Equine gamma herpesvirus presence and viral load are not associated with equine glandular gastric disease

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OBJECTIVE
To investigate the role of equine herpesvirus-2 (EHV-2) and equine herpesvirus-5 (EHV-5) in equine glandular gastric disease (EGGD) by visualizing and quantifying these gamma herpesviruses in EGGD-affected and normal glandular gastric mucosa of horses. A secondary objective was to describe the histopathological abnormalities in the equine gastric glandular mucosa in horses with EGGD.

ANIMALS
29 horses (n = 21 postmortem and 8 gastroscopy) categorized as normal (11), EGGD (12), or both EGGD and equine squamous gastric disease (6).

METHODS
Glandular gastric mucosal samples were collected from horses by gastroscopy or postmortem. Histopathology and in situ hybridization targeting EHV-2 and EHV-5 were performed on grossly normal and abnormal glandular gastric mucosa. The number of in situ hybridization-positive cells per millimeter squared of tissue was calculated. Evaluators were blinded to groups.

RESULTS
Glandular gastric tissues from horses without EGGD had higher viral loads in the mucosa than normal or abnormal tissues from EGGD horses. There was no difference in viral loads for EHV-2 or EHV-5 between grossly or endoscopically normal to abnormal gastric tissues within horses with EGGD. Lymphocytic plasmacytic gastritis was the most common histopathological abnormality, with only 3 horses having mucosal disruption (glandular ulcer or erosion).

CLINICAL RELEVANCE
Equine gamma herpesviruses are unlikely to play a role in the pathophysiology of EGGD. EGGD is frequently inflammatory with occasional mucosal disruption (ulcer or erosion).

Keywords: gastritis, equine herpesvirus 2, equine herpesvirus 5, ulcer, EHV

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understood, and antiacid treatments are less effective. Unlike the squamous mucosa, the glandular mucosa is designed to be in constant contact with gastric acid. Therefore, EGGD is thought to be a result of a failure of the normal glandular mucosal defense mechanisms. Stress could play a role in the breakdown of mucosal defenses, as horses with EGGD had increased adrenocortical responses to adrenocorticotropic hormone and novel stimuli. In humans, NSAID use can lead to gastric ulcers; however, studies in horses have not shown consistent induction of EGGD with NSAID treatment, and many horses with EGGD have not received NSAID treatment. Combined, these reports suggest NSAIDs at recommended doses may contribute to EGGD in individual animals but are unlikely to be the primary cause of disease at the population level. Helicobacter pylori infection is another known cause of gastric ulcers in humans, but Helicobacter-like pathogens do not appear to be associated with EGGD pathogenesis. Modest differences in the microbiome have been identified in association with EGGD, suggesting that bacteria may play a role in the development or persistence of glandular disease.

In humans, the development of a subset of peptic ulcers has been linked with herpesvirus infections. The alpha herpesviruses varicella zoster virus, herpes simplex virus type 1, and gamma herpesviruses Epstein-Barr virus have all been associated with gastric ulcers in humans. Stress is a critical factor in the development of peptic ulcers and chronic gastritis in humans, and herpesviruses are known to be reactivated by stress. Stress appears to be a contributing factor to EGGD in horses. Additionally, the equine gamma herpesviruses EHV-2 and EHV-5 have been detected in the gastric mucosa of horses. We hypothesized that equine gamma herpesvirus infections are associated with EGGD.

Little has been done to compare endoscopic or postmortem findings with histopathological descriptions of the abnormal equine glandular mucosa. Further description of changes in the gastric mucosal tissue on a microscopic level with EGGD may provide insight into the pathogenesis of EGGD. Our primary objective was to evaluate a potential link between gamma herpesviruses and EGGD. We did this by visualizing and quantifying EHV-2 and -5 by in situ hybridization (ISH) in gastric mucosal samples. We compared the viral load of these gamma herpesviruses in horses with versus without EGGD. In addition, we compared the viral load in EGGD lesions versus grossly normal tissue within individual horses. A secondary aim was to describe the histopathological findings of the abnormal glandular gastric mucosa observed during gastroscopy and postmortem examination.

