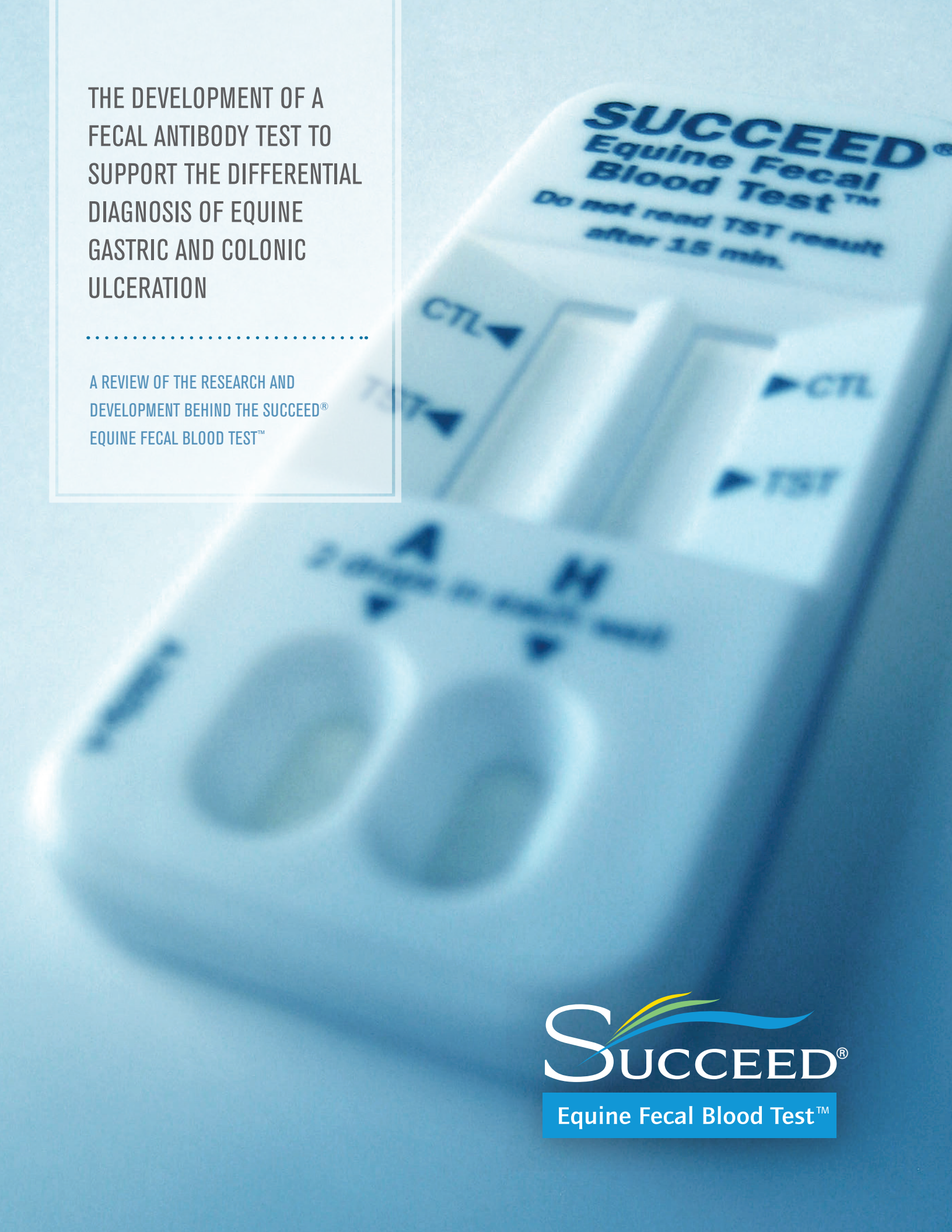
THE DEVELOPMENT OF A FECAL ANTIBODY TEST TO SUPPORT THE DIFFERENTIAL DIAGNOSIS OF EQUINE GASTRIC AND COLONIC ULCERATION

A REVIEW OF THE RESEARCH AND DEVELOPMENT BEHIND THE SUCCEED® EQUINE FECAL BLOOD TEST™



SUCCEED®
Equine Fecal Blood Test™

Over the course of eight years, we have observed a high rate of gastric and colonic ulcers in over 900 horses at abattoirs in the United States and Canada. Throughout, we have tested several technologies that attempt to detect these lesions using a fecal sample. We now report on an improved antibody test kit.

