

Characterization of equine vitamin D-binding protein, development of an assay, and assessment of plasma concentrations of the protein in healthy horses and horses with gastrointestinal disease

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OBJECTIVE

To purify and characterize equine vitamin D-binding protein (VDBP) from equine serum and to evaluate plasma concentrations of VDBP in healthy horses and horses with gastrointestinal injury or disease.

ANIMALS

13 healthy laboratory animals (8 mice and 5 rabbits), 61 healthy horses, 12 horses with experimentally induced intestinal ischemia and reperfusion (IR), and 59 horses with acute gastrointestinal diseases.

PROCEDURES

VDBP was purified from serum of 2 healthy horses, and recombinant equine VDBP was obtained through a commercial service. Equine VDBP was characterized by mass spectrometry. Monoclonal and polyclonal antibodies were raised against equine VDBP, and a rocket immunoelectrophoresis assay for equine VDBP was established. Plasma samples from 61 healthy horses were used to establish working VDBP reference values for study purposes. Plasma VDBP concentrations were assessed at predetermined time points in horses with IR and in horses with naturally occurring gastrointestinal diseases.

RESULTS

The working reference range for plasma VDBP concentration in healthy horses was 531 to 1,382 mg/L. Plasma VDBP concentrations were significantly decreased after 1 hour of ischemia in horses with IR, compared with values prior to induction of ischemia, and were significantly lower in horses with naturally occurring gastrointestinal diseases with a colic duration of < 12 hours than in healthy horses.

CONCLUSIONS AND CLINICAL RELEVANCE

Plasma VDBP concentrations were significantly decreased in horses with acute gastrointestinal injury or disease. Further studies and the development of a clinically relevant assay are needed to establish the reliability of VDBP as a diagnostic and prognostic marker in horses. (*Am J Vet Res* 2017;78:718–728)

Vitamin D-binding protein is part of the extracellular actin scavenging system that facilitates clearance of actin from the circulation.¹ Actin is an intracellular structural protein that is released into the circulation after cell injury and cell death. Circulating actin forms filaments that cause endothelial injury and microthrombi.^{2,3} Thus, high circulating concentrations of free actin are potentially lethal.^{2,4} The VDBP has a strong binding capacity for actin^{5,6}

and facilitates its clearance by the reticuloendothelial system in the liver,^{1,7} thereby potentially decreasing the risk of hypercoagulation, shock, and death.⁸ In people with severe physical trauma⁹ or sepsis¹⁰ and in rats with experimentally induced endotoxemia,¹¹ circulating VDBP concentrations were decreased $\leq$ 60 minutes after the event, and changes in serum concentrations of total and complexed VDBP were found to be indicative of acute tissue injury in human surgical patients.¹² In studies^{13,14} of people with trauma and liver failure, serum VDBP concentrations were also significantly lower in nonsurvivors than in survivors. Vitamin D-binding protein is mainly produced in the liver,¹⁵ and its production is stimulated by interleukin 6 and dexamethasone, and decreased by transforming growth factor β .¹⁵ Following an initial decrease, serum VDBP concentrations were increased by 35%, compared with baseline (preop-

ABBREVIATIONS

IR	Ischemia and reperfusion
mAb	Monoclonal antibody
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information
RaeqVDBP	Rabbit anti-equine vitamin D-binding protein
RahuVDBP	Rabbit anti-human vitamin D-binding protein
RIE	Rocket immunoelectrophoresis
VDBP	Vitamin D-binding protein

erative) values in human patients with major surgical trauma, rendering this protein to be classified as an acute phase protein.¹²

Vitamin D-binding protein is also present in horses^{16–18} and has a strong affinity to actin in this species.¹⁹ To the authors' knowledge, equine VDBP has not yet been characterized, and no data have been published on VDBP concentrations in healthy horses or horses with gastrointestinal injury. Horses with severe intestinal ischemia and inflammation are often susceptible to endotoxic shock and hypercoagulability.^{20–22} In a study²³ of ponies with experimentally induced small intestinal ischemia, hypercoagulation was evident within 15 to 18 hours after ischemia, followed by a fatal outcome after approximately 24 hours. Because of the short time frame between the onset of the ischemic lesions, development of a hypercoagulable state, and resulting death, fast-response biomarkers are required to assist in clinical decision-making for affected horses at the time of hospital admission.

The objective of the study reported here was to characterize equine VDBP, to develop an equine VDBP assay and establish a set of working reference values in healthy horses for its use, and to evaluate VDBP concentrations in horses with acute gastrointestinal diseases. We hypothesized that circulating VDBP concentrations would decrease in horses following brief experimentally induced intestinal IR, and that concentrations of the protein would be lower in horses with naturally occurring acute gastrointestinal diseases than in healthy control horses.

Materials and Methods

All animal experiments were performed in accordance with current ethical guidelines (Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals used for Scientific Purposes) and with permission (No. 2013-15-2934-00988) from the Danish Animal Experiments Inspectorate. Ethical approval for blood sample collection and surgical procedures on horses was further given by the University of Florida Institutional Animal Care and Use Committee and the University of Copenhagen Large Animal Teaching Hospital Ethical Committee. All data and samples from client-owned horses were obtained with the owners' permission as part of routine diagnostic evaluations.

Purification of native equine VDBP

Horse serum—Blood was collected from 2 healthy adult Standardbred mares for purification of VDBP from serum. The horses were 8 and 9 years of age and weighed 545 and 596 kg, respectively. The horses were owned by University of Copenhagen Large Animal Teaching Hospital, were kept under optimal housing conditions, and were regularly vaccinated and dewormed. Before collection of blood, both horses underwent a thorough clinical examination to rule out any evidence of inflammation or disease. A total volume of 4 L of blood (2 L/horse) was collected

aseptically via a temporary jugular venous catheter into 1-L sterile glass bottles without additives. After coagulation and sedimentation of RBCs by gravity, supernatant was collected, transferred into 100-mL sterile plastic bottles, and centrifuged at 2,000 X g for 10 minutes. The serum was collected and stored at -20°C in 50-mL plastic tubes until further processing (≤ 1 month later).

Ammonium sulfate fractionation—Ammonium sulfate^a was added to horse serum to a final concentration of 1.5M, and the mixture was stirred overnight at 5°C. After centrifugation at 16,300 X g for 1 hour at 5°C, the supernatant was dialyzed against three 10-L changes of 20mM tris buffer^b (pH, 8.0) to yield 2.4 L of dialysate.

