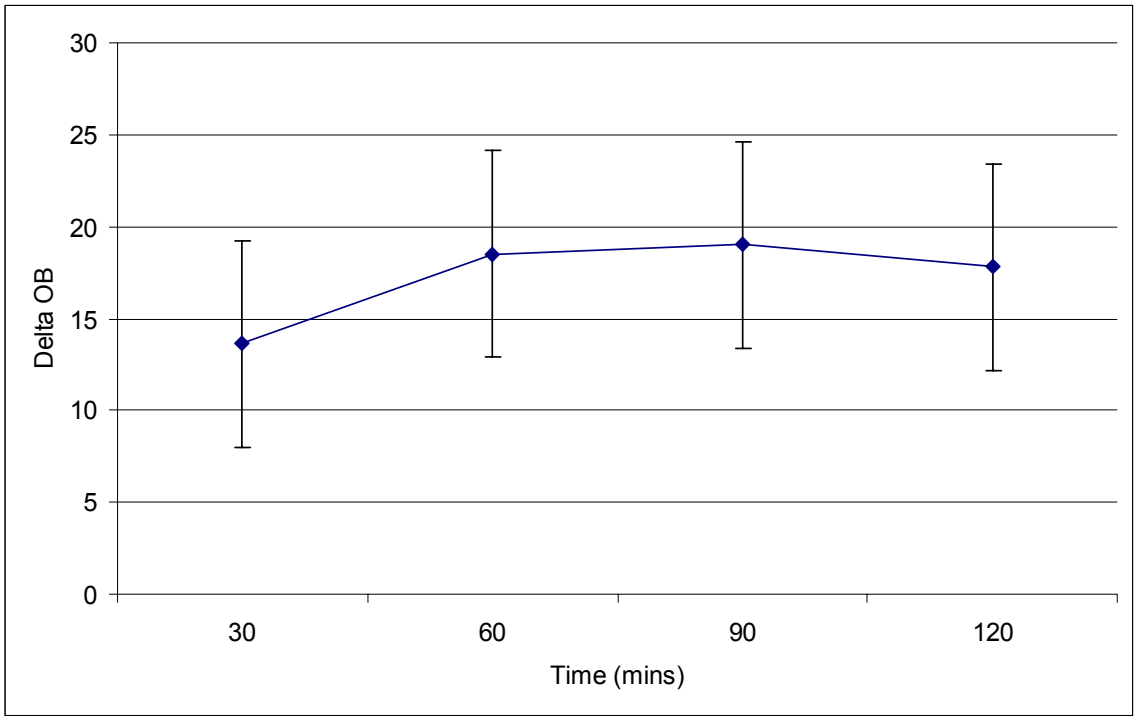


Fig 58. Graph of ΔOB mean±95%CL for IC Protocol

Effect	*n*	*Time*	*Time*	*P*	*α*
Time	*8*	*30*	*0*	*<.0001*	*0.05*
Time	*8*	*60*	*0*	*<.0001*	*0.05*
Time	*8*	*90*	*0*	*<.0001*	*0.05*
Time	*8*	*120*	*0*	*<.0001*	*0.05*

Table 28. Comparison of ΔC means by RMANOVA using the Dunnett-Hsu adjustment for the IC Protocol.

At all time points P <0.05 indicating that ΔC is different to the baseline value therefore the null hypothesis that the cecum contains no urease activity was rejected.