The SUCCEED® Equine Fecal Blood Test™ is highly accurate and sensitive and can help to differentiate gastric from colonic ulceration and related digestive tract pathologies. The kit is a two-part field test that is easy to employ and provides results in minutes. The test may also have some other important applications for the veterinarian.

The SUCCEED Equine Fecal Blood Test is available exclusively to veterinarians through major veterinary supply distributors in the U.S.:

- ***Butler Schein Animal Health***
- ***Milburn Equine***
- ***Midwest Veterinary Supply***
- ***MWI Veterinary Supply***
- ***Webster Veterinary***
- ***Animal Health International***

ULCER INCIDENCE IN PERFORMANCE HORSES

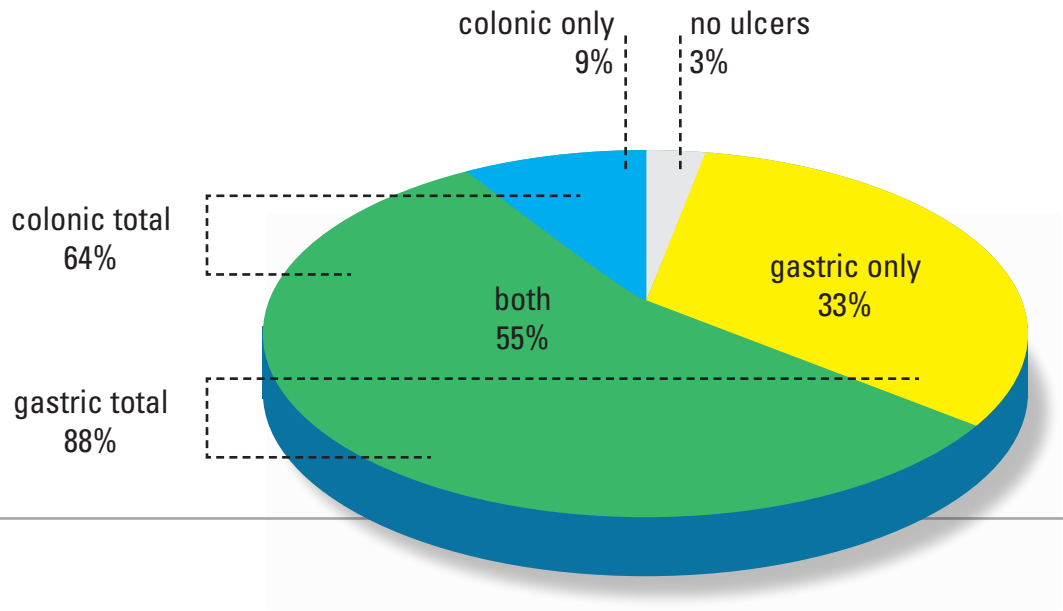


Figure 1: A 2003 study at a Texas abattoir found that only 3% of performance horses had no ulceration, and that a majority (55%) had both colonic and gastric lesions.

Horses may have lesions throughout their digestive tract, and those in the colon are not well understood. Most veterinarians are familiar with Equine Gastric Ulcer Syndrome (EGUS) which manifests primarily as lesions in the distal esophagus, the squamous area of the stomach and the proximal duodenum.[1, 2, 3] There are several possible causes of gastric bleeding, including ulcers, parasitism, infection and surgery. These problems, and the subsequent loss of blood, can adversely affect digestive health, resulting in pain, discomfort and impaired performance. Untreated, these issues can lead to anemia, colic and even death.[4]

Gastric ulcers can be visualized with a three-meter endoscope. However, the gastric area that is home to EGUS represents less than 10% of the equine GI tract. Equine digestion is dominated by hindgut action, but ulcers there are much harder to observe. Colonoscopies are impractical due to the difficulty of evacuating the equine colon without endangering the health of the horse. As a consequence, most equine vets are not familiar with colonic ulcers. However, it is now known that colonic ulcers are common in horses. The ability to accurately diagnose colonic ulcers and differentiate them from stomach ulceration is of particular importance since the treatment protocols are quite different. At the very least, treatments targeting stomach ulcers are likely to have little or no effect on conditions in the hindgut.

