

## Measurement of Carbonic Anhydrase I and II Isozymes in Feces as a Marker of Occult Blood in Horses with Intestinal Tract Bleeding

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*Although endoscopy is the definitive diagnostic method for the detection of colonic ulcers, the equipment required for performing the test is costly and difficult to use. Therefore, a simple cost-effective and reliable screening test for intestinal tract bleeding is needed. To this end, we measured carbonic anhydrase isozymes (CA-I and CA-II) originating from erythrocytes by ELISA in order to determine if they could be used as markers of occult blood in feces. For fecal extract preparation, 2 g of feces were mixed with 4 ml of 0.01 M Tris-HCl (pH 8.0) containing 0.01% thimerosal. The concentrations of CA-I and CA-II in the fecal samples of 13 clinically normal racehorses were found to be  $30.0 \pm 10.0$  and  $34.0 \pm 13.0$  ng/ml, respectively. Increased concentrations of CA-I were detected in the fecal samples of 5 horses after blood administration; however, no increase was observed in CA-II. The concentrations of CA-I and CA-II in the fecal samples of 88 racehorses with clinical signs of equine gastric ulcer syndrome (EGUS) were  $115.3 \pm 79.0$  and  $41.0 \pm 42.0$  ng/ml, respectively. Thus, our results indicate that CA isozymes can be useful as markers of occult blood in the fecal samples of horses with intestinal tract bleeding.*

**Key words:** carbonic anhydrase isozymes, ELISA, equine gastric ulcer syndrome, occult blood test

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Performance horses are at particularly high risk of developing equine gastric ulcer syndrome (EGUS). Endoscopic examination has shown that up to 100% of racehorses and 58% of other performance horses may have gastric ulcers [7, 9]. EGUS diagnosis is based on history, clinical signs, endoscopic examination, and response to treatment, but the only accurate method currently available for detecting

and monitoring EGUS is endoscopy, a technique which can determine the location and severity of the lesions, the type and duration of treatment required, and the response to treatment [8].

Endoscopes are costly, difficult to use in the field, and unsuitable for screening large populations. In addition, it is difficult to detect lesions in all parts of the intestinal tract. Pellegrini [13] reported that almost all performance horses studied had signs of gastrointestinal ulcers and that at least 60% had colonic ulcers. Therefore, a simple cost-effective and accurate method for detecting intestinal tract bleeding in horses is needed [12]. Currently, only a few studies have been performed on the use of hematological or biochemical markers for diagnosing gastric ulcers in horses [4, 13, 15].

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The objective of this study was to investigate whether the levels of carbonic anhydrase (CA) isozymes in fecal samples are useful as markers of occult blood in horses with alimentary canal bleeding. Equine erythrocytes contain 2 CA isozymes, CA-I and CA-II [3, 10], and only CA-II is found in equine digestive tracts [14]. Here, we determined the concentrations of CA-I and CA-II in fecal samples of horses using enzyme-linked immunosorbent assay (ELISA), and evaluated their usefulness as markers of the presence of occult intestinal blood.

## Materials and Methods

All experiments were performed and the animals were treated according to the guidelines of the Laboratory Animal Care Committee of Azabu University, Japan, and programs that are accredited by the Office of Laboratory Animal Welfare (OLAW) USA (#A5393-01) were used.

### *Equine feces samples used for the measurement of CA-I and CA-II*

Three groups of horses were analyzed for fecal content of CA-I and CA-II: 13 clinically normal horses (negative controls); 5 horses given blood orally (positive controls); and 88 horses with clinical signs of EGUS.

### *Negative controls*

Fecal samples from 13 clinically normal Thoroughbred horses (6–20 years old; four males and nine females) were used as negative controls. These horses had not expressed any clinical signs of EGUS for at least 1 month.