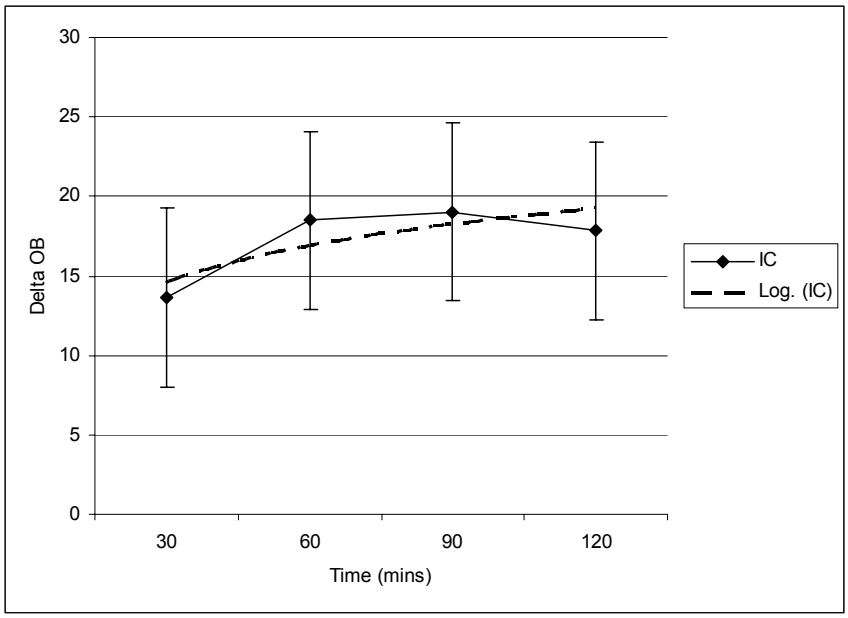


Fig 59. ΔOB Mean±95%CL at each time point for the Intra-Cecal Protocol, with a Logarithmic Trend Line Applied

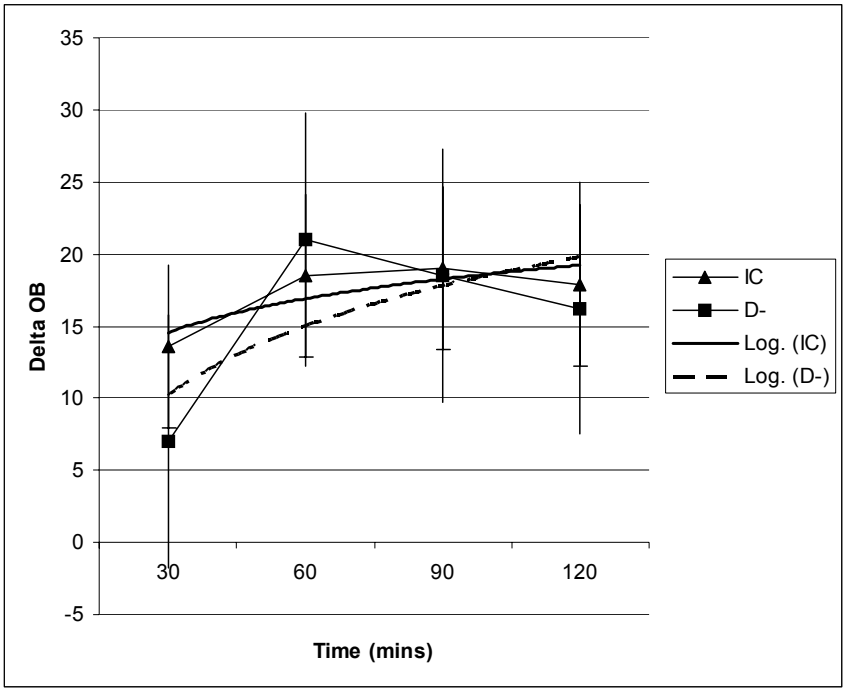


Fig 60. Comparison of ΔOB Mean±95%CL at each time point for the IC and D- Protocol, with a Logarithmic Trend Lines Applied

DISCUSSION

In the pilot study the goal was to determine the feasibility and logistics of ^{13}C-urea testing in the horse and to observe for any adverse affects from the test protocol. In order for a ^{13}C based test to be clinically useful it must be able to detect ^{13}C enrichment in excess of baseline variation.[302] In horses starved for 12 hours and stall confined, baseline ^{13}C variation can be considered negligible.[297] For a ^{13}C-urea test to be useful, sufficient substrate must be available to allow any urease present to produce labeled CO_2 in sufficient quantity to alter the $^{13}CO_2$:$^{12}CO_2$ ratio in the body CO_2 pool. Enough time must also be allowed to detect this change. In the pilot study no significant ^{13}C enrichment was found in either animal using both tests. This suggests that either gastric urease activity was not present, or that the test protocol was incorrect (a type II error). Two test protocol errors were possible. Firstly, as the peak level of $^{13}CO_2$ in human patients with *H.pylori* infection is detected 1.5 hours after a single dose of ^{13}C-urea, it is likely that sampling was performed too early.[294] For the principal study the sampling period was extended to 2 hours, with samples taken every 30 minutes. This resulted in detection of significant ^{13}C enrichment in all protocols. A second possible problem with the pilot protocol was rapid gastric emptying of the test solution. In man a fatty meal is given prior to the test solution in order to slow gastric emptying; and so increase contact time with *Helicobacter* urease. In the main study a separate protocol was added that included the α2 agonist sedative, detomidine (0.03mg/kg IV) to slow gastric emptying.[147,149]

In the pilot study there was no significant difference between the results from the breath and blood tests. The breath test relies on normal respiratory function, however the respiratory health of the two horses used in the pilot study was determined only through physical examination and auscultation during normal and rebreathing. These methods alone will not detect sub-clinical respiratory disease that may affect tidal volume and therefore CO_2 washout. As there was no difference between each method this was not a problem in the pilot study. In man the use of the ^{13}C-urea breath test is not recommended in patients with altered respiratory function, or in those incapable of forced expiration.[288]

In order to exclude the possibility of a type II error occurring due to subclinical respiratory in the principal study, we elected to use the blood test protocol.

