Histological assessment of -amyloid precursor protein immunolabelled rectal biopsies aids diagnosis of equine grass sickness

Histological assessment of β-amyloid precursor protein immunolabelled rectal biopsies aids diagnosis of equine grass sickness

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Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

Client consent was obtained through a standardised hospital consent form for archived pathology material and explicit written or verbal consent was given by owners for the use of tissue samples from horses that were subjected to euthanasia. The study was approved by the University of Edinburgh Ethical Review Committee

Source of funding

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Authorship

B. C. McGorum, S. Scholes and R. C. Jago were responsible for study design, determination of grading schemes and manuscript preparation. B. C. McGorum (ileum), R. C. Jago (rectum) and F. Coyle (CCG) analysed sections. B. C. McGorum, T. S. Mair, R. S. Pirie, G. R. Pearson and R. C. Jago provided samples. I. Handel contributed to statistical analysis. S. Scholes and E. M. Milne provided histopathological advice. S. Scholes and B. C. McGorum captured photos. All authors reviewed the final manuscript.

Summary

Background: An accurate, minimally invasive, ante-mortem, diagnostic test for equine grass sickness (EGS) is currently lacking. While histological examination of haematoxylin-eosin stained rectal biopsies for chromatolytic neurons is insensitive as a diagnostic test for EGS, it was hypothesised that the diagnostic accuracy could be improved by immunolabelling for β-amyloid precursor protein (β-APP) which has increased expression in cranial cervical ganglia (CCG) neuronal perikarya in EGS.

Objectives: To develop a grading scheme for assessing the distribution and intensity of β-APP immunoreactivity within individual rectal submucosal neurons and subsequently determine the diagnostic value of the distribution of different grades of neurons for EGS diagnosis.

Study design: Retrospective case-control diagnostic accuracy study.

Methods: Initially a standardised grading scheme was developed and β-APP immunoreactivity in individual neuronal perikarya and axons was compared in sections of CCG and ileum from EGS and control horses. The grading scheme was further refined before being blindly applied to submucosal neurons in rectal biopsies derived from 21 EGS and 23 control horses.

Results: β-APP immunoreactivity was increased in neuronal perikarya and axons in sections of CCG, ileum and rectum from EGS horses compared with controls. For rectal biopsies, a mean

immunoreactivity grade exceeding 1.1 was 100% specific and sensitive for EGS, and the presence of at least one neuron with diffuse labelling of the entire cytoplasm (grade 3) was 95% sensitive and 100% specific for EGS.

Main limitations: While the diagnostic criteria facilitated discrimination of the EGS and control biopsies evaluated in this study, further prospective validation using a larger sample set is required.

Conclusions: Histological assessment of β-APP immunolabelled rectal biopsies is more sensitive than conventional histological examination for EGS diagnosis. Further validation is required before this technique can be advocated for clinical decision making.

Introduction

A sensitive, minimally invasive, ante-mortem, diagnostic test for equine grass sickness (EGS) is currently lacking. Such a test would facilitate appropriate case management by aiding EGS diagnosis, which is currently challenging in some cases, and avoid the necessity for invasive ileal biopsy. The presence of chromatolytic neurons in rectal submucosa of EGS horses [1-4] indicates that histological assessment of rectal biopsies could potentially provide a relatively simple, minimally invasive and inexpensive ante-mortem diagnostic technique. Histological examination of two full-thickness, haematoxylin-eosin (H&E) stained sections of rectum collected post-mortem yielded a sensitivity of 71% and specificity of 100% for EGS diagnosis, when the diagnostic criterion was identification of at least three chromatolytic neurons [3]. However, a later study found that histological examination for chromatolytic neurons in (H&E) stained rectal biopsy sections is insensitive (21% sensitivity) for EGS diagnosis [4]. The diagnostic value of rectal biopsies in EGS diagnosis is potentially limited by small sample size, paucity of neurons in rectal submucosal plexi, difficulty in histological recognition of chromatolytic neurons, and crush artefacts. It was suggested that application of specific neuronal labelling may overcome some of these limitations thereby increasing the diagnostic utility [4]. Whilst synaptophysin immunolabelling of ileal sections facilitates correct differentiation of control and EGS cases [5] ,synaptophysin immunolabelling of rectal sections provides no further diagnostic sensitivity compared to H&E stained rectal sections (E. M. Milne, unpublished observation).