Ion exchange chromatography—All chromatography procedures were performed by use of a liquid chromatography system.^c A flow rate of 1 mL/min was used as described by Jørgensen et al.²⁴

Chromatography with a strong anion exchange cross-linked agarose medium was performed on a 2.6 X 20-cm column,^d which was equilibrated in 20mM tris buffer^b (pH, 8.0) and eluted with a linear gradient of 0 to 0.5M NaCl in the same buffer. Dialysate (1.2 L) from ammonium sulfate fractionation was loaded, and fractions of 5 mL were collected over a total of 6 column elution volumes and analyzed by SDS-PAGE, immunoblotting, and ELISA to identify fractions containing VDBP of adequate purity. These fractions were dialyzed against 20mM sodium acetate (pH, 4.5) and concentrated to a volume of 9 mL by use of a spin filtration column with a 30-kDa cutoff.^e

Chromatography with a strong cation exchange cross-linked agarose medium was performed by loading the dialyzed fractions from the strong anion exchange cross-linked agarose medium chromatography on a 1-mL column^f equilibrated in 20mM sodium acetate buffer (pH, 4.5), and elution was performed with a linear gradient from 0 to 1M NaCl in the same buffer. Fractions of 1 mL were collected over a total of 6 column elution volumes and analyzed by SDS-PAGE, immunoblotting, and ELISA for VDBP. Fractions of adequate purity were pooled and concentrated by use of a spin filtration column^e as previously described.

Production of recombinant equine VDBP

Owing to the small amount of native equine VDBP obtained with the described purification procedure, recombinant equine VDBP was produced for subsequent production of antibodies. The recombinant equine VDBP (NCBI sequence, XP_001489400) was expressed in *Escherichia coli* by a commercial source through use of proprietary technology^g involving gene synthesis, subcloning of the gene in expression vector E3, and induction of protein expression by isopropyl-β-D-thiogalactopyranoside. The recombinant equine VDBP was subsequently puri-

fied from inclusion bodies, yielding 3.2 mg of purified protein (0.8 mg/mL, determined by the Bradford method). The purity and identity of the protein was subsequently analyzed by SDS-PAGE and MS.

Production of antibodies

mAbs—Native equine VDBP was eluted from SDS-PAGE gel pieces with 10-fold (vol:vol) 1% SDS in PBS solution. The protein was mixed 1:1 (vol:vol) with an aluminum hydroxide gel adjuvant,^h prepared for injection by addition of thiomersal to 0.05%, and administered SC to 4 apparently healthy male Balb/c mice.ⁱ Each mouse was injected SC 6 times with 0.1 mL of the prepared vaccine at 14-day intervals. Blood samples (0.1 mL) were collected from each mouse every second week by venipuncture of the superficial temporal or submandibularis vein and tested for antibodies against equine VDBP by ELISA. A suitable mouse with a high titer of antibodies was selected for mAb production and received an additional injection of 0.1 mL of the vaccine IP. Mice were routinely monitored for adverse reactions and euthanized with CO₂ for collection of blood for antibody extraction at the end of the treatments. Serum was collected after blood coagulation and centrifugation.²⁵ After euthanasia, excision and gentle homogenization of the spleen for isolation of antibody-producing B cells and fusion and cloning of single antibody-secreting hybridoma cells by conventional technology,²⁶ a mAb (HYB 370-01) was obtained from the mouse that received the additional injection.

Another 4 healthy male Balb/c miceⁱ were injected 6 times SC at 14-day intervals with 10 µg of recombinant equine VDBP (prepared by mixing VDBP [0.1 mg/mL] 1:1 [vol:vol] with aluminum hydroxide gel adjuvant,^h and addition of thiomersal to 0.05%), resulting in 4 mAbs (HYB 378-01, HYB 378-02, HYB 378-03, and HYB 378-04) raised and collected in the same manner described for antibodies against the native protein. The antibodies raised against recombinant equine VDBP were used to verify the presence of VDBP in fractions during purification.

Polyclonal antibodies—Two apparently healthy male New Zealand White rabbits^j (body weight, approx 5 kg each) were injected SC with 25 µg of recombinant equine VDBP, mixed 1:1 (vol:vol) with aluminum hydroxide gel adjuvant (0.5 mL total volume prepared as described for mouse injections),^h 4 times at 14-day intervals. Blood samples (20 mL) were collected by venipuncture every other week, and blood was allowed to clot for collection of serum containing polyclonal RaeqVDBP antibodies. The rabbits were routinely monitored for adverse reactions and sedated with fentanyl and midazolam for collection of blood at the end of the treatments before euthanasia with pentobarbital. Another 3 healthy male New Zealand White rabbits^j weighing approximately 5 kg each were injected 4 times with commercially available human VDBP^k (0.1 mg in PBS mixed 1:1 [vol:vol]

with aluminum hydroxide adjuvant) following the same protocol used for the equine proteins to produce polyclonal RahuVDBP.

Assessment of VDBP purity

ELISA—A qualitative ELISA was performed on fractions collected by chromatography with mAb produced against recombinant equine VDBP. Briefly, microtiter plates^l were coated overnight at 5°C with collected fractions (1:1,000), and nonspecific protein was blocked by washing 3 times for 5 minutes at room temperature (approx 20°C) with buffer containing 50mM tris (pH, 7.5), 1% polysorbate 20 (non-ionic surfactant), and 0.3M NaCl that was specifically developed to minimize nonspecific binding and avoid blocking protein.²⁷ The samples were then incubated with primary anti-recombinant equine VDBP mAb (HYB 378-04, selected on the basis of reactivity testing described in the Results section), diluted 1:1,000 in tris-polysorbate-NaCl buffer for 1 hour at room temperature, washed with the same buffer as previously described, and incubated with a secondary antibody (alkaline phosphatase-conjugated goat anti-mouse IgG,^m diluted 1:1,000 in the same buffer) for 1 hour at room temperature. The washing step was then repeated. Finally, the presence of VDBP in the fractions was visualized by color development with para-nitrophenyl phosphate (1 mg/mL in 1M diethanolamine; 0.5mM MgCl₂; pH, 9.8). The absorbance at 405 nm with background subtraction at 650 nm was read with a microplate reader.ⁿ