Methods

Horses and case definitions

Samples were obtained from horses from 3 groups: (1) horses presented to hospital A (Cornell University Hospital for Animals) for gastroscopy examination between November 2018 and November 2020; (2) Cornell–owned horses euthanized for other reasons at hospital A between November 2018 and November 2020; and (3) horses donated to hospital B (Louisiana State University) for research between June and December 2018. Privately owned horses were included in the study with informed consent from clients. The study was approved by the IACUC at both Cornell (no. 2018-0107) and Louisiana State University (no. 18-053).

Horses were categorized as “normal” if no gross or endoscopic stomach lesions were observed. Horses were categorized as having EGGD if gross or endoscopic pathologic lesions, including hyperemia, erosions, hemorrhage, fibrinonecrosis, raised areas, or ulcers were observed. Tissue from EGGD horses was categorized as normal (EGGDN) or lesion (EGGDl) based on gross or endoscopic appearance. Horses with ESGD only were excluded from the control group, as ESGD could indicate underlying stress or other disease that might influence viral load in the glandular mucosa.

Biopsy

For client-owned animals, a routine procedure for sedated gastroscopy examination was performed by the attending clinician. The squamous mucosa along greater and lesser curvatures and the glandular mucosa at the pylorus were visualized. Endoscopic biopsies (2.3 mm) were obtained of normal pyloric mucosa and any lesions observed. The biopsy instrument was disinfected with a 10% bleach solution and rinsed with sterile saline in between each sampling site.

For necropsy samples collected at hospital A, gastric tissues were collected within 2 hours postmortem. Six-millimeter punch biopsies were obtained from grossly normal and abnormal tissue at the pylorus. Instruments were disinfected with 10% bleach solution and rinsed with sterile saline in between each sampling site. Postmortem samples at hospital B were collected by excision of grossly normal and abnormal tissues at the pylorus. Biopsies were fixed in 10% neutral buffered formalin and paraffin embedded.

Histology and ISH

Samples were evaluated using RNAscope ISH targeting the glycoprotein B (gB) gene of either EHV-2 or EHV-5, as previously validated. This assay detects both viral DNA and RNA and thus does not distinguish between latent and active infections. Positive (PPIB) and negative (DapB) control probes were applied for each assay and performed as expected. Slides were scanned digitally and the areas of the mucosa and lamina muscularis (LM) were measured using Animal Health Diagnostic Center web-based Pathology Center software. Cells with positive labeling were counted manually in the mucosa and LM separately. The entire slide was counted unless positive cells were too numerous to count, in which instances a smaller
area was measured out and counted, including both a minimum area of 1.0 mm² and a minimum of 100 positive cells. Individuals performing the counting of positive cells were blinded to horse and sample type (normal vs lesion). The number of positive cells per millimeter squared of tissue was calculated to determine viral load.

To differentiate between latent DNA and active replication when RNA is present, DNase pretreatment was applied on additional sections before labeling with EHV-2, EHV-5, and PPIB probes, as previously described.42 However, PPIB-positive control quality was poor, and the test could not be optimized within the cost and time constraints of this study. This analysis was, therefore, excluded.

To characterize the morphological changes in the glandular mucosa and verify our categorization of horses into normal and EGGD, H&E-labeled slides were evaluated by a board-certified veterinary pathologist (SPM), who was blinded to horse and sample type. Gastroscopic pinch biopsies were deemed insufficient for evaluation; therefore, only necropsy samples were assessed histologically. The glandular mucosa was described by severity, inflammatory cell type, depth, distribution, duration, fibrosis, glandular loss or dilation, erosion, ulceration, fibrinosuppurative inflammation, and any additional findings.

**Statistical analysis**

Viral load data (measured as the number of ISH-positive cells per mm²) was right skewed. The association between the presence of pyloric ulcers and the viral load with EHV-2 and EHV-5 in the pyloric mucosa and LM mucosa of normal and EGGD horses was evaluated using Wilcoxon rank-sum tests. The association between the amount of virus in EGGD versus EGGD tissues was assessed by Wilcoxon signed-rank test for nonparametric paired data. The analysis was performed with JMP Pro, version 17.0.0 (JMP Statistical Software). Significance was set at \( P < .05 \).

**Results**

**Horses**

At hospital A, 33 horses were examined. Twenty were included in the study, and 13 were excluded due to the presence of ESGD without EGGD. These 20 horses were considered to have normal stomachs (11) or stomachs with EGGD with or without ESGD (9). An additional 9 EGGD horses were included from banked tissues at hospital B. Horses with EGGD are denoted by letter (eg, horse A), and normal horses are denoted by number (eg, horse 1), and normal horses were obtained from 13 horses via necropsy and 7 horses via gastroscopy exams. At hospital B, samples were obtained from 8 horses via necropsy and 1 horse via gastroscopy exams.