This study tested the hypothesis that immunolabelling with β-amyloid precursor protein (β-APP), which accumulates in cranial cervical ganglion (CCG) neuronal perikarya in EGS [6], would improve the accuracy of histological assessment of rectal biopsies for EGS diagnosis. β-APP is an extensively post-translationally modified and proteolytically cleaved transmembrane protein, associated with synaptic formation and repair, present at high concentrations within neurons [7]. Increased β-APP expression is part of the acute phase response to neuronal injury [8], occurring in acquired diseases [9; 10], in various neurodegenerative conditions [11] including Alzheimer's disease [12], Down's Syndrome [13] and tauopathies [14] and in murine neurodegenerative disease models [15]. The expression of β-APP is therefore not specific for EGS.

The purpose of this study was to develop and refine a grading scheme for assessing the distribution and intensity of β-APP immunoreactivity within individual neuronal perikarya and axons in rectal biopsies and subsequently determine the best diagnostic predictor for discriminating EGS and control horses. Initially sections of ileum and CCG, were utilised to establish a grading scheme for assessing β-APP immunoreactivity prior to evaluating the rectal submucosal plexi which has fewer neurons [1; 2]. Neuronal pathology is not uniform throughout the gastrointestinal tract in EGS, and the ileum has consistently been reported to be most severely affected region [1; 2; 16]. Histopathological examination of formalin-fixed, H&E stained CCG is regarded as the "gold standard" diagnostic test for EGS [2; 17; 18], while histopathological examination of formalin-fixed, H&E stained ileal sections offers up to 100% sensitivity and specificity [16].

Materials and Methods

Collection of tissue samples

Tissue samples were collected ante-mortem from EGS and control horses for routine clinical diagnostic purposes or, with the horse owners' consent, post-mortem from horses subjected to euthanasia. All ileal [19], CCG [6; 19] and some EGS rectal biopsies [4] were available from previously reported studies. All the control and remaining EGS rectal biopsies were collected prospectively. Horses were of mixed breeds and sex. EGS was confirmed by post-mortem examination including conventional histopathological examination of H&E stained CCG and/ or ileum [2; 16-18; 20; 21], by specialist pathologists experienced in EGS diagnosis. EGS was categorised as previously described [22; 23]. In

summary, acute EGS cases had mild to moderate abdominal pain with gastric and small intestinal distension, subacute EGS cases had less severe signs, a more insidious onset and secondary large intestinal impactions, and chronic cases had none of these sequelae. All control cases with colic had a physical lesion identified at exploratory laparotomy. All other control horses had no ante-mortem clinical evidence of EGS and were subjected to euthanasia for unrelated reasons (see results). Thorough clinical examinations were performed, by clinicians experienced in EGS diagnosis, and specifically the control horses had no evidence of tachycardia, ptosis, muscle fasciculations, patchy sweating, dysphagia, base narrow stance or weight loss with 'tucked up' abdominal silhouette. The reported median age for EGS horses of 5 years [24] was considered when selecting cases for control rectal biopsies, such that there would be no significant inter-group difference in age. CCG and full thickness ileal samples were collected post-mortem within 3.5 h of death. Rectal biopsies were collected using uterine biopsy forceps[a] as described previously [4]. Post-mortem rectal biopsies were collected within 10 min of death. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and 4μm sections cut. A single section from each rectal biopsy contributed to a single histology slide for each horse.

Immunohistochemistry

Immunohistochemistry was done as previously reported [6] and as detailed in Supplementary item 1.

Grading of neuronal and axonal immunolabelling

Cranial cervical ganglion and ileum

See Supplementary item 2.

Rectum

Preliminary screening of rectal samples indicated that the grading scheme developed using the CCG and ileal sections was insufficiently precise to facilitate repeatable grading of the immunolabelling of individual neurons, and in particular to differentiate grade 2 and grade 3 neurons. Further refinement of the grading scheme to facilitate repeatableassessment of the intensity and distribution of immunolabelling of individual neurons was achieved by detailed assessment of the immunolabelling of individual neurons in a randomly selected subset of rectal sections, with no regard to disease status. Consequently, the derivation of the neurons (i.e. EGS or control horses) had no bearing on the grading refinement process. When applying the refined grading scheme, grade 3 neurons, in contrast to grade