Over the years we have developed several technologies to detect and hopefully differentiate gastric and colonic ulcers. This article describes the basic methodology and the results to date.

The 2003 Necroscopic Study

In 2003, Freedom Health conducted a large-scale necropsy of 180 performance horses. The resulting analysis (Pellegrini, 2005) revealed that 87% had gastric ulcers and 63% had colonic ulcers, with an overall ulceration rate of 97%.[5]

We knew that due to the length of the equine GI tract, many vets did not believe that gastric bleeding could be detected in horse feces. We wanted to test that belief, so we used a human-based guaiac fecal blood test (gFBT) to visualize it. Guaiac works by binding hemoglobin and turning blue in the presence of hydrogen peroxide.

In this study, a fecal sample was collected from each horse prior to euthanasia. For the necropsy, the digestive tract was removed and the stomach and colon were tied off for separate examination. Gastric ulcers were categorized according to the Practitioner's Simplified Scoring System[6] on a scale from 0 to 3:

Score	Description
0	Intact mucosal epithelium (can have mild reddening and/or mild hyperkeratosis)
1	Small single or small multifocal lesions
2	Large single or large multifocal lesions or extensive superficial lesions
3	Extensive (often coalescing) lesions with areas of apparent deep ulceration

Figure 2: A grade 3 gastric ulcer with major erosion.

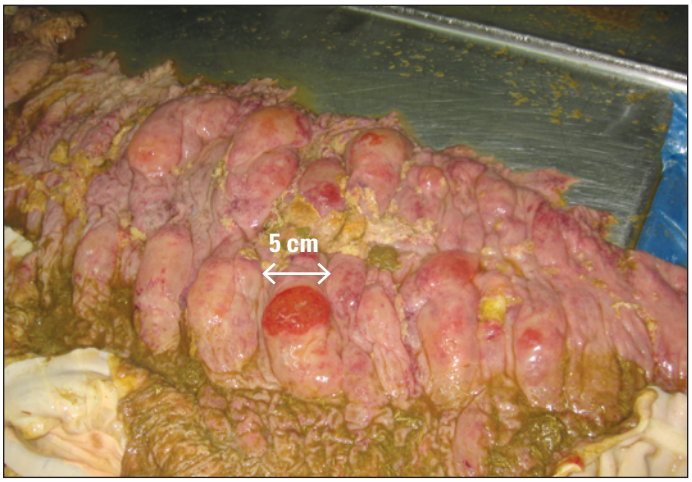


Figure 3: A grade 3 disseminated colonic ulcer with a large lesion.

Due to the dearth of research on colonic ulcers, a corresponding colonic ulcer grading scale did not exist. We therefore applied the Practitioner's Simplified Scoring System to the colon for purposes of this study.

The scoring system does not specifically reference bleeding or the presence of blood in its descriptions of ulcers at any score. We therefore assumed that ulcers of grade 2 or higher represent those where whole blood (hemoglobin) is present. Grade 1 ulcers, while not bleeding per se, may be associated with seeping of albumin at the point of injury.

The manure was tested with the gFBT and correlated to the gross examination of intestinal tissue. Overall, the gFBT proved to be highly specific and significant for the existence of an ulcer, but the existence of false negatives limited the overall accuracy of the test to 65%, roughly comparable to human outcomes with such a test.