### *Positive controls*

As positive controls, five clinically normal Thoroughbred horses (6–20 years old; two males and three females) were subjected to an experimental model of digestive tract bleeding. They were fed 450 g of hay cubes containing 200 ml of equine blood and 3.8% citric acid (Nippon Biotest Laboratories Inc., Tokyo, Japan) before being provided fodder in the evening. Two grams of feces was collected every 3 hr for a total of 54 hr after blood ingestion, and the samples were frozen at  $-20^{\circ}\text{C}$  until use.

### *Samples from 88 horses with clinical signs of EGUS*

In addition to the experimentally derived samples, single clinical fecal samples were obtained from 88 racehorses (4–6 years old). These horses showed clinical signs of EGUS such as inappetence, intermittent colic, lethargy, and suboptimal athletic performance.

### *Preparation of fecal extracts*

For fecal extract preparation, 2 g of feces were mixed

with 4 ml of 0.01 M Tris-HCl (pH 8.0) containing 0.01% thimerosal and shaken for 30 min. The samples were then centrifuged at  $16,000 \times g$  for 40 min at  $4^{\circ}\text{C}$ , and the supernatant was stored at  $-20^{\circ}\text{C}$  until use.

### *Optimization of the ELISA assay*

The specificity of the ELISA for equine CA-I and CA-II in the feces was established by three sets of experiments, that is, the recovery test, the interassay reproducibility test, and the dilution test. Recovery of 200 ng/ml of CA-I or 200 ng/ml of CA-II added to fecal samples was tested using fecal samples that had been obtained from a healthy horse, horse 1, and contained 20 ng/ml of CA-I and 10 ng/ml of CA-II. The coefficients of variation for the interassay reproducibility of the ELISA for CA-I and CA-II in the fecal samples from horse 1 were measured. To determine whether some fecal components interfered with the assay, several volumes of fecal samples (0.005 to 0.1 ml) were assayed for CA-I and CA-II, with the total volume being made up to 0.1 ml with 0.05 M Tris-HCl (pH 7.5) containing 0.3% BSA, 0.9% NaCl, 0.01% thimerosal, and 0.01 M EDTA (buffer A).

### *CA isozyme antigenicity*

To determine fecal changes in CA isozyme antigenicity, 2 g of feces from 5 other clinically normal Thoroughbred horses were incubated with 1 ml of equine blood containing 3.8% citric acid and were shaken at  $36^{\circ}\text{C}$  for 36 hr. CA isozymes were extracted at 6, 12, 24 and 36 hr, as described above.

### *Electrophoretic procedures and western blotting*

Electrophoresis and western blotting were performed as previously described [11].

Briefly, adequate volumes of purified equine CA-I and feces of horses with EGUS were separated using PhastSystem (Pharmacia Biotech, Uppsala, Sweden) and were transferred to Immobilon PVDF transfer membranes (Millipore Corp, Bedford, MA, USA). CA-I and CA-II were detected using rabbit antiserum to equine CA-I or CA-II; this antiserum had been previously produced in our laboratory [10]. After the membranes were incubated with the primary antibody, they were incubated with peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The membranes were washed again with PBS-Tween and then incubated for approximately 5 min in 0.05 M Tris-HCl (pH 7.6) containing 0.02%  $\text{H}_2\text{O}_2$  along with 0.2 mM 3,3'-diaminobenzidine-tetrahydrochloride (DAB-4HCl).

### *Standard procedure for CA-I and CA-II ELISAs of horse fecal samples*

The competitive ELISA method for equine CA-I and CA-II was previously established in our laboratory [10].