142 2 neurons, were classified as those with diffuse labelling of the entire cytoplasm, extending right up to
143 the perikaryonal margin, except when this was displaced by cytoplasmic vacuolation (Fig 1). This
144 grading system was then applied by a single observer (RJ, equine internal medicine senior clinical
145 scholar) in the blinded assessment of all rectal biopsy sections. In all sections, all submucosal plexus
146 neurons containing nuclei were graded, identifying neurons with x10 objective and grading individual
147 neurons with x40 objective.
148 All rectal biopsy sections were blindly assessed on a second occasion by the same author (RJ) and by
149 a specialist in veterinary pathology (SS), to assess intra- and inter-observer agreement, respectively,
150 to determine the repeatability of the proposed rectal biopsy grading scheme.
151

152 **Data analysis**

153 Age, number of rectal biopsies per horse and number of neurons counted per horse were described
154 using medians and interquartile ranges (IQR). For each horse the distribution of different grades of
155 neuron was determined and a mean immunoreactivity grade calculated.. Mann-Whitney U test was
156 used for inter-group (control vs EGS, ante-mortem vs post-mortem sample collection, chronic vs
157 subacute vs acute) comparisons of these variables. Using conventional histopathological examination
158 of H&E stained CCG [2; 17; 18; 20] and ileum [16; 21] as the reference tests for EGS diagnosis, the
159 sensitivity and specificity of EGS diagnosis using histological assessment of β-APP immunoreactivity
160 of rectal biopsies was calculated. The predictive value of the distribution of different grades of neurons
161 and of the mean immunoreactivity grade for EGS diagnosis was determined by constructing receiver
162 operating characteristic (ROC) curves including estimation of area under the curve (AUC). An optimal
163 cut-off was proposed by identifying a point that would give maximum sensitivity with an estimated
164 specificity of 1.0. A specificity of 1.0 was selected (i.e. no false positives within the study data) to
165 minimise the possibility of an erroneously diagnosed EGS horse being inappropriately euthanised. The
166 number of neurons required to be evaluated per horse to be confident that a grade 3 neuron was or
167 was not present, at the expected median prevalence was calculated using a surveillance design tool[b].
168 The total estimated number of neurons within the rectum required for this calculation was calculated
169 assuming that neuronal density is uniform throughout the length of the rectum [2], the diameter of
170 submucosal neuronal perikarya was 25μm, and rectal length and diameter was 30 and 7cm respectively
171 [25]. Using the criterion for EGS diagnosis as the presence of at least one grade 3 rectal neuron, kappa

statistics were calculated for the intra- and inter-observer agreement in the interpretation of cases (EGS/ control), using a diagnostic design tool[b]. GraphPad Prism[c] was used for all statistical analyses described above, unless specifically stated otherwise. $P<0.05$ was used as the threshold for statistical significance. This study conformed to Standards for the Reporting of Diagnostic Accuracy (STARD) guidelines where appropriate.

Results

CCG and ileal sections

See Supplementary item 2.

Rectal biopsy sections

Rectal biopsies comprised 21 EGS (6 acute, 12 sub-acute and 3 chronic) and 23 control horses. Eight EGS samples were collected ante-mortem and the remainder post-mortem. Samples from two control horses (one with weight loss and one with chronic diarrhoea) were collected ante-mortem and the remainder post-mortem. Reasons for euthanasia for controls collected post-mortem were behavioural (n=1), colic (n=4), neurological (n=4), recurrent uveitis (n=1), orthopaedic (n=7) and elderly horses donated for research (n=4). There was no significant difference in age of control and EGS horses from which rectal biopsies were collected (Supplementary item 3).

All rectal biopsy sections comprised mucosa, submucosa with the submucosal plexus, but no myenteric plexus. Significantly more rectal biopsies were collected from controls (median 4; IQR 4-5) than from EGS horses (median 2; IQR 2-2), resulting in a significantly higher total number of neurons counted in control sections (median 187; IQR 111-308) than in EGS sections (median 70; IQR 46-115). However, average number of neurons per section was not significantly different between EGS (median 37: IQR 23-58) and control (median 46; IQR 24-58) horses.

EGS horses had significantly lower percentages of grade 0 neurons and significantly higher percentages of grade 1, 2 and 3 neurons (Fig 1 & 2, Table 1, Supplementary items 4). Grade 3 neurons were not observed in control horses, but were observed in all but one EGS case. Very occasional adjacent nerve processes were labelled, but none were detected in the mucosa.