Perhaps most importantly, our observations clearly demonstrated that lesions within the equine digestive tract are not confined to the stomach. We saw several instances of right dorsal colitis, a known problem with horses taking NSAIDs. But in addition, we saw ulcers in all quadrants of the colon. This was unexpected, because colonic ulceration in these other quadrants was not

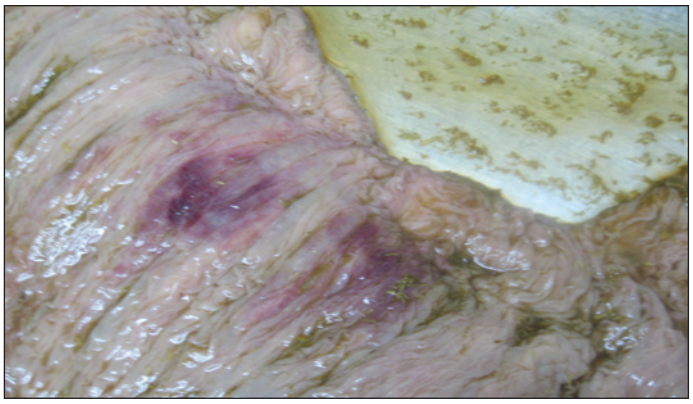


Figure 4: Right-dorsal colitis with a nodule (center).

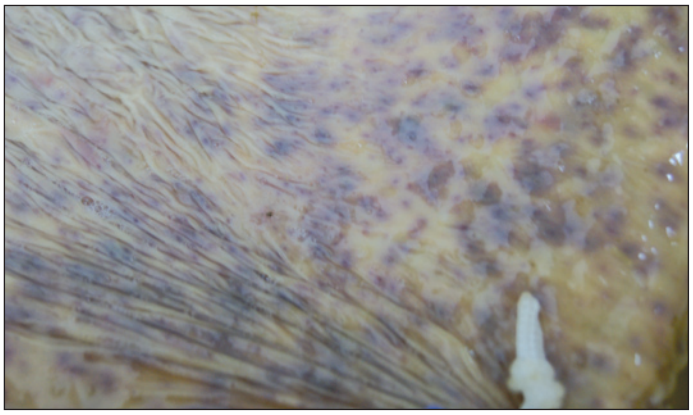


Figure 5: A severe ecchymosis of the left ventral colon.

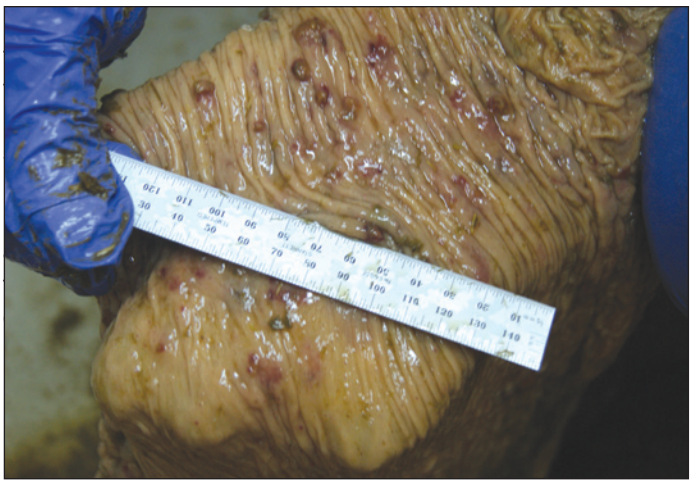


Figure 6: A grade 2 ulcer in the left ventral colon.

noted in the literature. In this study, and over the course of four additional studies over 7 years, we found large cysts, focal pinpoint ulcers, widely disseminated ulcers, petechiation, ecchymoses and purpura.

These first studies in 2003 demonstrated a high rate of colonic ulceration in performance horses. Our testing showed that blood can absolutely be detected in manure, although guaiac fecal blood testing proved to lack the sensitivity to definitively diagnose an ulcer.

Finding Marker Proteins for Antibody Detection

To improve sensitivity, we turned to the exacting technology of antibody binding, specifically lateral-flow immunoassays. These tests are easy to use in the field, yet still provide the high sensitivity and precision required to detect small quantities of blood products in fecal matter.

To detect and potentially localize equine ulcers, we undertook an analysis of two potential marker proteins found in blood that we hoped could distinguish foregut from hindgut lesions: albumin and hemoglobin. In an experiment conducted with researchers from Island Whirl Equine Colic Research Laboratory in Florida, equine blood was introduced through a gastric cannula to two experimental horses and fecal samples were then taken periodically for the subsequent 18 hours.