Briefly, each well of a flat-bottom micro-ELISA plate was coated with 0.1 ml of anti-CA-I or CA-II IgG dissolved in 0.1 M NaHCO<sub>3</sub> (pH 8.5) and left to stand for 16 hr at 4°C. The plates were then washed 3 times with 0.4 ml of 0.15 M phosphate-buffered saline (0.9% NaCl) solution containing 0.05% Tween 20 (PBS-Tween). After incubation at 23°C for 10 min with SuperBlock® Blocking Buffers (Thermo Fisher Scientific Inc., IL, USA), each well was washed 3 times. Purified CA-I or CA-II for standard (5–800 ng/ml) or fecal samples were diluted with buffer A, and subjected to ELISA, which was performed in duplicate. At the same time, 0.05 ml of biotinylated CA isozymes diluted with buffer A were added. After incubation at 4°C for 16 hr, 0.1 ml/well of avidin and biotinylated horseradish peroxidase complex (ABC reagent, Wako Pure Chemical Industries, Tokyo, Japan) were added. The peroxidase activity in the wells was measured after addition of 0.1 ml of ABTS peroxidase substrate (Kirkegaard & Perry Laboratories). After 10 min, 1% sodium dodecyl sulfate was added to terminate the enzyme reaction, and the absorbance at 405 nm was measured on an automatic ELISA reader (SH-1000; Corona Electric, Ibaraki, Japan).

#### *Measurement of fecal hemoglobin*

The hemoglobin (Hb) concentration in the fecal samples of horses was measured by the sodium lauryl sulfate-hemoglobin method (SLS) using a hemoglobin B test (Wako Pure Chemical Industries Ltd., Tokyo, Japan) and was compared to the concentrations of CA isozymes. Horse Hb was used as a standard (25–300 mg/ml). The hemoglobin B test was carried out according to the manufacturer's instructions.

#### *Statistical analysis*

Values are expressed as mean ± SD values. Statistical differences in the levels of CA-I in the feces of clinically healthy horses and horses with clinical signs of EGUS were analyzed using Student's *t*-test. *P* values of <0.01 were considered significant.

## **Results**

#### *Optimization of the ELISA assay*

Assay precision had been previously evaluated with 9 standard samples that were each assayed five times in one assay. The coefficients of variation for each assay were less than 5% [10]. When 200 ng/ml of CA-I or 200 ng/ml of CA-II were added, the average recovery rates were 96.1 and 97.1%, respectively. The coefficients of variation for the interassay reproducibility of the ELISA for CA-I and CA-II in the fecal samples were 6.8 and 6.2%, respectively (Table 1). When more than 0.04 ml of fecal sample were

used in the assay (more than 40% of the reaction mixture), CA isozyme levels that were slightly higher than expected were obtained (Fig. 1). Therefore, in the present study, fecal samples were diluted to 5 times with buffer A in order to measure the CA isozymes by ELISA.

#### *Antigenicity of CA isozymes in the feces*

The changes in the concentrations of equine CA-I and CA-II in the fecal samples incubated with blood at 36°C are shown in Fig. 2. The concentrations of CA-I and CA-II decreased from 100 to 88 and to 20% at 36 hr, respectively.

#### *Western blotting analysis*

A single band was obtained on the western blot probed with anti-equine CA-I serum from the fecal extracts of horses with EGUS and purified equine CA-I (Fig. 3A). Figure 3B shows a single band on the western blot probed with anti equine CA-II serum from the fecal extract from a horse with EGUS and purified equine CA-II. The molecular weight of CA-I and CA-II in the fecal extract from the horse with EGUS was similar to that of the purified equine CA-I and CA-II and was approximately 28,000.

#### *Concentrations of CA isozymes in feces as markers of occult blood*

The concentrations of CA-I and CA-II in the fecal samples of the 13 clinically normal horses were 30.0 ± 10.0 and 34.0 ± 13.0 ng/ml, respectively. Concentrations of CA-I, which slightly increased with time, were detected in the fecal samples of the 5 horses that had received 200 ml of blood orally with hay cubes but, the CA-II level did not increase (Fig. 4). The levels of CA-I and CA-II in the feces of the 5 horses before the experiment were 13.0 ± 3.0 and 3.0 ± 4.0 ng/ml, respectively.

The concentrations of CA-I and CA-II in the fecal samples of the 88 racehorses with clinical signs of EGUS were 115.3 ± 79.0 and 41.0 ± 42.0 ng/ml, respectively. Notably, the concentrations of CA-I in the fecal samples taken from racehorses with clinical signs of EGUS were 4.3 times higher than those of clinically healthy horses (*P*<0.001) (Fig. 5).