While most EGS and control rectal sections could be readily differentiated based on the intensity and distribution of neuronal β-APP immunoreactivity using the criteria of >5% grade 3 neurons (EGS), >40% grade 0 neurons (control) and high proportion of grade 2 neurons (EGS) (Fig 2), a sub-population of samples (n=9, highlighted in red in Supplementary item 4) were considered to be equivocal. The equivocal cases included 2 controls with a lower percentage of grade 0 neurons and higher percentage of grade 1 neurons, compared to the other controls. Both these cases were sampled ante-mortem; one for investigation of chronic diarrhoea and the other for investigation of weight loss. The 7 equivocal EGS cases were 1 acute, 3 subacute and 3 chronic EGS cases, which had <4% grade 3 neurons. Whilst these cases would be equivocal in a clinical situation there was no overlap in the mean immunoreactivity grade for neurons in EGS and control horses whereas the percentage of grade 1 neurons had the greatest degree of overlap between EGS and control horses, and all other diagnostic determinants were similar (Table 1). Consequently, the AUC of the ROC curves was highest for mean immunoreactivity grades (with a value exceeding 1.1 being indicative of EGS). When the specificity was set at 1.0, the highest diagnostic sensitivity for EGS diagnosis was a mean immunoreactivity grade exceeding 1.1 (1.0 sensitivity) and a percentage of grade 3 neurons exceeding 0.75% (0.95 sensitivity). Using the presence of at least one neuron with diffuse labelling of the entire cytoplasm (grade 3) in rectal biopsies as the criterion for EGS diagnosis yielded a 95% sensitivity and 100% specificity for EGS diagnosis.

The mean immunoreactivity grade for EGS samples collected ante-mortem (median 2.03; IQR 1.46-2.62) was not significantly different (p=0.089) to that for samples collected post-mortem (median 1.58; IQR 1.22-1.84). Further analyses were not performed for control samples as only 2 were collected ante-mortem. The median number of total neurons per section was higher in chronic cases (median 60; IQR 27-69.5) than acute/ subacute cases (median 34; IQR 23-49); however the difference was not significant.

The median prevalence of grade 3 neurons in EGS horses was 11.4% (Table 1). The total estimated population of submucosal neurons which could be biopsied within the rectum was 23-46,000,000 per horse, depending whether each nucleus was in 1 or 2 adjacent sections. If the total population of submucosal neurons that could be sampled by a rectal biopsy is >5000, grading of at least 30 neurons would be required to have 96% confidence that a grade 3 neuron was or was not present at the expected median prevalence of 11.4% (20 neurons = 89%, 50 neurons = 99.6% confidence).

The kappa statistic was 1.0 (1.0-1.0 lower and upper 95% limit) and 0.95 (0.87-1.04), respectively for the intra- and inter-observer agreement in the interpretation of each case.

Non-neuronal labelling and artefacts

Intimal asteroid bodies [26] were unlabelled in the antibody negative method control sections but were immunopositive in antibody positive sections (Fig 3a). This labelling was readily differentiated from positive neuronal labelling because of their location, protruding into the lumen of small arterioles and lack of nucleus. Pigment granules (haemosiderin and/ or lipofuscin) within macrophages were evident in the submucosa of some horses, and could be distinguished from immunolabelling by the characteristics of the pigment, presence in corresponding control sections and characteristics of cell morphology (Fig 3b). Oval particles of plant debris were occasionally translocated from the lumen during the biopsy procedure and could readily be differentiated by morphological characteristics including the absence of nuclei (Fig 3c).

Discussion

β-APP immunoreactivity was increased in neuronal perikarya and axons in sections of CCG, ileum and rectum from EGS horses compared with controls. These data extend previous findings of increased β-APP immunoreactivity in CCG sections and increased expression of β-APP in CCG protein extracts in EGS [6].