**Description of lesions**

Endoscopic and gross lesions for EGGD were described according to the European College of Equine Internal Medicine² consensus statement criteria (Table 1). Histopathology showed gastritis with an intact mucosal surface in 9/15 (60%) and an erosion and/or ulcer in 6/15 (40%). The erosions and ulcers confirmed histologically were seen as depressed or raised areas grossly. Frequently, the erosive areas were covered with a fibrinosuppurative layer. All horses with gastritis had lymphocytic or lymphoplasmacytic infiltrate, while neutrophils (27% [4/15]) and eosinophils (20% [3/15]) were noted in some. Fibrosis (20% [3/15]), glandular dilation (20% [3/15]), glandular loss (20% [3/15]), lymphoid aggregates (13% [2/15]), follicular lymphoid hyperplasia (7% [1/15]), suppurative adenitis (7% [1/15]), and submucosal mast cell tumor (7% [1/15]) were also observed. Horse 15 had multiple bots in the squamous portion of the stomach, and no other parasites were noted on gastroscopy. However, histopathology showed superficial nematodes, most consistent with Draschia megastroma, along with evidence of a mucosal barrier disruption with hair

<table>
<thead>
<tr>
<th>Description</th>
<th>No. of horses</th>
<th>No. of lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Severity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Distribution</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Focal</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Multifocal</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Epithelial appearance</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperemic</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Hemorrhagic</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Fibrinosuppurative</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Ulcerated</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>Mucosal contour</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depressed</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Flat</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Raised</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

For epithelial appearance and mucosal contour, some horses had more than 1 lesion described plus multiple descriptors per lesion.

Table 1—Gross description of equine glandular gastric disease in 18 horses.
and plant material and lymphoplasmacytic infiltrate; eosinophils were not seen. Horse 18 had multiple bots at the margo plicatus near the lesser curvature, and a long thin white parasite was noted in the glandular mucosa in the cardiac region on gastroscopy, which was not biopsied.

In situ hybridization findings

Postmortem and endoscopic biopsies were evaluated by ISH for viral load, although endoscopic biopsies included mucosa but not LM. All pyloric samples were positive for EHV-2, and all but 2 pyloric lesion samples were positive for EHV-5 (Figure 2).
Figure 2—In situ hybridization (ISH) identifies equine gamma herpesviruses in gastric glandular epithelium and smooth muscle. A—Photomicrograph of normal glandular mucosal epithelium from a horse that had equine glandular gastric disease (EGGD). Positive hybridization (pink to red staining, arrows) is observed scattered through the tissue. Horse 2; equine herpesvirus 2 (EHV-2) glycoprotein B (gB) ISH; bar = 500 µm; inset bar = 100 µm. B—Photomicrograph of ulcerated glandular mucosal epithelium with hybridization located deep in the glands. Horse 2; equine herpesvirus 5 (EHV-5) gB ISH; bar = 500 µm; inset bar = 100 µm. C—Photomicrograph of normal glandular mucosa from a horse with EGGD. Hybridization is present in the lamina muscularis mucosa. The mucosal surface is at the bottom right of this image and submucosa on top left. Horse 1; EHV-5 gB ISH; bar = 500 µm; inset bar = 100 µm. D—Photomicrograph of ulcerated glandular mucosal epithelium. EHV-5 hybridization is present in the nuclei of the gastric glandular epithelium. Horse 1; EHV-5 gB ISH; bar = 100 µm. E—Photomicrograph of normal glandular mucosal epithelium from a horse without EGGD. EHV-5 hybridization is present in the nuclei of gastric glandular epithelium. Horse A; EHV-5 gB ISH; bar = 100 µm. F—Photomicrograph of ulcerated glandular mucosal epithelium labeled with a negative control probe shows no background. Horse 2; DapB ISH; bar = 100 µm. G—Photomicrograph of ulcerated glandular mucosal epithelium labeled with a positive control probe for the ubiquitously expressed gene PPIB showed widespread cytoplasmic hybridization, as expected. Horse 2; EGGD pyloric ulcer; PPIB ISH; bar = 100 µm.
distribution and proportion of infected cells were similar for EHV-2 and EHV-5. Infected cells could be observed primarily in the outer mucosal layer, with fewer horses showing infection in the deeper gastric glands and in the LM. Most cells showed nuclear hybridization. The proportion of positive cells was generally low, with occasional samples where nearly every cell was ISH positive. In the mucosa, gastric glandular tissues from normal horses had significantly higher median viral loads for both EHV-5 and EHV-2 than EGGDN or EGGDL tissues, although there was a large amount of overlap between groups (Table 2 and Figure 3). In the LM, gastric glandular tissues from normal horses had significantly higher EHV-5 viral loads than EGGDN (Table 3). Within an individual horse with EGGD, there was no significant difference in viral loads in EGGDN versus EGGDL tissues in either mucosa or LM (Supplementary Figure S1).