Albumin is known to be degraded by enzymes such as pepsin and trypsin in the stomach and duodenum. As a consequence, we expected that any albumin detected in fecal matter must emanate from a hindgut lesion caudal to the duodenum. The study also looked at hemoglobin, which our previous guaiac research had shown can survive both gastric and colonic degradation. Taken together, we realized that detection of these two proteins could provide a novel technique for helping to distinguish these two disjoint areas of ulceration.

These two protein markers were analyzed using an Enzyme-Linked ImmunoSorbent Assay (ELISA). When the results were plotted, we saw that the levels of hemoglobin peaked and then slowly fell over the 18-hour period, while albumin levels remained consistently low due to the gastric source of the serum. This provided strong support for the utility of these two markers in a differential diagnosis.

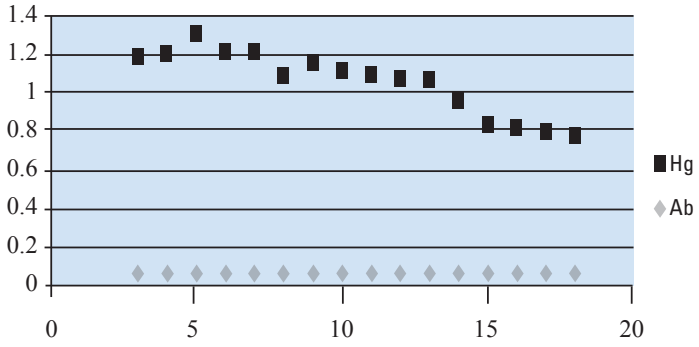


Figure 7: OD450 results from an averaged ELISA time-course assay on two horses injected with blood via gastric cannulation at Island Whirl. Note the slow decay of detected hemoglobin and the consistently low levels of albumin.

Preliminary Results

From this experiment, we determined that albumin could serve as a proxy for hindgut lesions, while the stability of hemoglobin should allow its use as an indicator of either foregut or hindgut lesions. The following table illustrates its use as a differential diagnostic.

Results	Negative albumin	Positive albumin
Negative hemoglobin	No detectable bleeding	Hindgut bleeding
Positive hemoglobin	Foregut bleeding	Hindgut and possible foregut bleeding

Creating an Antibody Test

We next set out to determine useful diagnostic levels of these two blood components in compromised GI tissue. Based on the albumin and hemoglobin experiments at Island Whirl, we designed an immunoassay field kit using purified antibodies against albumin and hemoglobin. To prepare the kit, a peptide sequence unique to each equine protein was chosen and synthesized in the lab, further conjugated to enhance immunogenicity, and then injected into rabbits. At two and three months, the 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 from the blood, and all the serum samples were pooled.[7]

Antibodies were then purified using an affinity column containing the original peptide sequence, ensuring that only antibodies to the chosen sequence were in the final antibody preparations. These proteins have peptide sequences that are uniquely equine, and thus are only present in the equine digestive tract from either ingested equine blood (e.g. from the lungs) or from bleeding occurring at some point in the digestive tract.

The test has one antibody well tuned to detect above-baseline albumin and another for hemoglobin. A couple of drops of diluted fecal matter are placed in each well and after a few minutes, the presence of albumin and hemoglobin are indicated and a diagnosis can be made on the spot.

Antibody Testing Methodology

To test and incrementally improve the functionality of the immunoassay kit, from 2007 to 2011, we conducted four additional necroscopic studies at Canadian abattoirs. These tests were carried out using a protocol similar to the original guaiac studies: upon euthanasia, fecal balls were collected and tested, and these results were then correlated to the visual observations of the horse GI tract. These studies were run blind; the grading of the tests was independent of the bowel dissection. The results of the antibody tests were correlated to the anatomical observations to check their accuracy and positive predictive value.

For hemoglobin, the antibody test was correlated to the overall level of observed GI ulceration, where the positive gastric and colonic cutoff was set to grade 2 and above (where the ulcer is producing whole blood and, thus, hemoglobin is likely to be present). In our tests, the kits improved in accuracy over time, going from about 75% to over 90%. Sensitivity was always good, as antibodies can be made highly responsive. The positive predictive value, which is an indicator of how likely a horse is to have an ulcer given a positive result, has always been good, rising with each test kit formulation from around 80% to well over 90%.