β-APP is synthesised in the endoplasmic reticulum and transported through the Golgi apparatus, plasma membrane and axons [7]. The pattern of labelling observed in control rectal submucosal neurons (Fig 1: grade 1) is consistent with this normal cellular processing of β-APP, and is similar to that observed in other neuronal populations. In contrast, some neurons in EGS horses had intense granular to diffuse labelling throughout the cytoplasm (Fig 1), indicating abnormal accumulation of β-APP; consistent with dysfunction and degradation of membrane-bound compartments. This study confirms previous work [6] that EGS is associated with accumulation of β-APP in perikarya of degenerate neurons, in adjacent nerve processes and in intra-ganglionic axons, but not in larger nerve fascicles. Potentially this may reflect (a) upregulation of neuronal synthesis of these proteins, (b) reduced catabolism of β-APP, (c) dysfunction of glycoprotein processing in the Golgi network, and/or (d) failure of axonal transport of protein-containing vesicles to the nerve terminal. Accumulation of β-

APP in the perikarya and intra-ganglionic axons, but not in larger nerve fascicles is consistent with the latter. Consistent with the latter two hypotheses, ultrastructural loss of a recognizable Golgi structure is a likely early event in EGS, and EGS is associated with major perturbations in the cytoskeleton of autonomic neurons resulting in accumulation of dopamine-β-hydroxylase and presynaptic proteins in neuronal perikarya [19; 27; 28]. β-APP has been described as a marker of early axonal injury prior to apparent histological changes in routine H&E sections [29].

Evaluation of β-APP immunolabelled rectal biopsy sections using a standardised grading scheme can aid diagnosis of EGS. Indeed, for the sections evaluated in this study, a mean immunoreactivity grade exceeding 1.1 was 100% specific and sensitive for EGS, and the presence of at least one neuron with diffuse labelling of the entire cytoplasm (grade 3) was 95% sensitive and 100% specific for EGS. This diagnostic accuracy is comparable to that of conventional histological examination of ileal biopsies [16; 21] which has a sensitivity and specificity of 100% [16] and is significantly higher than that of conventional histological examination of two rectal biopsies (sensitivity 21%) [4]. However, it should be stressed that further validation of this technique is required prior to its application to clinical cases.

A subset of CCG, ileal and rectal biopsy samples were pre-screened in order to establishgrading schemes and to identify non-neuronal staining, prior to blinded evaluation. Individual neuronal perikarya and axons were used as examples of β-APP immunolabelled neurons to facilitate development and refinement of the grading schemes, with absolutely no bearing on whether the neurons were derived from control or EGS sections. The grading scheme was then applied blindly to determine which grading parameter had the greatest diagnostic accuracy in discriminating EGS and control sections.

A limitation of the study was that although the diagnostic criteria facilitated differentiation of EGS and control rectal samples in this sample set, further development and prospective validation of both the grading scheme and the diagnostic criteria using larger numbers of EGS and control samples is necessary before the diagnostic value of this approach can be fully advocated.

Whilst the sub-population of equivocal samples would have been correctly classified using the criteria of mean immunoreactivity grade exceeding 1.1, in a clinical situation, there would be a degree of uncertainty in their assessment. Consequently, the use of these criteria in a clinical situation would have reduced the confidence in a diagnosis based solely on this diagnostic approach, a significant consideration in light of the fact that a false positive result could prompt euthanasia of a horse without

EGS. It is important to note however, that these samples would have been highlighted as equivocal and not erroneously diagnosed. Further work is therefore required to optimise differentiation of the equivocal sub-population of samples. While a mean immunoreactivity grade exceeding 1.1 was 100% specific and sensitive for EGS, there are limitations to the use of this criterion for EGS diagnosis. Firstly there was very little difference between the highest mean immunoreactivity grade for control horses (1.057) and the lowest mean immunoreactivity grade for EGS horses (1.137) (Supplementary item 4); consequently, it is possible that this criterion may not be fully discriminatory when a larger sample size is prospectively evaluated. Further limitations of this criterion are that it is laborious to determine and likely subject to a degree of inter-observer variability.

Similarly, while the presence of at least one grade 3 neuron was 95% sensitive and 100% specific for EGS, use of this criterion is potentially limited by errors in differentiating grade 2 and grade 3 neurons. To reduce this error, very repeatable grading criteria were developed and applied, such that grade 3 neurons had diffuse labelling of the entire cytoplasm, extending right up to the perikaryonal margin, except when this was displaced by cytoplasmic vacuolation (Fig 1). This potential limitation is confounded by the relative paucity of grade 3 neurons in rectal biopsies of EGS cases (median 11.4% of neurons, IQR 2.8-42) compared to CCG (median 35.5, IQR 21-41). Consequently, EGS diagnosis was based on the presence of only a few grade 3 neurons, and in occasional cases only 1 grade 3 neuron (n=2 horses). Chromatolytic neurons have been reported in the coeliacomesenteric ganglia, jejunum, ileum and small colon in clinically normal horses [2]. Whilst the assessment of chromatolysis is subjective, and relatively objective immunolabelling grading criteria may reduce the likelihood of incorrectly classifying neurons, a larger sample size is required to determine if grade 3 neurons are ever present in control horses. Although increasing the threshold for the diagnosis of EGS to the presence of ≥4 grade 3 neurons would increase the diagnostic certainty, it would also reduce the sensitivity to 62% (Supplementary item 5).