Assessment of antibody reactivity

All anti-equine and anti-human VDBP antisera were tested for reactivity against equine and human VDBP, human albumin, and human α₁-antitrypsin by Ouchterlony diffusion and crossed immunoelectrophoresis as described by Jørgensen et al.²⁴ Ouchterlony immunodiffusion was performed with 1% agarose^o gels in tris-tricine buffer^b (0.37M and 0.13M, respectively) with 9mM calcium lactate (pH, 8.6); antibody suspensions were placed in the center wells and samples in the outer wells. Crossed immunoelectrophoresis was also conducted in 1% agarose gels with the same buffer as described for immunodiffusion. The first dimension was performed with a current of 10 V/cm, and for the second dimension, 1% agarose gels with 110 µL RaeqVDBP antibody/22 mL gel (specific antibody concentration unknown) and a current of 3 V/cm were used for overnight incubation as described by Jørgensen et al.²⁸ Further, SDS-PAGE followed by immunoblotting was carried out as previously described by Jørgensen et al.²⁴ and Christiansen et al.²⁹ with premade 4% to 20% polyacrylamide gels.^p Gels were stained with Coomassie brilliant blue^q or silver stain.^{24,29} Immunoblots were labeled with 1 mAb against recombinant equine VDBP (HYB 378-04) or a commercially available polyclonal antibody against human VDBP^r as primary antibodies, alkaline phosphatase-conjugated sec-

ondary antibodies (goat anti-mouse^s or anti-rabbit^t IgG, respectively), and 5-bromo-4-chloro-3-indolyl phosphate with nitro blue tetrazolium as alkaline phosphatase substrates. Antibodies were diluted 1:1,000 (vol:vol).

For assessment of antibody cross-reactivities, an ELISA was carried out as previously described for purification of equine VDBP, with microtiter plates coated with recombinant equine VDBP or commercially available purified human VDBP^k (1 µg/mL in 50mM sodium carbonate buffer; pH, 9.6), respectively.

Characterization of equine VDBP by MS

To verify the protein sequence and assess for presence of potential isoforms, 3 types of VDBP were separated by gel electrophoresis and analyzed by MS. Single Coomassie brilliant blue-stained protein spots for identification were excised from an SDS-PAGE gel loaded with approximately 4 µg of each of the following samples of VDBP: purified human VDBP,²⁴ commercially available purified human VDBP,^k and recombinant equine VDBP. Excised gel sections containing proteins were washed in 50% acetonitrile in water, followed by washing in 100% acetonitrile solution. After drying and in-gel digestion with porcine sequencing grade modified trypsin,^u proteins were redissolved in 0.1% trifluoroacetic acid. Flexible gel-loading tips^v packed with poly (styrene-divinylbenzene) particle resin^w were used to wash the peptides in 0.1% trifluoroacetic acid before elution with an acetonitrile and 0.1% formic acid solution (70:30 ratio [vol:vol]). The samples were dried and redissolved in 0.1% formic acid before MS analysis. A second sample of recombinant equine VDBP was reduced with dithiothreitol, alkylated with iodoacetamide, digested with modified trypsin,^u and analyzed by MS in parallel with the 3 samples above without separation by SDS-PAGE.

The desalted peptides were loaded onto a 100-µm-inner-diameter, 18-cm reversed-phase capillary column (packed with ultrapure phase sorbent^x) in buffer A (0.1% formic acid and 5% acetonitrile), and run on a nano-liquid chromatography system.^y The peptides were eluted over 50 minutes with a gradient from 0% to 34% B-buffer (95% acetonitrile and 0.1% formic acid) at 350 nL/min flow rate and via nano-electrospray introduced into an MS system.^z

An open-source search engine^{aa} was used with an MS analysis interface^{bb} for peptide identification. The data were searched against sequences for human VDBP (NCBI Protein database accession No. P02774) or equine VDBP (NCBI Protein database accession No. XP_001489400) as applicable through use of the following search parameters: parent ion precision, 10 ppm; fragment ion precision, 0.5 Da; maximum expect value, 0.01; variable modifications, oxidation of methionine; fixed modifications, carbamidomethylation of cysteine; enzyme, trypsin; maximum number of missed cleavages, 2; and refinement, semicleavage.

RIE assay for measurement of VDBP concentrations in equine plasma

Rocket immunoelectrophoresis²⁸ was performed on 1% agarose gels with RaeqVDBP (unknown concentration; 110 µL/22 mL gel) and a current of 3 V/cm overnight. The polyclonal antibody was selected on the basis of crossed immunoelectrophoresis findings (described in Results). The recombinant equine VDBP was used to calibrate a pooled standard of serum from 5 healthy horses (25 mL each). A VDBP concentration of 1,040 mg/L relative to recombinant equine VDBP was determined by RIE, and the calibration was verified by use of purified equine VDBP. Standard dilutions of this serum (ie, a standard curve) were included twice on all gels (70, 35, 17.5, and 8.75 mg/L) along with 2 positive control samples (both at concentrations of 50 µg/mL) and study samples (from healthy horses and horses with experimentally induced intestinal IR or naturally occurring gastrointestinal disease), all diluted (1:50 [vol:vol]) in electrophoresis buffer (tris-tricine^b [0.37M and 0.13M, respectively] with 9mM calcium lactate [pH, 8.6]). Each sample was analyzed twice on 2 separate gels (5 µL/well), and the VDBP concentration was calculated by comparison with the standard curve.

Sample collection for evaluation of plasma VDBP concentrations in healthy horses and horses with gastrointestinal injury or disease

Healthy horses—Jugular venous blood was collected into 10-mL heparinized tubes from a total of 61 healthy adult horses of various breeds and both sexes as described by Pihl et al.³⁰ Plasma was stored at -80°C for ≤ 6 years (storage time of > 15 years at -20°C was shown not to influence concentrations of VDBP in human serum³¹). Horses were considered healthy on the basis of results of clinical examination, routine hematologic and serum biochemical analyses, and, in some cases, postmortem inspection. Samples were obtained from horses that were part of a teaching herd (n = 11), client-owned horses admitted for elective surgery at the University of Copenhagen Teaching Hospital for Large Animals (n = 15), and horses from an abattoir (n = 15). Blood samples (collected prior to induction of anesthesia) were also obtained from 20 horses that participated in 3 studies³²⁻³⁴ on experimentally induced intestinal IR at the University of Florida.

The horses involved in this part of the study resided in 2 climate zones (maritime climate [between latitudes 45° and 60°; n = 41] and humid subtropical [between latitudes 25° and 40°; n = 20]), and samples were collected at various times of day over a period of 1 year. The feeding and parasite control regimes were not standardized in these horses.