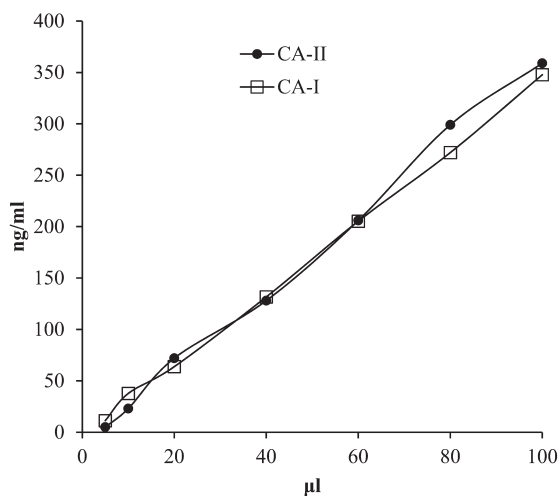
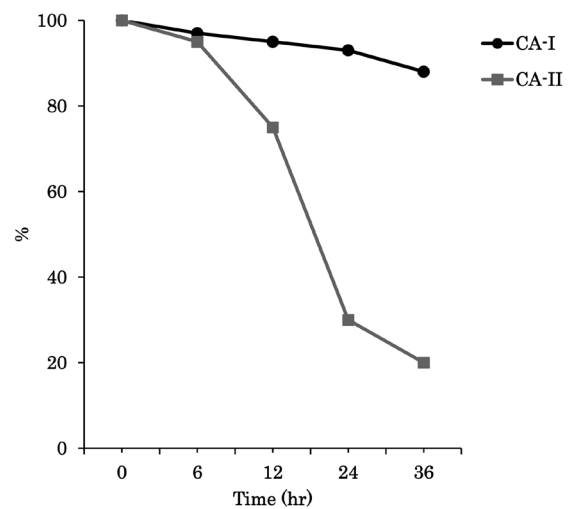
#### *Fecal hemoglobin*

Increased concentrations of Hb were not detected in the fecal samples from the horses that had received 200 ml of blood. The average level of Hb in these horses was 3.0 ± 0.6 mg/ml. The concentrations of Hb in the fecal samples incubated with 1 ml of blood at 36°C were 17.3 ± 2.5 mg/ml (100%) at 0 hr, 16.5 ± 0.8 mg/ml (95.6%) at 6 hr, 16.4 ± 0.9 mg/ml (94.7%) at 12 hr, 16.9 ± 1.2 mg/ml (97.9%) at 24 hr, and 17.1 ± 1.8 mg/ml (99.0%) at 36 hr after the start of the experiment.

**Table 1.** Inter-assay reproducibility and recovery of CA-I and CA-II ELISAs using equine fecal samples

Assay day	CA-I (ng/ml)				CA-II (ng/ml)			
	Mean*	SD	CV (%)	Recovery (%)	Mean*	SD	CV (%)	Recovery (%)
1	189.2	6.4	3.4	94.6	183.4	13.2	7.2	91.7
2	203.2	3.9	1.9	101.6	191.6	12.4	6.5	95.8
3	180.3	10.1	5.6	90.2	193.8	10.9	5.6	96.9
4	209.5	13.3	6.3	104.8	215.5	7.6	3.5	107.8
5	194.9	20.7	10.6	97.5	198.5	14.6	7.4	99.3
6	175.7	7	4	87.9	182.8	14.7	8	91.4
Mean	192.1	13	6.8	96.1	194.3	12	6.2	97.1

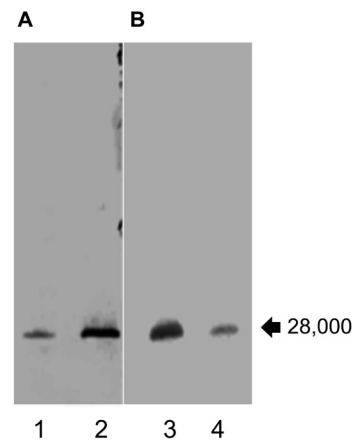
\*: Mean of 5 determinations. CV: Coefficient of variation.