CCG, ileal and rectal neurons from EGS horses, but not control horses, had pyknotic nuclei (Fig 1c). Further study is required to determine whether this conventional histopathological analysis for morphologic features of neurodegeneration, together with chromatolysis and neuronal swelling, could be incorporated into the grading scheme to improve diagnostic accuracy of rectal biopsies.

There was complete intra-observer and very good inter-observer agreement in the interpretation of cases. The single case that was not agreed upon was an equivocal EGS case which had only 1 grade 3 neuron. In a clinical situation, it is unlikely that a diagnosis of EGS would be made upon observation of a single grade 3 neuron and further criteria or assessment of additional sections are required to give greater confidence differentiating the equivocal sub-population of samples.

Further work is required to assess the effect of EGS category on neuronal β-APP immunoreactivity. Consistent with previous work [1; 2; 17; 18], a higher proportion of normal neurons was found in chronic cases; all 3 chronic EGS sections had significantly fewer grade 3 rectal neurons than acute and sub-acute cases. Consistent with a further study [2], the median number of total neurons per section was higher in chronic cases than acute/ subacute cases; however the difference was not statistically significant and data from more chronic cases should be assessed to further investigate these findings. Acute and subacute forms of the disease are invariably fatal, while some cases of chronic EGS survive with appropriate nursing [24; 30-33]. A larger data set of chronic cases is required to investigate the potential prognostic value of β-APP immunolabelling.

Whilst estimations indicate that at least 30 neurons must be examined to be 96% confident that a grade 3 neuron was or was not present, at the expected median prevalence of 11.4%, it is likely that the diagnostic value of rectal biopsies and the confidence in the diagnosis could be improved by increasing the number of biopsies collected from each horse, and by examining multiple non-serial sections cut from individual biopsies. Previous studies indicate that the density of submucosal neurons does not have a consistent circumferential pattern in rectal samples [3], indicating that collection of biopsies from specific locations around the circumference of the rectal wall cannot reliably maximise the number of neurons sampled. While the total number of neurons identified in control rectal cases exceeded that of EGS cases, this reflected the increased number of biopsies collected from controls. Consequently there was no intergroup difference in the median number of neurons per section. The median number of rectal biopsies collected for EGS horses was comparable to the previous study by Mair *et al.* [4]. More biopsies were collected from control horses to increase the confidence of the calculated specificities.

The effect of ante-mortem versus post-mortem sampling must be further evaluated. Both control samples that were collected ante-mortem had lower percentages of grade 0 neurons and were the only control cases that were classified as equivocal. Whilst there was a difference of 0.45 between the

median mean immunoreactivity grade of EGS samples collected ante- and post-mortem, the difference was not significant.

The use of relatively non-invasive rectal biopsies versus ileal biopsies collected at laparotomy to diagnose EGS offers economic, welfare and time benefits. A disadvantage is the increased time required for immunolabelling of rectal biopsies compared with haematoxylin-eosin staining of ileal biopsies; total sample fixing and processing times being, respectively, a minimum of 12h and 6h. The timescale may be appropriate for ante-mortem diagnosis of sub-acute and chronic EGS cases and post-mortem confirmation of EGS in horses that are subjected to euthanasia in the field when the invasive removal of CCG or ileum is not feasible. However, this technique may be unsuitable for rapid ante-mortem diagnosis of acute EGS, unless the time requirement can be reduced, perhaps by using accelerated fixation protocols, frozen sections and employing rapid immunolabelling techniques.

In conclusion, this work has demonstrated the potential diagnostic value of immunolabelled rectal biopsies for EGS diagnosis. However, further prospective studies are required before the use of this technique can be fully advocated in clinical decision making.