Horses with experimentally induced IR of intestines—As part of other studies,³²⁻³⁴ ischemia of the jejunum, colon, or both was experimentally induced in

12 horses under general anesthesia for 30 minutes or 1 hour followed by 4 hours of reperfusion as described by Graham et al (2011)³⁴ and Freeman et al (1988).³⁵ Briefly, following standard aseptic preparation and draping of the abdomen, a 25-cm ventral midline incision was performed. Venous and arterial occlusion were used to render the applicable tissues (a 40-cm segment of jejunum, starting 4 arcades proximal to the ileum; two 20-cm-long segments of colon at the pelvic flexure; or both) ischemic. When the jejunum and colon were both subjected to the experimental surgical treatment, occlusions were initiated simultaneously. Occlusion was released to allow reperfusion after the predetermined interval. The horses were euthanized while under general anesthesia by IV administration of sodium pentobarbital solution.³²

The present study included 5 horses with colonic ischemia of 60 minutes, 5 horses with colonic ischemia of 60 minutes and jejunal ischemia of 30 minutes, and 2 horses with jejunal ischemia of 60 minutes. One hour of colonic ischemia caused mild mucosal injury characterized by cellular and interstitial edema, mild hemorrhage, epithelial cell lifting, and single cell necrosis. Restitution of the epithelial cell lining was evident after 4 hours of reperfusion. The mucosal damage was similar in the jejunal segments after 30 minutes of ischemia, and epithelial cells recovered during reperfusion. In contrast, 60 minutes of jejunal ischemia caused severe irreversible mucosal damage characterized by complete loss of the villus epithelium, severe hemorrhage, and crypt cell necrosis.³⁵

Jugular venous blood samples were collected via catheters before ischemia, at the end of ischemia induction, and after 1 hour and 4 hours of reperfusion. Blood samples were collected into 10-mL heparin-containing tubes, which were centrifuged for 10 minutes at 2,000 X *g* to collect plasma. Plasma samples were stored at -80°C until analyses (≤ 6 years).

Horses with naturally occurring gastrointestinal diseases—A total of 59 adult horses with acute gastrointestinal diseases admitted to the Large Animal Teaching Hospital of the University of Copenhagen were included in the study. At admission, blood (10 mL) was collected from a jugular vein into 10-mL vacuum tubes containing heparin.^{cc} Plasma was collected and stored as described for healthy horses. All horses underwent a thorough clinical examination at admission including rectal examination, nasogastric intubation, abdominocentesis, venous blood gas analysis, hematologic and serum biochemical analysis, and fecal examination for the presence of sand or parasite eggs. A final diagnosis was established on the basis of clinical findings, laboratory data, response to medical treatment, and, when available, surgical and necropsy findings.

Only horses with acute colic (signs of moderate to severe abdominal pain), a verified diagnosis, and confirmed duration of colic were included in the

present study. For study purposes, horses were retrospectively assigned to various groups on the basis of colic duration before admission (< 12 hours vs ≥ 12 hours) and diagnosis (strangulation obstructions, simple obstructions, or enterocolitis). The diagnosis of strangulation obstruction was confirmed by either surgery or necropsy. Enterocolitis included duodenitis-proximal jejunitis and acute colitis or typhlocolitis. Duodenitis-proximal jejunitis was diagnosed in horses with excessive amounts of gastric reflux (> 24 hours' duration with a cumulative volume > 20 L) that responded to medical treatment, or when no concomitant mechanical obstructions could be identified at surgery or necropsy. Acute colitis or typhlocolitis was diagnosed at necropsy or clinically in horses with severe diarrhea on the basis of signs of dehydration, endotoxemia, and cardiovascular failure including tachycardia, hypothermia, mucous membrane discoloration, and increased capillary refill time. Horses with diarrhea that did not have signs of abdominal pain were not included in the study.

Statistical analysis

Samples from the 61 healthy horses were used to establish a set of working reference values for plasma VDBP concentration. Results of a Shapiro-Wilk test for normal distribution of the data revealed data were parametric. Ranges were calculated nonparametrically as recommended in the guidelines for establishing reference intervals³⁶ with freely available reference interval calculation software^{dd} for use with a commercial spreadsheet.^{ee} The program was also used to calculate the 90% confidence intervals of the upper and lower limits of the calculated ranges with the bootstrap method, and outliers were identified with Tukey and Dixon tests. To evaluate the influence of sex on the plasma VDBP concentrations for healthy horses, the ratio between subgroup distribution widths (ie, the SDs) was analyzed for female horses (mares) versus male horses (geldings and stallions combined) as recommended by Harris and Boyd,³⁷ although the establishment of true subpopulation reference intervals requires sample sizes of > 50 for each subgroup.³⁸ Mean plasma concentrations of VDBP in healthy horses were compared among mares, geldings, and stallions by ANOVA and between breed types (cold-blooded [Icelandic horses, ponies, and draft horses] vs hot-blooded [Thoroughbreds, Standardbreds, and warmblood-type horses]) by a Student *t* test.

Associations between VDBP concentrations and IR-related time points were tested with repeated-measures ANOVA followed by a Tukey multiple comparisons test. Comparisons of plasma VDBP concentrations between horses with naturally occurring gastrointestinal disease and healthy horses as well as between horses with < 12 hours and ≥ 12 hours of colic duration were performed with Student *t* tests with Welch correction for unequal variances. Associations between VDBP concentration and diagnosis type (strangulating obstruction, simple obstruction,

or enterocolitis) in horses with acute gastrointestinal diseases were tested by ANOVA. Values of $P < 0.05$ were considered significant. Statistical analyses were performed with commercially available software packages.^{ff,gg}

Results

Purification and characterization of equine VDBP

Most of the purification methods attempted did not yield relevant quantities of purified native equine VDBP, although the protein could be identified in spots excised from Coomassie brilliant blue-stained SDS-PAGE gels by MS. However, 1 purification scheme (ammonium sulfate fractionation, strong anion exchange chromatography, and strong cation exchange chromatography) resulted in a small amount (40 µg) of equine VDBP (from 2 L of serum), with a purity of approximately 90% as determined by visual assessment of SDS-PAGE gels and nitro blue tetrazolium-stained immunoblots (**Figure 1**). Results of SDS-PAGE and immunoblotting showed that the relative molecular weight of the native equine VDBP was approximately 50 kDa and slightly higher than that

of purified human VDBP obtained in another study²⁴ (**Figure 2**) likely attributable to glycosylation, as the sequence-derived molecular weight of equine VDBP is lower than that of human VDBP (51.088 kDa vs 51.214 kDa; molecular weights calculated from NCBI molecular sequences XP_001489400 and P02774, respectively).