The ideal diagnostic test should also be easy to perform. Whilst no adverse effects were observed with tracheal sampling in the pilot study, only two samples were taken from each horse. As the principal study was to include multiple sample points and multiple protocols this was further reason to accept the ^{13}C-urea blood test as the test protocol.

The prevalence of gastric lesions found by gastroscopy was low (squamous 27.8%, glandular 33.3%) and very similar to the level previously reported for horses not in training.[5] This low prevalence reflected the fact that all the horses were pasture fed and not in work.[3] Serial gastroscopy was not performed however given the repeated periods of starvation throughout the gastric ^{13}C-urea study it is likely that a degree of peptic injury resulting in squamous erosion may have occurred.[10] Experimental feed deprivation will increase gastric acidity, lowering the median pH of gastric juice to 1.55 from pH 3.1 found in horses fed free choice hay.[53] Increased acidity has been shown to upregulate *Helicobacter* urease activity and UreI expression and activity.[26,166] It is therefore possible that increased urease activity may have developed during the study protocol and introduced the duration of the study period as a variable.

No serious complications of the polypectomy snare biopsy technique were observed, making this an excellent technique for obtaining large specimens from the glandular mucosa.[34]

The use of PCR with primers specific for *H.pylori* has an optimal specificity of 100%, when compared to culture as a gold standard, for the diagnosis of *Helicobacter* infection in man.[274] The primers used in this study were for the *Helicobacter* specific *ureI* gene that encodes the urea channel UreI which is vital to gastric colonization and acid tolerance.[163] In this study all 12 horses from which antral biopsies were submitted for PCR were positive for the *Helicobacter* specific gene material. This method is widely accepted in man and has been applied to post mortem specimens from the stomach of horses on two prior occasions.[26,31,33,162,163,166,274,279,303] In both equine reports gene material was detected in 91-100% cases, which corresponds to the 100% prevalence seen

in this study. As such we believe this is the first description of the identification of *Helicobacter* gene material from the stomach of live horses. The antral biopsies were all obtained using the polypectomy snare technique rather than the traditional fenestrated biopsy cup.[34] This technique yields a larger sample with less crush injury to the tissue. As *Helicobacter* is typically present in the mucus overlying the mucosa the use of the polypectomy technique may have helped retain this layer and enhance sensitivity. Submission for PCR of samples obtained using a biopsy cup would obviously be required to assess this effect. It must be noted that the loop was thoroughly cleaned with protein degrading solution, or replaced between procedures to limit contamination of samples.

Glandular ulceration was found in 42% of the horses in the PCR study group, and when present it was mild (median grade 1, mean 1.2, range 1-2). This prevalence is slightly below that previously reported from a mixed population of horses (58%), which may be explained in two ways.[18] Firstly, the only currently known etiology for glandular ulceration in the horse is NSAID administration and none of our horses had received any for at least 6 weeks prior to the study.[125,126] Secondly, whilst peptic injury is not an inciting factor in glandular injury it is responsible for tissue damage once mucosal defenses are breached. As previously described, risk factors responsible for increasing gastric acidity and causing peptic injury were not present in this study population prior to inclusion. No association between the presence of glandular ulceration and PCR status was attempted, as this was not the purpose of this study. It is however interesting to observe that all the horses were asymptomatic and the observed lesions, if any, were mild. In man the majority of *Helicobacter* infections are asymptomatic.[25,28] Further work is required to assess the importance, if any, of *Helicobacter* as a cause of gastric ulceration in the horse.