For albumin, the antibody test was correlated exclusively to the level of observed colonic ulceration, where the cutoff was set to grade 1 and above (where albumin is likely to be present). As with the hemoglobin tests, these have continually improved in accuracy as we have better understood the normal baseline rate of albumin loss in the hindgut.

Two statistical measures remain low – negative predictive value (75% for Albumin and 57.9% for Hemoglobin) and specificity (27.3% for Albumin). The negative predictive value is an indicator of how likely a horse is clear of ulcers given a negative test result. In this case, the specificity is affected by the very low rate of ulcer-free animals, which makes the correlation rare and difficult. Specificity, which indicates the percent of true negatives correctly identified, is measured as: true negatives / true negatives + false positives. Again, the low number of true negatives certainly affects the value of this measure. Further, it is possible that some false positives may have been true positives simply overlooked during necropsy examination. (In vitro studies indicate that the FBT replicates precisely.)

Due to the nature of antibody chemistry, the test is generally very sensitive, leading to a low number of false positives. That has led to good correlations, especially on metrics such as accuracy, specificity and positive predictive value.

SCHEMATIC REPRESENTATION OF ANTIBODY UPPER LIMITS

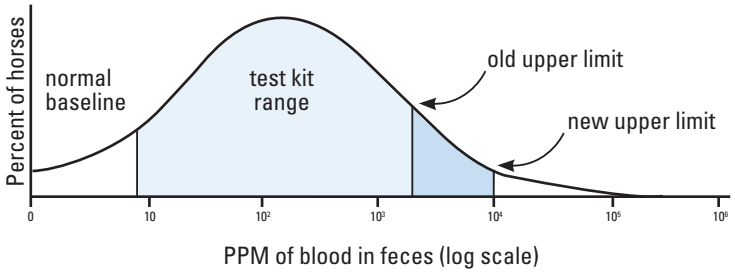


Figure 8: The test kit requires a lower bound, above baseline bleeding, to prevent false positives. But the upper bound is often constrained by a combination of lateral flow physics and biochemical binding that can flood the kit and may lead to false negatives. Our goal has been to extend the upper boundary without affecting the low-level sensitivity of the original test. Note that this graph is logarithmic and that capturing more of the flooding region requires a many-fold improvement in range.

The lower level of sensitivity is adjusted to three times the normal baseline level – that is, the level of blood present in the feces of normal, healthy horses which does not represent a pathological condition. This correlates well with our observations. However, in those cases where there is frank blood or large lesions, we have noted that antibody test kits can "flood" or exceed the upper range of antibody sensitivity, producing false negatives. This has become the central focus of our attempts to refine the test. With each iteration of the test, we have been able to extend the upper range of sensitivity to better capture the blood loss levels associated with the entire array of ulceration and related conditions. As such, the new test kit is unlikely to be flooded in normal use.

While the antibody test is reliable and precise, it is important to remember that horses themselves are somewhat variable. Normal horse blood can have from 11-19 g/dL of hemoglobin and 2.4-4.2 g/dL of albumin, and variations in fecal output can also affect blood concentrations. These measures may change over time for any given subject, so blood volumes cannot be rigorously computed from a single measurement. As a result, repeated testing over an extended period can help to build a better picture of actual blood loss.

Discussion

The latest iteration of our antibody test kit, called the SUCCEED® Equine Fecal Blood Test™ (FBT), is in the form of a two-part wicking rapid-test specific to equine blood proteins. There are two wells in the kit, one to detect albumin and one for hemoglobin. Against a fecal background, the sensitivity of the test is 8 parts per million (ppm) for albumin and 8 ppm for hemoglobin, based on whole blood equivalents. The upper limit for both is approximately 10,000 ppm, with the albumin antibodies likely exceeding this, and the hemoglobin antibodies likely falling just short of this level.