After in-gel digestion with trypsin, elution of the cleaved peptides, and analysis by MS, all proteins were identified as their respective species VDBP with sequence coverages of 90.9% to 91.3% for human and 77.5% to 85.4% for equine protein (**Supplementary Figure SI**, available at <http://avmajournals.avma.org/doi/suppl/10.2460/ajvr.78.6.718>). The lower degree of coverage for equine VDBP was attributable to a missing basic residue (Lys59), resulting in a large tryptic peptide remaining undetected. In both human and equine VDBP, arginine is the third residue and lysine is the fourth residue from the end; the result in our analysis was that the first and last 3 residues were not identified because the peptides resulting from a tryptic digest were too small for detection in our MS system. In 1 sample of human VDBP, a peptide with a missed cleavage site allowed the identification of the C-terminus.

Recombinant equine VDBP, which was produced in *E coli* and therefore not glycosylated, migrated to a slightly lower relative molecular weight in SDS-PAGE, compared with human VDBP, most

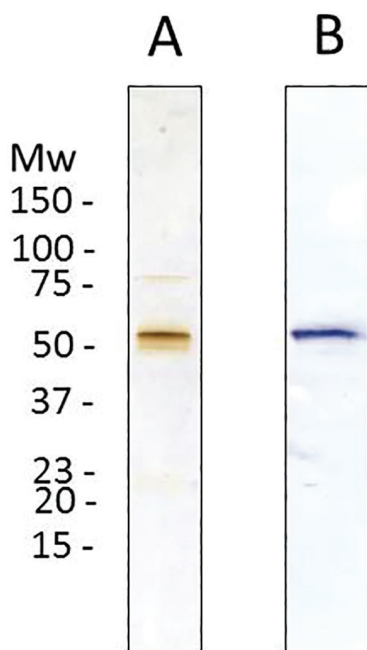


Figure 1—Composite photographic image showing results of SDS-PAGE (A) and immunoblotting (B) of purified equine VDBP obtained from serum of healthy horses by a protocol that included ammonium sulfate fractionation, strong anion exchange chromatography, and strong cation exchange chromatography. The SDS-PAGE was run under reducing conditions, and the gel was silver stained. The immunoblot was labeled with monoclonal anti-equine VDBP (HYB-378-04) primary antibody and alkaline phosphatase-conjugated goat anti-mouse secondary antibody; bound antibody was visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium as substrate. Markings on the left indicate molecular weight (Mw) in kDa.

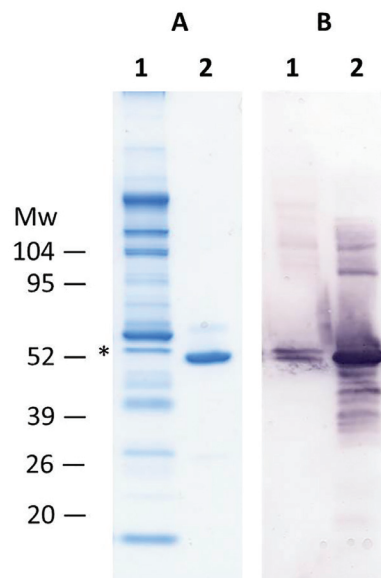


Figure 2—Composite photographic image showing results of SDS-PAGE (A) and immunoblotting (B) of the eluted fraction following chromatography of ammonium sulfate-fractionated equine serum (lanes marked with 1), compared with purified human VDBP^{24,25} (lanes marked with 2). The serum was subjected to strong anion exchange chromatography followed by strong cation exchange chromatography. The immunoblot was labeled with a commercially available RahuVDBP primary antibody and goat anti-rabbit secondary antibody, and bound antibody was visualized as described for the samples in Figure 1. Asterisk indicates the band identified as equine VDBP by immunoblotting and MS. See Figure 1 for remainder of key.

likely because of its lower molecular weight of 51.088 kDa versus 51.214 kDa (**Figure 3**). Because 2 distinct bands were produced on SDS-PAGE of equine VDBP, both were excised following electrophoresis of the protein and analyzed separately (Supplementary Figure S1). No differences were found in the primary structures or the terminals, indicating that the double band might have represented an artifact of the gel electrophoresis or that a protein residue with unknown posttranslational modification might have been present. Assessment by MS following electrophoresis revealed sequence identity and showed that the purity of the recombinant equine protein was 95% to 99%.

Antibody reactivity

The 5 mAbs (1 [HYB 370-01] raised against purified equine VDBP and 4 [HYB 378-01, HYB 378-02, HYB 378-03, and HYB 378-04] against recombinant equine VDBP) and 2 polyclonal antibodies (1 raised against recombinant equine VDBP [RaeqVDBP] and 1 against human VDBP [RahuVDBP]) were found to have medium or strong reactivity with the corresponding VDBP antigen when used with SDS-PAGE followed by immunoblotting or on ELISA (**Supplementary Table S1**, available at <http://avmajournals.avma.org/doi/suppl/10.2460/ajvr.78.6.718>). One mAb against recombinant equine VDBP (HYB 378-04) reacted equally with human VDBP on ELISA and im-

munoblots and was used for identification of equine VDBP during purification. The mAb raised against purified equine VDBP (HYB 370-01) cross-reacted with human VDBP, but also with albumin (not shown), which limited its further use. The polyclonal RaeqVDBP antibody cross-reacted with human VDBP on ELISA, but not on immunoblots, whereas the polyclonal RahuVDBP antibody had a weak cross-reactivity with equine VDBP for both methods.

The polyclonal RahuVDBP and RaeqVDBP antibodies did not cross-react with equine and human VDBP, respectively, in Outcherlony immunodiffusion (data not shown). The polyclonal RahuVDBP antibody had no cross-reactivity with human albumin, suggesting it was very specific for human VDBP; however, the antiserum reacted with α_1 -antitrypsin, which was known to be present in the VDBP preparation used for immunization.²⁹

Results of crossed immunoelectrophoresis indicated that the polyclonal RaeqVDBP antibody was monospecific and reacted with both recombinant and purified native equine VDBP (**Figure 4**). For this reason, this antibody was used to perform the RIE assays.