The use of the Pyloritek™ RUT demonstrated urease activity in 7/15 (47%) of horses (Pyl+). None was detected in the remaining 8/15 (53%) (Pyl-). The majority of samples were obtained using the polypectomy snare (14/15), the remaining one was obtained using the fenestrated cup. Of the positive results 6/7 were obtained using the polypectomy technique and 1/7 using the fenestrated cup. Obviously no effect of the differing techniques could be assessed from these numbers. False RUT positive results

can occur with gross blood contamination of the biopsy sample, however blood contamination was not observed to be present in any case. In man, false negative results can occur as the distribution of *Helicobacter* on the glandular mucosa is not uniform. To limit this effect when using the Pyloritek™ RUT, 2-3 specimens are placed on each test strip, with each specimen coming from a different region of the stomach. A combination of antrum and body yields a sensitivity (92%) that exceeds body (86%) or antrum (78%) alone.[264] In this study all samples were obtained only from the pyloric antrum. This may represent a type II error in that a positive test result may have been obtained if the glandular body of the stomach had also been tested. Recently it has been shown that the prevalence of lesions in this portion of the stomach may historically have been underestimated and so future investigation into use of RUTs in the horse should include specimens taken from the glandular body as well as antrum.[19]

Ten horses had both PCR and the RUT performed, of which all were positive by PCR, but only 4 were positive by RUT. If PCR is then taken as a gold standard (100% specific)[274] in this study, a sensitivity of 40% is obtained for the Pyloritek™ RUT. This is intriguing as the specimens for PCR and Pyloritek™ were obtained from the same polypectomy snare sample in 14 cases. The basis of RUT is bacterial urease and urea uptake mechanisms, the activity of which are not constant, but are upregulated by a prolonged, acidic environment.[26,166] In order to increase bacterial urease action it would be interesting to repeat the RUT in individuals who had been feed deprived according to an accepted protocol for increasing gastric acidity to induce squamous ulceration, and to couple this with gastric pH measurement.[11] This may enhance the sensitivity of the RUT in the horse. The specificity of the RUT could obviously not be assessed as no negative results were obtained by PCR.

As one purpose of this study was to identify the presence of *Helicobacter* in the equine stomach, the RUT succeeded as it showed urease activity to be present in gastric glandular mucosal biopsies from 7 horses. The low sensitivity of the test in this study would limit its use as a screening test to identify *Helicobacter* urease activity as a means to select cases suitable for bacteriologic culture. Obviously further study is required if RUT are to be used in the horse.

As opposed to the localized nature of RUTs, the ^{13}C-urea blood test provides a global assessment of gastric urease activity. In this study the baseline variation in ^{13}C enrichment was slightly higher 25.88±1.05 per mil (mean±SD) than that previously reported (20.8±5.03ppm). The difference reflects geographical location and individual diets. The British ponies were fed hay for 1 month prior to the test period, whereas our horses were grass fed and then maintained on alfalfa hay. This type of variation is well described in man.[304] The individual variation in ^{13}C enrichment in this study was so small as to be considered negligible (mean±SD 0.31±0.24per mil, range 0.046-0.943per mil), which conforms with a previous report of basal ^{13}C variation in the horse.[297]

Statistically significant (P<0.05) ^{13}C enrichment of the body CO_2 pool was shown by a less negative ΔC per mil values at all time points in all test protocols, when compared to baseline levels. The timing and values of peak enrichment of the body $^{13}CO_2$ pool during all the ^{13}C-urea blood test protocols is shown in table 24. Given that the normal $t_{1/2}$ for fluid phase gastric emptying in the horse is approximately 30-35minutes, the significant increase in ΔOB at 30 and 60 minutes in the D-, D+ and H protocols indicates that urease activity is present within the equine stomach. Gastric biopsy specimens from all of these horses were positive by PCR for the presence *Helicobacter* specific UreI gene material. As *Helicobacter* is currently the only gastric urease/UreI positive microorganism known to science, I believe that this is extremely strong evidence for the presence of an equine gastric *Helicobacter* species and as such is the first report of active *Helicobacter* infection in the horse. Culture and subsequent bacterial identification are by definition the gold standard when demonstrating novel infections and are still required to identify which member of the *Helicobacter* genus is present.

Two different cut off values are used for interpretation of the ^{13}C-urea blood test in man. Firstly, an absolute value of >-17Δ^{13}C per mil at 60 minutes is used. This method has the advantage of requiring only one blood sample and as such is less expensive and yields greater patient compliance.[289] As baseline ^{13}C enrichment is not known this technique can only accurately be applied to a certain population (western diet, no prior *H.pylori* therapy for 1 year prior to testing, no antibiotics, bismuth or proton pump inhibitors for 1 month prior to testing). A second approach is to use a ΔOB cut off value

of >6 at 60 minutes.[287,295] This method requires double the blood sampling and is correspondingly more expensive and invasive, however it is unaffected by inter-individual baseline variation and so can be applied to multiple populations. In this study both $\Delta^{13}C$ and ΔOB values are presented, however analysis of ΔOB is more applicable to this novel population.