### Table 2—Equine gamma herpesviral load in the pyloric mucosa of horses with and without equine glandular gastric disease (EGGD).

<table>
<thead>
<tr>
<th>Mucosa</th>
<th>EHV-2</th>
<th>EHV-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>11 9.6</td>
<td>7.3</td>
</tr>
<tr>
<td>EGGD_</td>
<td>18 0.82</td>
<td>0.87</td>
</tr>
<tr>
<td>EGGDN</td>
<td>18 0.43</td>
<td>0.63</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Normal vs EGGDN (P value)</th>
<th>EGGDN vs EGGDL (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.023ª</td>
<td>0.018ª</td>
</tr>
<tr>
<td>Normal</td>
<td>0.026ª</td>
<td>0.021ª</td>
</tr>
<tr>
<td>EGGDN</td>
<td>1</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Median (range) in situ hybridization–positive cells/mm². Viral load was compared between normal and EGGD horses by Wilcoxon rank-sum test. Viral load was compared between normal (EGGDN) and lesion (EGGDL) tissues within EGGD horses by Wilcoxon signed-rank test for paired data. EHV-2 = Equine herpesvirus 2. EHV-5 = Equine herpesvirus 5.

### Table 3—Equine gamma herpesviral load in the pyloric lamina muscularis mucosa of horses with and without equine glandular gastric disease (EGGD).

<table>
<thead>
<tr>
<th>Lamina muscularis</th>
<th>EHV-2</th>
<th>EHV-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5 2.3</td>
<td>7</td>
</tr>
<tr>
<td>EGGD_</td>
<td>15 0.38</td>
<td>1.3</td>
</tr>
<tr>
<td>EGGDN</td>
<td>15 0.27</td>
<td>0.19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Normal vs EGGDN (P value)</th>
<th>EGGDN vs EGGDL (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>.41</td>
<td>.081</td>
</tr>
<tr>
<td>Normal</td>
<td>.14</td>
<td>.044ª</td>
</tr>
<tr>
<td>EGGDN</td>
<td>.93</td>
<td>.89</td>
</tr>
</tbody>
</table>

Median (range) in situ hybridization–positive cells/mm². Viral load was compared between normal and EGGD horses by Wilcoxon rank-sum test. Viral load was compared between normal (EGGDN) and lesion (EGGDL) tissues within EGGD horses by Wilcoxon signed-rank test for paired data. EHV-2 = Equine herpesvirus 2. EHV-5 = Equine herpesvirus 5.

### Discussion

Our hypothesis that the presence of equine gamma herpesviruses in gastric tissues would be positively associated with EGGD was not supported. In fact, there appeared to be higher viral loads in horses with normal gastric mucosa instead. This is in direct contrast to studies in humans showing an association between the detection of herpesviruses in peptic lesions only.

EGGD more closely models human peptic ulcer disease than does ESGD. Efforts to identify causes of EGGD have considered common causes of human peptic ulcers, including NSAIDs and *H pylori*, however, these have not been well substantiated in horses. In this study, we explored a potential relation with herpesviruses. Most horses are infected with EHV-2 and EHV-5 within the first 6 months of life. These gamma herpesviruses can be detected in both healthy and sick horses in nasal and genital swab samples, conjunctiva, lacrimal gland, optic nerve, leukocytes, lymphoid, and nervous tissues. However, infection with EHV-2 and -5, but not the alpha herpesvirus EHV-1,
have been detected in gastric mucosa, and a small sample set was suggestive of a higher infection rate in horses with ulcers.42 Our larger dataset, albeit still a small number of horses with EGGD, confirmed the frequent presence of gamma herpesviruses in equine gastric mucosa but found no association with EGGD. This resembles recent investigations of equine keratoconjunctivitis and EHV-2, whereby 28.6% (22/77) of ocular swabs from horses without eye disease were positive by PCR and only 8.3% (4/48) with keratoconjunctivitis.51 However, positive clinical response to antiviral treatment suggests EHV-2 might still play a role in equine keratoconjunctivitis.