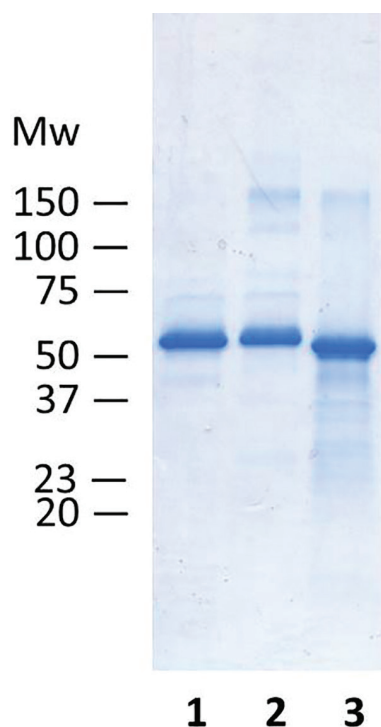


Figure 3—Photograph showing results of SDS-PAGE of human and recombinant equine VDBP with reducing sample preparation and Coomassie brilliant blue staining. Lane 1: purified human VDBP²⁴; lane 2: commercially available purified human VDBP; lane 3: recombinant equine VDBP. See Figure 1 for remainder of key.

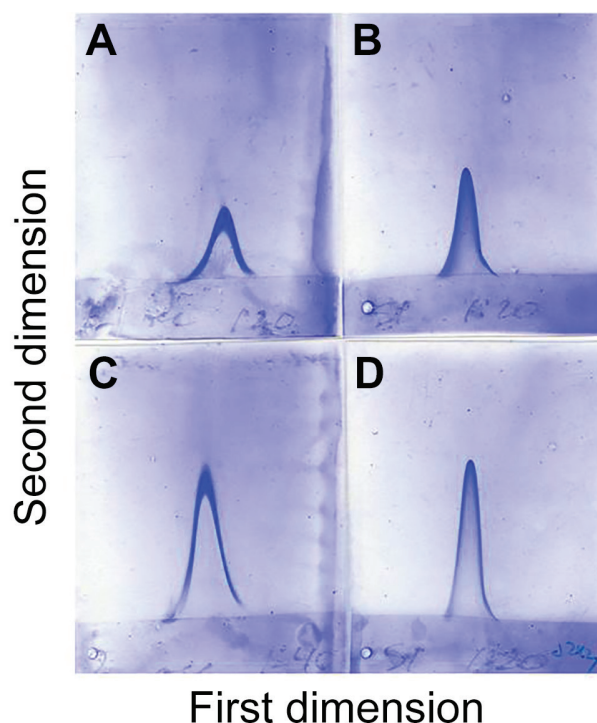


Figure 4—Photographs of Coomassie brilliant blue–stained agarose gels showing results of crossed immunoelectrophoresis of equine VDBP with a polyclonal antibody raised against recombinant equine VDBP (RaeqVDBP). Sample material loaded in the wells for the first-dimension separation included 4 μ g of recombinant equine VDBP (A), 5 μ L of equine serum containing 1,040 mg of VDBP/L (B), 8 μ g of recombinant equine VDBP (C), or 10 μ L of equine serum containing 1,040 mg of VDBP/L (D). For the second dimension, 1% agarose gels with 110 μ L RaeqVDBP antibody/22 mL gel and a current of 3 V/cm were used for overnight incubation.

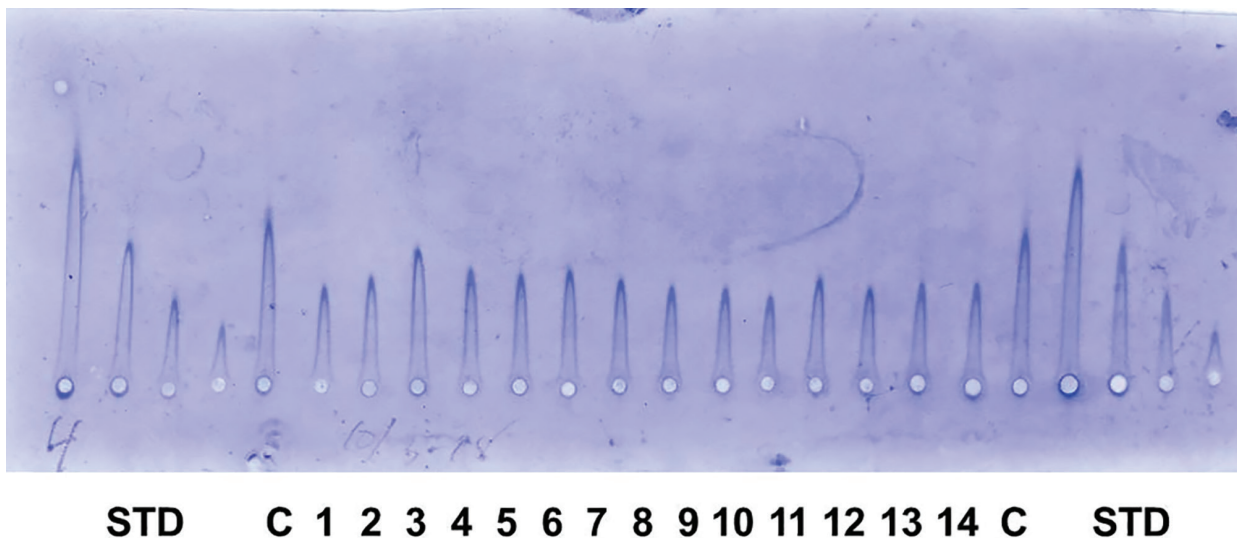


Figure 5—Photograph of a Coomassie brilliant blue-stained agarose gel showing results of RIE of equine serum samples with RaeqVDBP (unknown concentration; 110 μ L/22 mL gel). Standard dilutions (STD) of equine VDBP in pooled serum (70, 35, 17.5, and 8.75 mg/L, respectively, as viewed from left to right) were included twice on each gel. C = Positive control sample (concentration, 50 μ g/mL). Lanes numbered 1 to 14 represent patient samples.

Concentrations of VDBP in plasma samples from healthy horses and horses with gastrointestinal disease or injury

Healthy horses—Healthy horses enrolled in this part of the study ($n = 61$) had a mean $\pm$ SD age of 8 ± 6 years (range, 1 to 26 years). The group comprised 53 (87%) hot-blooded and 8 (13%) cold-blooded horses, including 28 (46%) mares, 21 (34%) geldings, and 12 (20%) stallions with mean $\pm$ SD body weight of 493 ± 118 kg (range, 120 to 665 kg).