The greatest degree of ^{13}C enrichment occurred using the D^- protocol, with both peak $\Delta^{13}C$ and ΔOB values, at 60 minutes. 75% horses exceeded the $\Delta^{13}C$ cut off (-17$\Delta^{13}C$ per mil) and 87.5% the ΔOB cut off (≥6). The addition of detomidine to the test protocol resulted in peak enrichment of the body CO_2 pool that was of less magnitude and delayed to 120 minutes. The $\Delta^{13}C$ cut off was not reached using this protocol and only 37.5% horses had a ΔOB ≥6. The use of only 250mg ^{13}C-urea resulted in no horses reaching the $\Delta^{13}C$ cut off and only 2 having ΔOB ≥6. The timing of peak enrichment was identical to that found with the detomidine protocol. The addition of bethanechol resulted in 75% horses reaching the $\Delta^{13}C$ and ΔOB cut offs at 90 and 120 minutes, with peak ΔOB occurring at 120 minutes.

The ΔOB mean±95% CL at each time point are shown for the D- and D+ protocols in figure 53 and the H and B protocols in figure 54. A logarithmic trend line has been applied to each data series to show the pattern of ^{13}C enrichment of the body CO_2 pool with the different protocols.

The greatest degree of ^{13}C enrichment was detected using the D- protocol. This was an unexpected finding as the D+ protocol most closely resembles that performed in man and was expected to be most likely to demonstrate urease activity and ^{13}C enrichment. There are two possible explanations for the difference in measured $^{13}CO_2$ enrichment between the two protocols. Firstly, in the D- protocol the horses were unsedated and so resented nasogastric intubation and repeated blood sampling. The anticipation and act of needle puncture, and the act of catching each horse every 30 minutes increased respiratory rate and may have elevated resting metabolic rate. This could have resulted in increased CO_2 expulsion and so $^{13}CO_2$ enrichment at each sampling point. Conversely, the administration of detomidine in the D+ protocol resulted in easy handling and sampling of each horse. However, detomidine as an α-2 agonist also

causes respiratory depression and persistent bradypnea.[305] This will result in CO_2 retention and reduced $^{13}CO_2$ enrichment at each sample point. To validate these hypotheses either arterial blood gas analysis, or continued sampling until ^{13}C enrichment had returned to baseline, would be required. Using the latter, total CO_2 enrichment could then be calculated from the area under curve of the enrichment/time graph. Neither of these procedures were performed in this study. To limit the effect of excitement in future application of the ^{13}C-urea blood test, an in-dwelling jugular catheter should be use to facilitate blood sampling. Ideally gastric emptying should be delayed using a fatty meal, although this effect has not been described in the horse.

Comparison of the means at each time point of the D- and D+ protocols was performed using the two sampled *t*-test function of Minitab release 13.1[A]. A Statistical difference ($P<0.05$) between the mean ΔOB was only found 60 minutes ($P=0.014$) (fig. 55). At all other time points there was no statistical difference between the mean ΔOB of either protocol. Application of a logarithmic trend line to the data from the two protocols, using the stats package of Microsoft Excel[B], (fig. 53) shows that the overall pattern of $^{13}CO_2$ enrichment to be similar in each protocol, although the slope of the D- trend line is obviously steeper than that of the D+ protocol.

With PCR as a gold standard for the diagnosis of *Helicobacter* infection and using a cut off value of ΔOB >6, the maximum sensitivity of the various protocols of the ^{13}C-urea blood test are show in table 25. The D- protocol had a sensitivity of 75% at 60 minutes. The mean ΔOB at this time point was also the greatest in the entire study. The specificity of the test is unknown as no negative results were obtained using PCR. Obviously further work is required to accurately apply this test to the horse.

Gastroscopy of the eight horses used for ^{13}C-urea testing demonstrated three to have mild glandular hyperemia (EGUS grade 1/4), the remainder were normal (grade 0/4). To examine if ^{13}C enrichment was greater in horses with glandular hyperemia a two sample *t*-test was performed. No significant difference ($P=0.37$) was found in ^{13}C enrichment between horses with or without mild glandular pathology in this study. None

[A] Minitab v13.1, Minitab Inc.,
[B] Excel 2002 (sp3), Microsoft Corporation,

of the horses who took part in the ^{13}C-urea blood test had clinically significant glandular pathology. Further study is required to assess if a link exists between severity of glandular pathology, degree of ^{13}C enrichment and hence *Helicobacter*.