The results of these new versions of the antibody tests were compared to 178 anatomical dissections to check their predictive values. For albumin, the antibody test was correlated exclusively to the level of colonic ulceration, where the cutoff was set to grade 1 and above:

Albumin as an indicator of colonic ulcers grade 1 or worse, N=178		
test	Ulcers >=1	Ulcers < 1
positive	TP=166	FP=8
negative	FN=1	TN=3
	accuracy:	94.9%
	sensitivity:	99.4%
	specificity:	27.3%
	predictive val pos:	95.5%
	predictive val neg:	75.0%
	p-value:	.045

Note the high levels of accuracy and sensitivity for the albumin component, as well as a good statistical significance (p = .045).

For hemoglobin, the test correlated well to the overall level of observed GI ulceration when the positive gastric and colonic cutoff was set to grade 2 and above.

Hemoglobin as an indicator of any ulcer, grade 2 or worse, N=178		
test	Ulcers >=2	Ulcers < 2
positive	TP=154	FP=5
negative	FN=8	TN=11
	accuracy:	92.7%
	sensitivity:	95.1%
	specificity:	68.8%
	predictive val pos:	96.9%
	predictive val neg:	57.9%
	p-value:	.028

Again, note the high levels of accuracy and sensitivity and the significant p-value of .028.

The SUCCEED FBT lets you conduct a simple test on a horse stall-side, without invasive or costly diagnostic procedures or referrals. The test provides a quick objective measure of possible foregut and hindgut lesions and related conditions without relying solely on symptomology and other subjective inputs. The two-part diagnostic can be performed in the barn in a few minutes with no extra equipment – a fecal sample and approximately 3 oz. of clean tap water, along with the contents of a single kit, are all that is required to test one horse. The results are easy to read directly from the window of the rapid-test kit, and appear in minutes.

Because the test can be performed in minutes, it is possible to test a number of horses in a barn or other boarding environment in the course of a typical client visit. Given the ease and affordability of the SUCCEED FBT, practitioners can easily test all of their clients' horses on a consistent schedule. Regular testing is especially important for performance horses, or whenever the care, feeding and general husbandry are less than ideal for digestive health, including intermittent feeding, high-grain diets, stall confinement, trailering, etc.

Other Applications

As well as the detection of ulcers, the test may also be useful to rule in or rule out Protein Losing Enteropathy (PLE). The albumin part of the test is a sensitive indicator of albumin loss, set high enough to ignore normal baseline levels.

The presence of hypoproteinemia and/or hypoalbuminemia on a CBC/chem profile means the horse may be losing proteins, mainly albumin. A common cause is PLE, which the FBT results can buttress if the horse tests positive for albumin. A negative result might indicate a protein losing nephropathy (PLN), which may be verified by running an FBT (Test A) on a urine sample from the subject horse. A positive result verifies this. (Practitioners should be aware that a negative result may reflect a level of albumin in the urine below the lower detection limit of the test.)

The test can also be used with the horse's history to examine possible consequences of NSAID usage and possible colonic lesions in the right dorsal quadrant.

Testing a client's horses, especially those assumed to be in good digestive health, can provide an opportunity for the veterinarian to educate their clients about these hidden GI issues. It can help you to provide a proper physiological context for many of the performance or behavioral issues horse owners and trainers face regularly, but which are often attributed to poor training or the horse's individual attitude or ability.

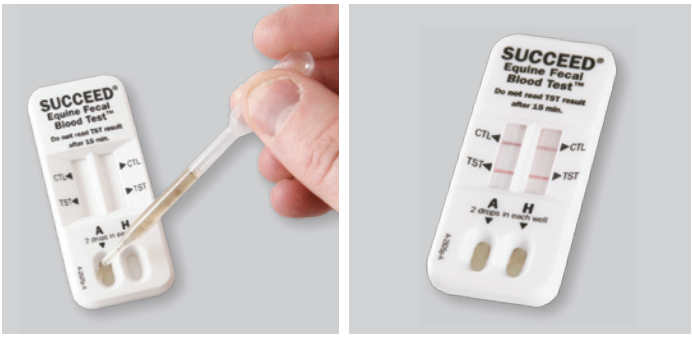


Figure 9: The SUCCEED Equine Fecal Blood Test requires a fecal sample from the subject horse and about 3 oz. of clean tap water. Results are visible within 15 minutes.

SUCCEED®
Equine Fecal Blood Test™

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