Plasma concentrations of VDBP measured by RIE in samples from healthy horses were used to determine a working reference range for plasma VDBP concentration of 531 to 1,382 mg/L. An example of RIE results is shown (**Figure 5**). The 90% confidence intervals for the upper and lower limits of this range were 495 to 642 mg/L and 1,311 to 1,401 mg/L, respectively. The 90% confidence intervals of the upper and lower limits exceeded the width of the working reference range by up to 28% and thus exceeded the established recommendation of 20%.³⁶ No outliers were identified with the Tukey method. According to the Harris and Boyd³⁷ test for partitioning of reference intervals, mares had a higher working reference range (483 to 1,498 mg/L) than did geldings and stallions combined (544 to 1,267 mg/L). However, no significant differences were found when mean VDBP concentrations were compared among mares, geldings, and stallions (962, 946, and 835 mg/L, respectively; $P = 0.2$). There was also no significant ($P = 0.7$) difference in mean VDBP concentrations between cold-blooded (899 mg/L) and hot-blooded (936 mg/L) breeds.

Horses with experimentally induced intestinal IR—Plasma samples obtained from horses during and af-

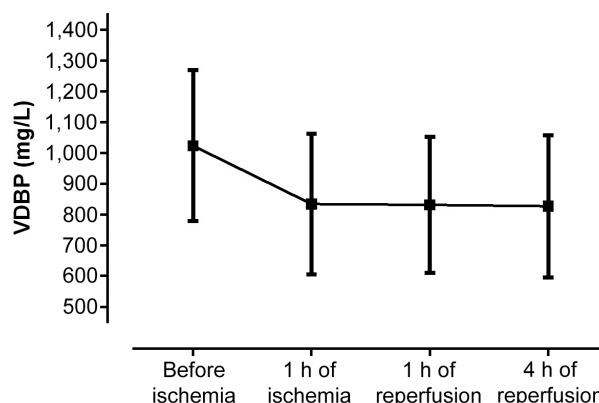


Figure 6—Mean $\pm$ SD VDBP concentrations in plasma collected from jugular venous blood samples before and during experimentally induced IR in 12 horses. Plasma concentrations of the protein measured after induction of ischemia and during reperfusion were significantly ($P < 0.001$ for all comparisons) lower than those measured before ischemia.

ter intestinal IR had significantly ($P < 0.001$) lower VDBP concentrations, compared with samples collected from the same horses before induction of IR. Mean $\pm$ SD results for samples collected at baseline, after 1 hour of ischemia, and after 1 and 4 hours of reperfusion were summarized (**Figure 6**).

Horses with naturally occurring gastrointestinal disease—Horses in this group ($n = 59$) had a mean $\pm$ SD age of 11 ± 6 years (range, 1 to 27 years). There were 27 (46%) hot-blooded and 32 (54%) cold-blooded horses, including 22 (37%) mares, 32 (54%) geldings, and 5 (8%) stallions, with a mean $\pm$ SD body weight of 491 ± 126 kg (range, 205 to 760 kg).

The mean $\pm$ SD plasma VDBP concentration in horses with naturally occurring gastrointestinal dis-

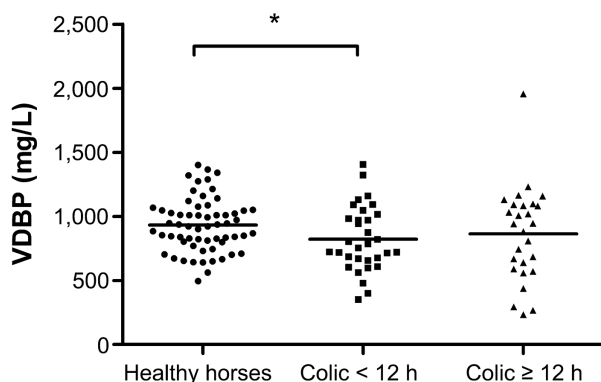


Figure 7—Scatterplot of plasma VDBP concentrations in healthy horses ($n = 61$) and horses with colic of short (< 12 hours; $n = 32$) and long (≥ 12 hours; 27) duration. Plasma concentrations of VDBP were significantly ($P = 0.04$; asterisk) lower in horses with colic of short duration than in healthy horses, whereas concentrations were not significantly ($P > 0.05$) different between healthy horses and horses with colic of long duration.

eases was 841 ± 309 mg/L, compared with 931 ± 208 mg/L for healthy horses. These values did not differ significantly ($P = 0.06$). However, horses with a colic duration of < 12 hours (18 horses with strangulating obstruction, 9 with enterocolitis, and 5 with simple obstruction) had a significantly ($P = 0.04$) lower mean plasma VDBP concentrations than did healthy horses (827 ± 255 mg/L vs 931 ± 208 mg/L, respectively; **Figure 7**). In contrast, plasma VDBP concentrations in horses with a colic duration ≥ 12 hours (862 ± 368 mg/L; 12 horses with strangulating obstruction, 14 with enterocolitis, and 1 with simple obstruction) did not differ from those for healthy horses ($P = 0.3$).

Discussion

Equine VDBP was successfully purified, but with more difficulties than anticipated, compared with previously published protocols for purification of equine¹⁹ and human VDBP.^{24,29} Only 40 μ g of protein could be purified from 2 L of serum from healthy horses, corresponding to 0.00004% of the estimated amount of 1 g of VDBP present in that volume. The purity was approximately 90% as judged by visual inspection of SDS-PAGE gels and thus was lower than expected. The insufficient amount of VDBP obtained by purification may have been caused by a diffuse, broadly eluting characteristic of equine VDBP during chromatography. This might have been attributable to heterogeneous glycosylation, although the glycosylation status of equine VDBP is not known. Moreover, the relatively low concentration of VDBP in serum, compared with the high concentrations of other serum proteins (eg, albumin, which is partially homologous to VDBP, and α_1 -antitrypsin, which contaminates all commercial human VDBP preparations), might have affected the specificity of the antibodies used and thus complicated the purification procedure and the assessment of VDBP in collected fractions.

Because we encountered difficulties in purification of sufficient amounts of VDBP from equine serum, the recombinant protein was successfully produced as an alternative approach. Sufficient quantities of highly pure equine VDBP were obtained to allow the production of monoclonal and polyclonal anti-equine VDBP antibodies as well as the development of an analyzing assay.

Evaluation by SDS-PAGE and immunoblotting revealed that recombinant equine VDBP had a slightly lower molecular weight, compared with the human equivalent, in agreement with the slight differences in their theoretical molecular weights of 51.088 and 51.214 kDa, respectively. The identities of the natural human and recombinant equine VDBPs were verified by enzymatic digestion and MS peptide mapping. Whereas human VDBP obtained from 2 sources formed single sharp bands on SDS-PAGE, the equine VDBP formed double bands. Mass spectrometric analyses of the 2 bands did not discern whether the second band was an artifact or a different molecular formulation of the protein.