All of the test horses were positive for gastric urease activity using the ^{13}C-urea blood test and yet only 40% were positive using the Pyloritek™ RUT. This may be explained in one of two ways: either the Pyloritek™ positive horses had greater urease activity or the presence of *Helicobacter* is not uniform across the glandular mucosa. If greater urease activity was present we would expect Pyl+ horses to have greater ^{13}C enrichment values than Pyl- horses. This effect was analyzed using the two sample *t*-test (fig. 56). No significant difference was found in the ^{13}C enrichment at 60 minutes in the D- protocol between Pyl+ and Pyl- horses (P=0.873). The disparity in demonstration of urease activity between the two methods possibly reflects an uneven population density of *Helicobacter* across the gastric mucosa. In man the sensitivity of a RUT is affected by the number and location of samples tested, and by the bacterial density.[264] In this study we took multiple samples from only one site, the pyloric antrum. In man a variety of sites are recommended, with a combination of antrum and body providing the greatest sensitivity (92%).[264] Given the disparity in normal gastric anatomy between man and the horse further study would be required to identify the ideal sites for sampling if RUTs are to be applied to the horse with similar sensitivity. One potential use for RUT in the horse is the selection of samples suitable for bacteriologic culture, as a positive result demonstrates bacterial viability.

In the D- and H protocols peak enrichment occurred at 60 minutes and reduced after that. This pattern is typical of the ^{13}C-urea blood test in man, where peak enrichment also occurs at 60 minutes.[287,294,295] The timing of peak enrichment and ΔOB at 120 minutes in both the D+ and B protocols was interesting. In the D+ protocol it is most likely that the detomidine induced bradypnea was causing CO_2 retention and that the increase in enrichment at the end of the test period was due to the sedation wearing off. As detomidine slows gastric emptying by a factor of approximately 1.68, at 120 minutes approximately 25% of the test meal will still be in the stomach and so prolonged enrichment should be expected.[149] The low frequency of positive results with the

Pyloritek™ RUT suggests that urease activity is of low density in the stomach, which further supports continued gradual hydrolysis of the test solution when gastric emptying is delayed.

The peak enrichment in the bethanechol protocol at 120 minutes was intriguing as this parasympathomimetic speeds up the fluid phase gastric emptying $t_{1/2}$ to 17.74minutes.[148] By 120 minutes almost 7 half lives will have occurred and less than1% of the test solution will remain in the stomach. Two possibilities could explain the late peak in this group. Firstly, bethanechol is a potent muscarinic agonist and as such will produce contraction of bronchial smooth muscle.[305] This could result in bronchial constriction and retention of CO_2, so delaying peak ^{13}C enrichment. To the author's knowledge, there are no reports quantifying this effect of bethanechol in the horse. Secondly, as the horse is a hindgut fermenter, capable of enterohepatic recycling of urea, it was considered possible that cecal urease activity may produce the delayed peak in this group. It was also considered important to assess the affect that cecal urease may have on the clinical validity of the ^{13}C-urea blood test in the horse as a whole. The D+ protocol was repeated with the ^{13}C-urea administered directly into the cecum under laparoscopic guidance. Significant ^{13}C enrichment of the body CO_2 pool was found at all time points, when compared to baseline, indicating that cecal urease activity was present. Peak mean enrichment occurred at 90 minutes (-11.45$\Delta^{13}C$ per mil). Correspondingly the peak mean ΔOB value was 19.04 at 90 minutes, after which the degree of enrichment declined (fig. 56).

As the IC protocol was based upon the D+ protocol a similar degree of respiratory depression and CO_2 retention would be expected from the administration of detomidine. Comparing the trend lines of each protocol shows them to be very similar (fig. 59), suggesting that CO_2 retention was indeed occurring.

In order for cecal urease to activity to affect the results of the gastric study a rapid increase in ^{13}C enrichment would have to occur once the test solution reached the cecum, requiring a great deal of cecal urease activity to be present. As peak activity was not reached until 90 minutes after direct intra-cecal administration of the test solution, this is

unlikely to have happened. The declining level of enrichment seen at 90 and 120 minutes in both the D- and H protocols makes any cecal involvement also very unlikely.