Manufacturers' addresses

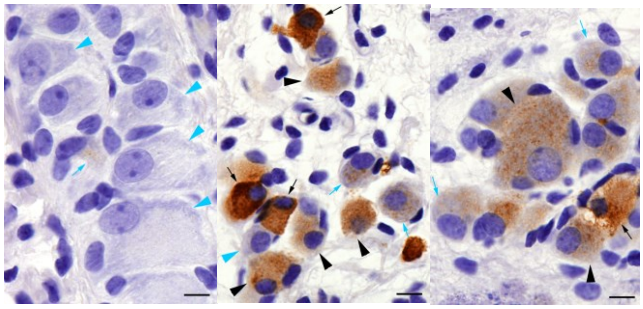
[a]Equivet uterine biopsy forceps; Kruuse UK Ltd, Sherburn in Elmet, UK

[b]http://epitools.ausvet.com.au

[c]GraphPad Software, La Jolla, California

Figures

Fig 1: Grading scheme used to assess β-amyloid precursor protein immunolabelling of neuronal perikarya in rectal submucosal ganglia. Grade 0= no labelling (blue arrowheads); 1= sparse labelling involving less than half of the cytoplasm (blue arrows); 2= greater than half of the cytoplasm is labelled but areas of unlabelled cytoplasm are still discernible (black arrowhead); and 3= diffuse labelling of entire cytoplasm right up to perikaryonal margin with no discernible unlabelled cytoplasm (black arrows).



A: Control horse: the majority of neurones are grade 0; the example of grade 1 labelling (blue arrow) was not included in the analysis as the nucleus of the neurone is not in the plane of the section.

B: Grass sickness horse: examples of all immunolabelling grades present.

C: Grass sickness horse: immunolabelling of neurones varies from grade 1-3; note the grade 3 neurone has peripheral cytoplasmic vacuolation and a shrunken (pyknotic) nucleus compared with the other neurones in the ganglion.

Bars = 10µm.

Fig 2: Percentage distribution of rectal submucosal neuronal grades for individual horses (Control = blue dot, equine grass sickness (EGS) = green dot and red cross = median).

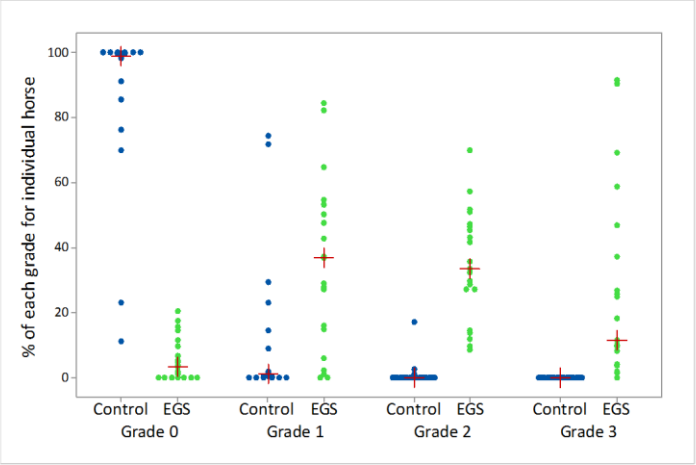
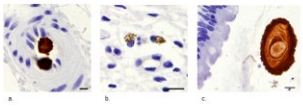


Fig 3: Examples of non-neuronal labelling and pigment artefacts (a) vascular intimal asteroid bodies (bar = 10µm), (b) macrophages containing intracytoplasmic pigment (bar = 10 µm), and (c) foreign (plant) material adjacent to mucosa (bar = 20µm).



Tables

Table 1: Diagnostic determinants with associated areas under the receiver operating characteristic curve (AUC), and highest achievable sensitivity, when specificity was set at 1.0, and the thresholds that were set to achieve this, for the diagnosis of EGS.

EGS = equine grass sickness, IQR = interquartile range, CI = confidence interval

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Supporting Information Items

Supplementary item 1: Immunolabelling methodology

Supplementary item 2: Cranial cervical ganglia and ileal sections

Supplementary item 3: Number of samples collected for each clinical category.

Supplementary item 4: Total number of neurons counted in combined rectal biopsy sections for individual horses and grades of β-amyloid precursor protein immunolabelling (% of total neurons).

Supplementary item 5: Receiver operating characteristic curve for number of grade 3 rectal submucosal neurons required for the diagnosis of equine grass sickness.