The RIE assay was established with RaeqVDBP and was used successfully for measurements of VDBP concentrations in plasma from healthy horses, horses with experimentally induced intestinal IR, and horses that had various gastrointestinal diseases with colic of short (< 12 hours) or long (≥ 12 hours) duration. However, the RIE format is not suitable for implementation in routine clinical diagnostics. For routine evaluation of circulating VDBP concentrations, assay formats that can be automated are preferred.

In the present study, we identified a working reference range for plasma VDBP in healthy horses of 531 to 1,382 mg/L, which was higher than values reported for healthy human subjects (176 to 623 mg/L)³⁹ and generally higher than results found for rats, where males had higher circulating concentrations than females (656 ± 52 mg/L vs 472 ± 46 mg/L).³⁴ In our study, mares had higher plasma concentrations of the protein than did stallions and geldings (combined); however, evaluation of samples from a larger number of horses would be needed to confirm differences between the sexes. The reasons for the apparent differences in circulating concentrations of VDBP between horses and humans or rats were not determined. There may be true species differences, or the finding could be related to methodological differences or different reactivity of the assay antibodies against the recombinant and native equine VDBP.

The tissue injury created by experimentally induced intestinal IR³²⁻³⁴ in the horses of the present study was sufficient to cause a significant decrease in plasma VDBP concentrations within 60 minutes after induction of intestinal ischemia, compared with results for the same horses prior to IR. This corresponded to results of a human study,⁹ where circulating VDBP concentrations were found to be significantly lower than in healthy human subjects within 60 minutes after injury caused by severe trauma, and results

of a study in rats,¹¹ where this variable was significantly decreased from the pretreatment value by 60 minutes after administration of endotoxin to induce shock. The very rapid decrease detected in the present study was likely a result of VDBP binding to actin released by injured intestinal cells and clearance of the VDBP-actin complex by the reticuloendothelial system in the liver.¹ These findings suggest that VDBP measurements might be suitable for monitoring intestinal injury over time in experimental settings. Analysis of circulating VDBP concentrations could be beneficial in future studies, such as investigations to evaluate treatment options for horses with this type of injury.

Horses with colic of short duration (< 12 hours) had significantly lower plasma concentrations of VDBP than did healthy horses. These findings were in agreement with those for the IR experiment as well as the findings in published studies that involved human subjects⁹ and rats.¹¹ The slightly higher concentrations of the protein in horses with colic of long duration (≥ 12 hours) could have resulted from resynthesis of VDBP in the liver as has been shown for other acute-phase proteins in human patients after acute trauma¹³ or surgery.¹² The very small differences between horses with colic of short versus long duration could have reflected a modest amplitude of response. The concentration difference could, however, also be a consequence of the different group compositions. Horses with a colic duration ≥ 12 hours mainly included patients with enterocolitis (14/27 [52%]), whereas those with a colic duration of < 12 hours included a smaller proportion with enterocolitis (9/32 [28%]). We chose a cutoff time period of 12 hours for this investigation, and it is possible that use of a different cutoff time would yield different results.

The wide range of working reference values found in this study and the modest change in concentrations of VDBP, even in horses with severe gastrointestinal diseases, indicated that measurement of this protein would not be a useful diagnostic tool in individual patients because the values of healthy and diseased horses would be expected to overlap. Repeated measurements of VDBP in horses with severe gastrointestinal disease might be useful as a prognostic indicator, as found in humans with severe tissue trauma.¹³ The current assay format is, however, not suitable for a routine clinical setup, and further development and larger studies are needed to fully explore the diagnostic and prognostic potential of circulating VDBP in horses.

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The authors declare that there were no conflicts of interest.

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Footnotes

- a. Merck, Darmstadt, Germany.
- b. Sigma, St Louis, Mo.
- c. Äkta FPLC system, GE Healthcare, Uppsala, Sweden.
- d. Q Sepharose Fast Flow, GE Healthcare, Uppsala, Sweden.
- e. Ultracel, Millipore, Bedford, Mass.
- f. Mono S, GE Healthcare, Uppsala, Sweden.
- g. BacPower protein expression, GenScript, Hong Kong, China.
- h. Alhydrogel, Brenntag Biosector, Frederikssund, Denmark.
- i. Envigo, Horst, The Netherlands.
- j. Charles River Laboratories, Saint-Germain-Nuelles, France.
- k. Gc-globulin from human plasma, G8764, Sigma, St Louis, Mo.
- l. NUNC, Roskilde, Denmark.
- m. Goat anti-mouse IgG, Sigma, St Louis, Mo.
- n. Thermomax, Molecular Devices, Menlo Park, Calif.
- o. Cambrex, Copenhagen, Denmark.
- p. Novex, Carlsbad, Calif.
- q. Coomassie Brilliant Blue, Thermo Scientific, Rockford, Ill.
- r. Polyclonal rabbit anti-human Gc-globulin, A0021, Dako, Glostrup, Denmark.
- s. Goat anti-mouse IgG, Sigma, St Louis, Mo.
- t. Goat anti-rabbit IgG, Sigma, St Louis, Mo.
- u. Promega, Southampton, Hampshire, England.
- v. GELoader, Eppendorf AG, Hamburg, Germany.
- w. POROS R2, Applied Biosystems, Foster City, Calif.
- x. ReproSil Pur C18 AQ, 3 μ m, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany.
- y. Proxeon 1000 easy-nLC, Proxeon Biosystems, Odense, Denmark.
- z. LTQ-Orbitrap XL, Thermo Fisher Scientific, Bremen, Germany.
- aa. X! Tandem, offline version 2013.09.01.1. Available at: www.thegpm.org. Accessed Feb 6, 2016.
- bb. GPMW, version 10.0, Lighthouse data, Odense, Denmark.
- cc. BD Vacutainer system, Becton, Dickinson and Co, Plymouth, Devon, England.
- dd. Reference Value Advisor, version 2.1, Biostatistiques Ecole nationale Veterinaire de Toulouse, France. Available at: www.biostat.envt.fr/spip/spip.php?article63. Accessed Jan 26, 2016.
- ee. Excel 2010, Microsoft Corp, Redmond, Wash.
- ff. Prism, version 5.0, GraphPad Software Inc, San Diego, Calif.
- gg. SAS, version 9.3, SAS Institute Inc, Cary, NC.

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