The possibility of cecal urease activity contributing to the peak enrichment in the B protocol was investigated using the two sampled *t*-test (fig. 60). The ΔOB values at 30 minutes in the IC protocol were compared to the 120 minute ΔOB values from the B protocol. No significant difference was found (P=0.69), indicting that the two mean enrichment values were not different. This suggests that cecal urease activity may have contributed to the enrichment seen in the B-protocol

SUMMARY AND CONCLUSIONS

Currently the reported evidence for *Helicobacter* infection in the horse combines seroconversion in weanlings, identification of *Helicobacter* specific gene material using PCR and histologic evidence of a neutrophilic gastritis in post mortem stomach specimens.[31-33] Histologic and bacteriologic evidence to define the presence of an equine gastric *Helicobacter* is still lacking. Both of these methods rely on sufficient bacterial density in the biopsy specimen, and as such sensitivity is reduced after eradication therapy in man. Maintenance of bacterial viability is also important for accurate culture results, particularly as *Helicobacter* is fastidious and quickly dies. Because of these problems alternative methods of *Helicobacter* detection have developed. The purpose of this study was to apply three of these to the horse, all of which were directed at identifying urease activity in the equine stomach.

All horses tested had evidence of gastric *Helicobacter* gene material by PCR. The primers used in this test specifically identify the *ureI* gene that encodes the UreI channel that is vital to gastric colonization. This makes erroneous detection of species that are merely transiting the stomach highly unlikely. In man PCR has a specificity of 100%, this test in particular is 100% specific for gastric dwelling *Helicobacter*, as *ureI* negative mutants are not capable of gastric colonization.

Urease activity was then demonstrated in these horses by two different methods. Firstly the Pyloritek™ rapid urease test demonstrated urease activity in glandular mucosal biopsy specimens taken from the pyloric antrum of 7 horses. Secondly the ^{13}C-urea blood test demonstrated urease activity in stomach of 8 horses. We believe that the demonstration of urease activity in the stomach of horses which are positive for gastric *Helicobacter* by PCR is the strongest evidence currently reported for the presence of an equine gastric *Helicobacter* species. Culture and subsequent bacterial identification are, by definition, the gold standard when demonstrating novel infections and are still required to identify an equine *Helicobacter* species. Fulfillment of Koch's postulates would then be needed to fully define the nature of the infection. Further study is also required to define the pathogenicity, if any, of *Helicobacter* in the horse.

When compared to PCR, the use of the ^{13}C-urea blood test in the horse was most sensitive when a ΔOB cut off, rather than a Δ^{13}C cut off, was used. In two protocols peak ^{13}C enrichment occurred at 60 minutes, as is the case in man. The use of sedation to slow gastric emptying also affected respiratory function and resulted in CO_2 retention and reduced ^{13}C enrichment. This resulted in reduced sensitivity of this protocol. The use of a prokinetic enhanced gastric emptying and delivery of the test meal to the cecum, resulting in peak ^{13}C enrichment at 120 minutes. The ^{13}C-urea blood test was repeated in 8 horses, with the test solution directly instilled into the cecum under laparoscopic guidance. Peak ^{13}C enrichment was found at 90 minutes. Whilst cecal urease activity was shown to be present, it is considered unlikely that it contributed to the significant ^{13}C enrichment demonstrated by the use of the ^{13}C-urea blood test in the horse.

APPENDIX

EQUATIONS

Δx (per mil) = $(R_{sa}-R_{st})/R_{st}$ x 1000

Where x is the element, carbon, and R is the ratio of $^{13}C/^{12}C$ in the sample (sa) and standard (st).

$\Delta OB = \Delta^{13}C_{tx} - \Delta^{13}C_{t0}$

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CURRICULUM VITAE

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DATE OF BIRTH: 29th October 1972 **BIRTHPLACE:** Bristol, UK

FAMILY STATUS: Married, one son **NATIONALITY:** British

ACADEMIC ATTENDANCE AND QUALIFICATIONS:

7/2001 – 6/2004 Marion duPont Scott Equine Medical Center,
Virginia-Maryland Regional College of Veterinary Medicine
Residency in Equine Internal Medicine
Master of Science degree in Mechanisms of Disease in Veterinary Medicine.

10/1992 – 6/1997 University of Liverpool, Faculty of Veterinary Science
Batchelor of Veterinary Science (BVSc) with credit in Equine Medicine and Surgery.
Elective study in Equine Orthopaedics
Finalist 1996 Rhone Merieux National Veterinary Student of the Year Award

9/1989 – 6/1991 Chew Valley School
A Level – Biology A, Physics A, Geography A, Chemistry B

9/1980 – 6/1989 Bristol Grammar School
GCSE – 11 Subject passes

SPECIALITY BOARD CERTIFICATION:

American College of Veterinary Internal Medicine (Large Animal):
General Examination – Passed June 2003
Credentials - Accepted December 2003
Eligible to sit Certifying Examinations June 2004

Royal College of Veterinary Surgeons, Certificate in Equine Internal Medicine:
Practical experience - Approved November 2003
Eligible to sit Certifying Examinations Summer 2004

