

The Use of Novel Antibody Tools to Detect the Presence of Blood in Equine Feces

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Overview: The equine digestive tract can suffer from a variety of injuries that can result in internal blood loss such as parasitism, bacterial infection, and gastric and colonic ulcers. The presence of these injuries can compromise overall digestive health and function, and could relate to larger issues such as internal pain, feed refusal, colic and death. Of these injuries, gastric ulcers are perhaps the best studied in the horse due to the use of endoscopy techniques for direct observation of gastric lesions. The ability to detect and identify the source of blood loss throughout the equine digestive tract would, therefore, be of value in understanding the horse's health beyond gastric ulceration.

Other than direct observation via endoscopy, few techniques exist to diagnose the presence of digestive tract bleeding. While staining techniques using guaiac acid have been used, the sensitivity of this test is poor and specificity is potentially low and prone to interference. The use of equine blood-specific antibodies would offer a higher degree of specificity and sensitivity, but those technologies have not been developed at present.

The purpose of this experiment is to use ELISA techniques to test the specificity and sensitivity of newly developed antibody technologies specific for components unique to equine blood as a foundation for readily detecting digestive tract bleeding in horses. After verifying sensitivity, these antibodies were then used in a "real world" situation to detect the presence of fecal blood 24 hours after blood was introduced to the stomach of a horse.

Methods:

Antibody Production: Two different polyclonal antibodies specific for two antigenic proteins unique to equine blood were prepared (AB1, AB2) in a rabbit model. A specific peptide sequence for each antigenic protein was chosen and synthesized in the lab, further conjugated to enhance immunogenicity, and injected into rabbits. At 2 and 3 months, rabbits were given booster injections of the peptide sequence to further enhance their immune reaction and maximize antibody production. At the conclusion of three months on the protocol, bleeds were taken from each rabbit, serum was separated, and all serum samples were pooled.

Antibodies were then purified using an affinity column containing the original peptide sequence, ensuring that only antibodies to our chosen sequence were in the final antibody preparation.

AB1: Prepared using the 35 AA C-terminus of target antigen 1

AB2: Prepared using both the 22 AA N-terminus and 36 AA C-terminus of target antigen 2

Sample Collection: In experiment 1, equine blood (40mls) was introduced through a gastric cannula to 2 experimental horses. Representative fecal samples were taken periodically for the next 24 hours, mixed with a 1X TRIS buffered saline, and stored frozen. In experiment 2, representative fecal samples were taken from 25 active racing horses and 25 pleasure horses, mixed with a 1X TRIS buffered saline, and stored frozen.

ELISA: Standard ELISA methodology was used to prepare each sample and adhere to test wells. For detection, samples were first exposed to either AB1 or AB2 for primary detection, and then further reacted with an anti-rabbit antibody labeled with peroxidase for colorimetric detection. All samples were read using a standard microwell plate reader.

Results:

Experiment 1: Figures 1 and 2 (next page) represent data collected from time course assay on two cannulated horses. Data points have been corrected for background and represent a full 24 hours of fecal collection.

Experiment 2: Figure 3 (next page) represents single time point data for race and pleasure horses. As the results indicate, antigen 1 was detected in approximately 28% of all horses and antigen 2 was detected in approximately 60% of all horses. For race horses, antigen 1 was detected in approximately 47% of horses and antigen 2 was detected in approximately 48% of horses. Antigen 1 was detected in approximately 8% of all pleasure horses, while antigen 2 was detected in approximately 68%.

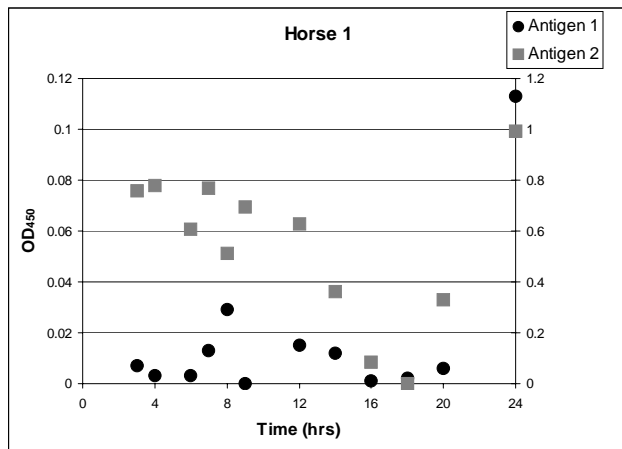


Figure 1. OD₄₅₀ results from ELISA time course assay, horse 1. Background corrected readings indicate early detection of antigen 1 and possibly antigen 2 during first 12 hours, with a clear final peak of both antigen 1 and 2 during the final 24 hour timepoint.

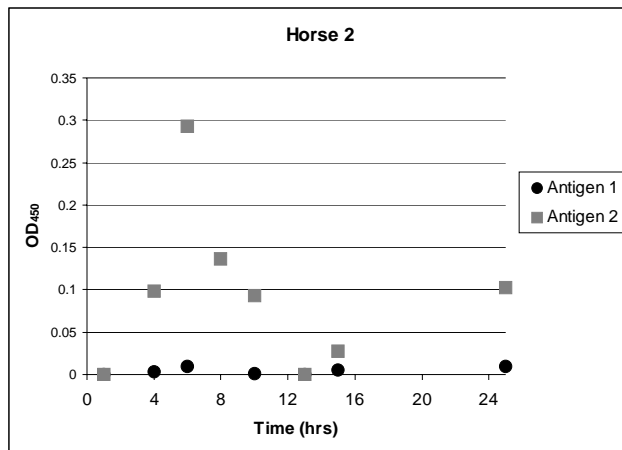


Figure 2. OD₄₅₀ results from ELISA time course assay, horse 2. Background corrected readings indicate early detection of antigen 2 but not antigen 1 during the first 12 hours. Unlike horse 1, only antigen 2 was seen to rise again at 24 hour timepoint.