It is possible that the apparently ubiquitous nature of gamma herpesvirus infection is masking a disease effect or disease interaction. In this study, we detected viral DNA as well as RNA and could not confirm whether positive cells reflected latent or active infections. Although it is possible that reactivation occurs locally associated with EGGD lesions, this seems unlikely as we would have expected to have similar or higher viral loads in these lesions. In people with gastroduodenal ulcer disease, herpes simplex virus was detected by PCR in 9.5% of patients, and the virus was only detectable in lesions and not in normal tissue nearby.52 Few studies29,31,43,44,53 have compared the gross appearance of the glandular stomach of the horse during endoscopy exams or postmortem to histopathology findings. Here, we report on the histopathological findings in 18 horses with EGGD. Abnormal histopathology was noted for all the gross lesions. Of the horses in the EGGD group, 40% had erosion or ulceration and 60% had gastritis with a intact mucosal surface. The presence of nematodes in the gastric mucosa but found no association with EGGD. This resembles recent investigations of equine keratoconjunctivitis and EHV-2, whereby 28.6% (22/77) of ocular swabs from horses without eye disease were positive by PCR and only 8.3% (4/48) with keratoconjunctivitis.51 However, positive clinical response to antiviral treatment suggests EHV-2 might still play a role in equine keratoconjunctivitis.

In summary, EGGD is characterized histologically by gastritis with a lymphoplasmacytic infiltrate and sometimes has disruption of the mucosal epithelium by gastritis with a lymphoplasmacytic infiltrate and sometimes has disruption of the mucosal epithelium and raised lesions corresponded with gastritis only. One horse that was categorized as normal on postmortem evaluation had mild, multifocal, chronic lymphoplasmacytic gastritis and multifocal interglandular fibrosis on histopathology. This finding suggests that visualization of the glandular mucosa during postmortem examination, and presumably endoscopically, can miss mild gastritis in the glandular region of the equine stomach. Gastroscopy can also miss glandular lesions due to the presence of feed or fluid in the stomach, inadequate insufflation of the stomach, or clinician experience. Gastroscopic biopsy can be a useful ancillary diagnostic tool for EGGD, although current limitations of pinch biopsy instruments (small samples, crush artifact) present a diagnostic challenge to the histopathologist where the mucosa to submucosal layers can be incomplete and might preclude the diagnosis of inflammatory infiltrate in gastric mucosal samples.53,56 The presence of nematodes in the gastric mucosa is a reminder that parasites might play a role in EGGD in horses. These parasites might also suggest whether positive cells reflected latent or active infections. Although it is possible that reactivation occurs locally associated with EGGD lesions, this seems unlikely as we would have expected to have similar or higher viral loads in these lesions. In people with gastroduodenal ulcer disease, herpes simplex virus was detected by PCR in 9.5% of patients, and the virus was only detectable in lesions and not in normal tissue nearby.52 Few studies29,31,43,44,53 have compared the gross appearance of the glandular stomach of the horse during endoscopy exams or postmortem to histopathology findings. Here, we report on the histopathological findings in 18 horses with EGGD. Abnormal histopathology was noted for all the gross lesions. Of the horses in the EGGD group, 40% had erosion or ulceration and 60% had gastritis with an intact mucosal surface. The presence of nematodes in the gastric mucosa but found no association with EGGD. This resembles recent investigations of equine keratoconjunctivitis and EHV-2, whereby 28.6% (22/77) of ocular swabs from horses without eye disease were positive by PCR and only 8.3% (4/48) with keratoconjunctivitis.51 However, positive clinical response to antiviral treatment suggests EHV-2 might still play a role in equine keratoconjunctivitis.


Supplementary Materials
Supplementary materials are posted online at the journal website: avmajournals.avma.org