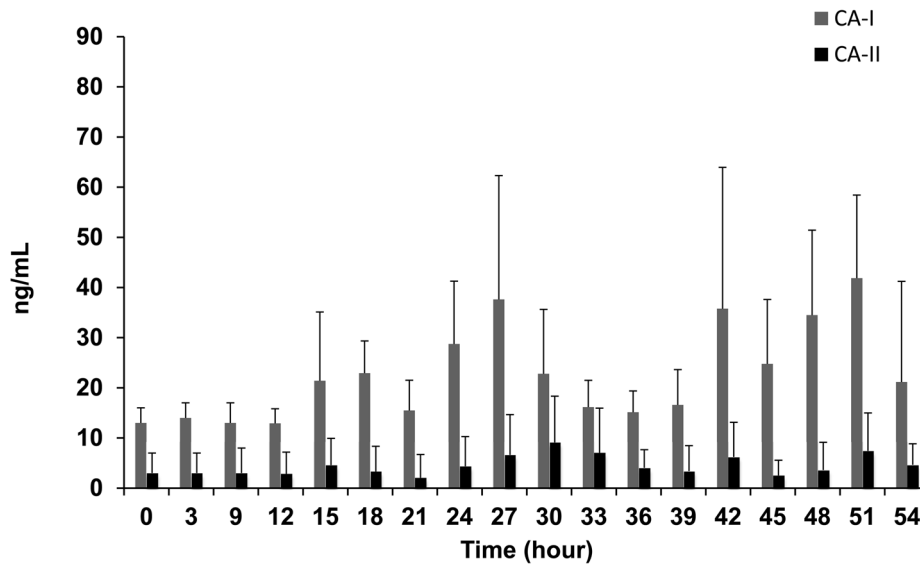
**Fig. 1.** Effect of fecal sample dilution on CA-I and CA-II ELISAs.**Fig. 2.** Changes in the antigenicity of equine CA-I and CA-II in the fecal samples incubated with blood at 36°C for 36 hr.

The concentration of Hb in the fecal samples from the negative control horses (13 samples) and horses with clinical signs of EGUS (88 samples) were  $1.6 \pm 1.7$  and  $3.0 \pm 1.9$  mg/ml, respectively.

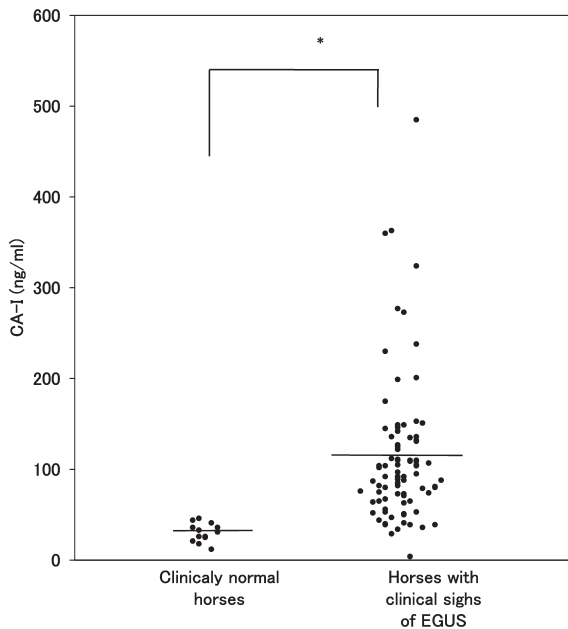
## Discussion

In human medicine, chemical and immunological tests for occult blood are currently employed in early screening for hemorrhagic diseases associated with intestinal cancer [1, 6]. Immunological tests for occult blood are specific for Hb derived from erythrocytes [15]. A few reports have described the use of immunological tests for Hb in fecal occult blood in dogs [5]. To our knowledge, few studies have been published on immunochemical measurement of equine Hb in fecal samples. Pellegrini [13] reported that the guaiac-based fecal occult blood test (gFOBT) for equine gastric and colonic ulceration has high specificity but fairly low sensitivity. In this study, the immunological measure-

**Fig. 3.** Western blotting after SDS-PAGE. CA-I and CA-II were detected by use of anti-equine CA-I (A) and anti-equine CA-II (B), respectively. Lane 1, Feces of horse with EGUS; Lane 2, Purified equine CA-I; Lane 3, Feces of horse with EGUS; Lane 4, Purified equine CA-II.