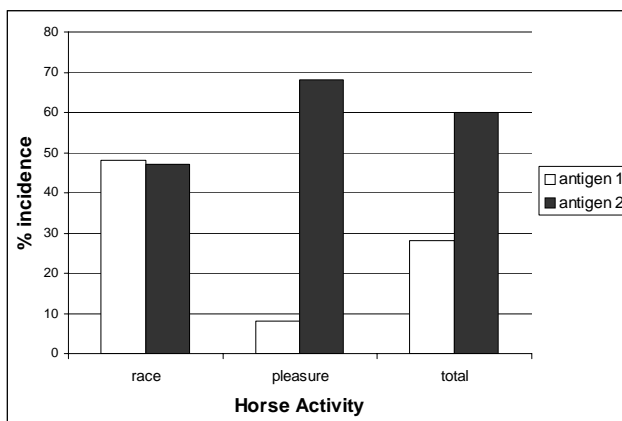


Figure 3. Detection of antigen 1 and 2 in horses of different activity levels. As in experiment 1, both antigens were positively detected in fecal samples after correction for background.

Discussion: This paper details the use of novel antibody techniques to detect the presence of proteins derived from equine blood. Based on their genetic and peptide sequences, these proteins are unique to the equine, and would only be present in the equine digestive tract from either ingested equine blood or from bleeding occurring at some point in the digestive tract.

Currently, equine veterinarians rely on endoscopy, symptomology and/or treatment response as diagnosis. All of these approaches have limitations – endoscopy only accounts for the gastric region and a 3-meter endoscope may not be readily available; gross observation is highly subjective and is hindered by the fact that external symptoms may be attributable to a variety of causes; and treatment response takes time and may do more harm if misdirected.

The ability to reliably detect equine blood with high sensitivity based on manure sampling can have major benefit in the equine practitioner’s diagnosis, leading to more timely and accurate treatment options. Examples include any situations where anemia or subclinical anemia is present, where endoscopy has ruled out gastric bleeding, or where a 3-meter endoscope is unavailable, or in situations where gross observation may point to a variety of potential conditions. Further, the ability to rapidly detect blood in feces would enhance the veterinarian’s ability to monitor an equine patient following intestinal resection, where blood loss is of particular concern and may not be detected for 24 hours or more running serial packed cell volumes.

Of further importance would be the use of a rapid test that could distinguish between blood of cranial origin (bleeding prior to the duodenum) and of caudal origin (bleeding located in the cecum and colon). To accomplish this differentiation a double antigen test, as described above, has been employed. The use of antibodies for detection of human fecal occult blood has been well established for the diagnosis of cancer and ulcers (Barrows, 1978), however this technology has not as of yet been used in equines. Additionally, this technology has been limited to the positive or negative detection of blood, without differentiation of caudal or cranial bleeding. By using two antigens, one derived from a quickly degrading protein, and one that is more recalcitrant to degradation in the digestive tract, the source of bleeding should be determined. In this experiment, antigen 1 is believed to be quickly degraded in the digestive tract, while antigen 2 is more stable to digestive degradation and thus will be resident in digestive material much longer. Thus, detection of antigen 1 indicates caudal bleeding, while detection of antigen 2 could indicate either caudal or cranial bleeding.

While these results have initially been confirmed *in vitro* using blood added to manure, experiment 1 establishes the validity of the technique *in vivo*. As

the results show, it is possible to detect the presence of both antigens using this technology, and that the presence of these antigens changes over time. Furthermore, as shown by horse 1, after an expected 24 hour transit time through the digestive tract, the blood introduced gastrically is evidenced by a major spike in both antigens. These results were not repeated in animal 2, but that could simply be due to a longer transit time through the digestive tract indicating that the peak was missed. Finally, the results from both time trials indicate potential digestive tract bleeding, not unexpected in animals fitted with gastric and colonic cannulas.

Experiment 2 was conducted to further establish the validity of this dual antibody approach. 50 horses were selected at random, 25 from an active racing population, and 25 from a leisure horse population, and single time point fecal samples were taken and evaluated using ELISA techniques identical to experiment 1. As indicated by the results, approximately 60% of all horses in the study have indications of cranial bleeding or caudal bleeding, while approximately 38% of all horses had indications of caudal bleeding only. This roughly corresponds to

previous work looking at incidences of gastric and colonic ulcers during necropsy. (Pellegrini, 2005, Bedding, Pellegrini, 2006)

When looked at as a whole, both experiments confirm the validity of both detection of fecal occult blood in equine manure, as well as the potential differentiation of cranial and caudal bleeding in horses. The implications of this research are far reaching and set the stage for development of diagnostic aids that can help the veterinarian more precisely and quickly diagnose digestive tract health issues in equine patients.

References:

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Pellegrini, Franklin L. Results of a Large-Scale Necroscopic Study of Equine Colonic Ulcers. *J Equine Vet Sci* 2005; 25 (3) 113-117.

Barrows, GH, Burton, RM, Jarett, DD, Russell, GG, Alfor, MD, Songster, CL. Immunochemical Detection of Human Blood in Feces. *American Journal of Clinical Pathology*. 1978, Mar; 69(3): 342-6.