PROFESSIONAL MEMBERSHIP:

Member of the Royal College of Veterinary Surgeons, MRCVS 7/1997 - present
Member of the British Equine Veterinary Association, 1995 - present
Member of the Veterinary Council of New Zealand, 2001 - present
American College of Veterinary Internal Medicine – Candidate 7/2001 – present
Phi Sigma National Biological Honour Society, 2003 - present

EMPLOYMENT:

7/2001 – 6/2004	Marion duPont Scott Equine Medical Center, Virginia-Maryland Regional College of Veterinary Medicine, Leesburg, Virginia, USA Resident in Equine Internal Medicine, ACVIM approved residency program in equine internal medicine Program advisors: Dr. MJ Murray DVM MS DACVIM, Dr. MO Furr DVM PhD DACVIM
3/2001 – 6/2001	Waikato Equine Hospital, Hamilton Veterinary Services, Hamilton, New Zealand. Assistant Equine Veterinarian Contact: Dr. DJ Black BVSc MACVSC MRCVS
9/2000 – 2/2001	Sabbatical – honeymoon backpacking in SE Asia, India, Dubai and New Zealand
1/1998 – 9/2000	Cinder Hill Equine Veterinary Clinic, Horsted Keynes, West Sussex, UK. Assistant Equine Veterinarian Contact: CD Ginnett BVM&S MRCVS, MA Livesey BVMS MSc DACVS MRCVS
7/1997 – 12/1997	Furniss, Morton and Partners, Weston-s-Mare, North Somerset, UK. Assistant Mixed Animal Veterinarian

EXTERNSHIPS:

4/1997 – 5/1997	Dubai Equine Hospital, Dubai, UAE.
6/1996 – 8/1996	Dr.'s Baker and McVeigh, Littlefields Equine Hospital, Durban, South Africa

PUBLICATIONS:

Hepburn RJ. Colitis X in the Horse. *Grayson Jockey Club Foundation Research Today* 19(5) 2002

Lopes MA, **Hepburn RJ**, McKenzie HC and Sykes BW. Enteral Fluid Therapy for Horses. *Compendium Continuing Education for the Practicing Veterinarian* 25(5) 2003

Hepburn RJ, Furr MO. Sinonasal Adenocarcinoma Causing CNS Disease in a Horse. *Journal of Veterinary Internal Medicine* 18(1) 2004

Murray MJ, **Hepburn RJ**, Sullins KE. Endoscopic Biopsy of the Equine Gastric Antrum. *Equine Veterinary Journal* 36(1) 2004

Hepburn RJ, Furr MO. Cerebrospinal Fluid Analysis in the Horse. *In progress*

PRESENTATIONS:

Hepburn RJ, Murray MJ, Furr MO, McKenzie HM and Ward DL. Demonstration of Urease Activity in the Equine Stomach and Application of the ^{13}C-Urea Blood Test to the Horse. *ACVIM Forum 2004*

TEACHING ACTIVITIES:

2003 – 2004 4th year Veterinary Student Lecture (VMS 9544) – *The Joy of Cardiac Auscultation in the Horse.*
Foal Watch Volunteer Instruction. *An introduction to the Theories and Techniques of Equine Neonatal Critical Care*
Graduate Student Seminar (VMS 5944) – *Equine Gastric Ulcer Syndrome*

2003 Graduate Student Seminar (VMS 5944) – *Cerebrospinal Fluid Analysis in the Horse*
Graduate Student Seminar (VMS 5944) – *Calcium and Magnesium Homeostasis and the Effect of Gastrointestinal Disease*
Continuing Education for Equine Veterinary Technicians - *Practical Medical Records*
Foal Watch Volunteer Education - *An introduction to the Theories and Techniques of Equine Neonatal Critical Care*

2002 – 2003 3rd year Veterinary Student Practical (VMS 8695) – *Equine Respiratory Examination Techniques Lab*

2002 Graduate Student Seminar (VMS 5944) – *Aminoglycoside Pharmacokinetics and Therapeutic Monitoring in the Horse*
Graduate Student Seminar (VMS 5944) – *Parenteral Nutrition in the Horse*

2001 Graduate Student Seminar (VMS 5944) – *Hairy Ponies and Mutant Mice, Current Thoughts on Etiology, Diagnosis and Treatment of Equine Cushing's Like Disease*

RESEARCH:

Identification of Urease Activity in the Gastrointestinal Tract of the Horse and Investigation of the Utility of ^{13}Carbon Urea Tests
Murray MJ, **Hepburn RJ,** Furr MO
Stewart-Bonsall Award $9000