**Fig. 4.** Changes in CA-I and CA-II concentrations in the fecal samples of 5 clinically normal horses after oral equine blood administration.



**Fig. 5.** Concentrations of CA-I in the fecal samples of 13 clinically normal horses and 88 horses with clinical signs of EGUS. The solid lines represent the median levels of each group. \*:  $P < 0.01$ .

of the ELISA for CA isozymes in the fecal samples in the current study was 100 ng of CA-I/g of feces. The ELISA assay for CA-I and CA-II in fecal samples has sufficient sensitivity and satisfactory precision.

The concentrations of CA-I and CA-II in the erythrocytes taken from healthy racehorses were  $1.7 \pm 0.5$  and  $1.0 \pm 0.1$  mg/g Hb, respectively [10]. The CA-I/CA-II ratio in the erythrocytes was 1.8. However, the CA-I/CA-II ratio in the feces of horses with EGUS was approximately 2.0–11.2. Furthermore, CA-II was almost undetectable in the feces samples obtained from the horses administered blood infusions. In the antigenicity test, the levels of equine CA-I in the feces of the horses decreased to 88% at 36 hr after the start of the experiment, whereas the levels of equine CA-II in the feces decreased to 20% at 36 hr. These results suggest that the CA-II in the feces, which passed through the gastrointestinal tract, is strongly influenced by digestion or resolution due to digestive juices and intestinal bacteria. The molecular weight of CA-I and CA-II in the feces of horses with EGUS was similar to that of purified CA isozymes. We consider that the difference in the antigenicity of CA isozymes is dependent on the difference in the molecular structure. The antigenicity of CA isozymes was not completely lost for 36 hr. These results prove that CA isozymes in equine fecal samples can be detected using an immunochemical method.

ment of CA-I and CA-II was found to be more sensitive than the SLS method. The sensitivity of the gFOBT [13] and the SLS method in measuring Hb in the fecal samples of the present study was 10 mg of Hb/g of feces. The sensitivity

Increasing levels of CA-I were detected in feces at 32 and 37 hr after the start of blood ingestion, whereas, slightly increased levels of CA-I were detected in feces at 27 and 51 hr after the horses were given blood with hay cubes.

Immunohistochemical studies have shown the presence of CA-II in the equine digestive tract, whereas the presence of CA-I has not been demonstrated [14]. CA-II is localized in surface epithelial and parietal cells in the glandular region of the stomach, in absorptive duodenal columnar cells covering the villi, and in columnar cells in the upper part of the colonic crypts. Although CA-I has not been found in the intestinal tract by immunohistochemistry, the concentrations of CA-I in the fecal samples of clinically normal horses (negative controls) were approximately 30 ng/ml. The source of this fecal CA-I is unknown.

Pellegrini [13] reported that almost all the horses examined had signs of intestinal ulcers and that at least 60% had colonic ulcers. Furthermore, physiological bleeding, as reported in humans [2], and exercise-induced pulmonary hemorrhage should be carefully considered in the clinical application of a CA isozyme assay for fecal occult blood in horses.

In the present study, the concentrations of CA-I in the fecal samples taken from racehorses with clinical signs of EGUS were 4.3 times higher than those of clinically healthy horses. The results of this study indicate that CA isozyme concentrations determined by ELISA can be useful as markers of occult blood in the fecal samples of horses with intestinal tract bleeding. Based on the present preliminary data, it is apparent that additional studies should be conducted to clarify the clinical diagnostic value of measuring CA isozyme concentrations in equine fecal samples.

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