—Full Paper—

Optimisation of a serum albumin removal protocol for use in a proteomic study to identify the protein biomarkers for silent gastric ulceration in horses

Kanaporn POLTEP[1,6], Parichart TESENA[2], Yodying YINGCHUTRAKUL[3], Jane TAYLOR[4] and Tuempong WONGTAWAN[5,6]*

[1]The Monitoring and Surveillance Centre for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom 73170, Thailand
[2]Department of Clinical Science and Public Health, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom 73170, Thailand
[3]Proteomics Research Laboratory, Genome Technology Research Unit, National Centre for Genetics Engineering and Biotechnology, Pathum Thani 12120, Thailand
[4]Biomedical Teaching Organisation, Biomedical Sciences, Edinburgh Medical School, University of Edinburgh, Edinburgh, EH8 9AG, U.K.
[5]Department of Preclinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom 73170, Thailand
[6]Laboratory of Cellular Biomedicine and Veterinary Medicine, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom 73170, Thailand

Silent gastric ulceration occurs without evidence of clinical signs and is common in horses. There is currently no a simple and effective method to diagnose this disease. Proteomics can be used to identify serum biomarkers, but the most abundant serum protein, albumin, could conceal candidate biomarkers. Therefore, it is recommended to remove albumin before a proteomic study; however, there is no specific albumin depletion kit or standard protocol available for horse samples. The objectives of this study were to optimise a protocol to remove equine serum albumin and to use albumin-depleted serum to identify the protein biomarkers for silent gastric ulceration. Gastroscopy was used to identify gastric ulceration, and serum was obtained from horses with either a healthy gastric mucosa or gastric ulceration. Serum albumin was removed using the trichloroacetic acid (TCA) protein precipitation method, and this protocol was optimised by varying the concentration of TCA, type of organic solvents, ratio of serum to protein precipitation solution, and incubation times. Electrophoresis and image analysis were used to compare the amounts of albumin, immunoglobulins G (IgG), and protein degradation before and after TCA precipitation. The best protocol was chosen to remove albumin for a proteomic study (electrophoresis and mass spectrometry). The results revealed that protocol 2 (ratio of serum to solution 1:5, 10% TCA in acetone, and 90 min incubation) was the most efficient protocol to remove albumin (98%) and IgG heavy (80%) and light (98%) chains without degrading other proteins. After electrophoresis and mass spectrometry analysis, KRT1, KRT6A and KRT18 were identified as potential markers for silent gastric ulceration.

Key words: *albumin removal, biomarker, gastric ulceration, horse, proteomics*

J. Equine Sci.
Vol. 29, No. 3
pp. 53–60, 2018

Received: January 31, 2018
Accepted: May 24, 2018
*Corresponding author. e-mail: tuempong.wan@mahidol.edu

Gastric ulceration is one of the most common diseases found in horses [12, 32, 34]. Some horses exhibit clinical signs, but others develop silent gastric ulceration (also known as subclinical or non-clinical gastric ulceration), and both types can affect a horse's performance and health [1, 16, 35].

The most accurate method of diagnosing equine gastric ulceration is gastroscopy [32], but this technique is unsuitable as a screening test due to the need for expensive equipment, the complicated procedure, and the fact that it is time-consuming to perform [15]. A faecal blood test and sucrose permeability test can be used for screening the disease, but their sensitivity and specificity are much lower than gastroscopy [11, 39].

Detection of disease-specific proteins (biomarkers) in blood is rapid, practical, and effective, and many commercial biomarker kits have been developed for detecting human diseases [21]. Proteomic technologies including electrophoresis and mass spectrometry have been used to identify protein biomarkers for gastric diseases such as *H. pylori* infection associated with gastric ulceration and cancer in humans [29] and gastric ulceration in foals [33]. A study in foals revealed alpha (1)-antitrypsin as a candidate marker for gastric ulceration [33], but this protein has not been developed for clinical use. Other equine diseases such as laminitis and osteoarthritis have also been investigated to identify disease markers by using proteomic technologies [5, 31].

However, albumin is the most abundant protein in serum [24], and it can conceal the candidate biomarker proteins, which are usually low-abundance proteins [18]. Therefore, it is essential to eliminate albumin before proteomic analysis [9]. In human analyses, serum/plasma albumin is usually depleted by using column immunoaffinity purification or commercial albumin removal kits [30]. Unfortunately, there is no commercial kit available specifically for horses, and most kits for human samples inefficiently remove equine albumin [22]. Additionally, there is also a lack of published reports relating to the protocol of serum albumin removal in horses.

Trichloroacetic acid (TCA) protein precipitation is a common method used for proteins precipitation [20]. It is an analogue of acetic acid, and at the optimal concentration in suitable organic solvents, it selectively binds to albumin [26]. The TCA protein precipitation method has been used to remove or purify albumin from serum from several species [3, 9, 14, 23]. The objectives of this study were to optimise the protocol to remove horse serum albumin and to use albumin-depleted serum to identify protein biomarkers for silent gastric ulceration.

Materials and Methods

A summary of the experimental design is shown in Fig. 1.

Animals

Equine serum was obtained from a previous study, in which we investigated the occurrence of gastric ulceration in Thoroughbred horses by using gastroscopy [35]. Horse ages were between 7–15 years old, and body weights were 400–450 kg. Recorded videos and images of gastric ulcerations were obtained from the previous study [35] and graded as recommended by the Equine Gastric Ulcer Council [32]. Horses were sedated with 0.5–1 mg/kg xylazine hydrochloride intravenously before performing video gastroscopy (Model GVE 2100A2, Huger Medical Instrument, Shanghai, China). Ten millilitres of blood were collected from the jugular vein, and samples from 10 horses with a normal gastric mucosa and 10 horses with silent gastric ulceration (at non-glandular stomach) were collected.

This research project was approved by the Animal Care and Use Committee of the Faculty of Veterinary Science, Mahidol University. The horse owner (Veterinary and Remount Department of the Royal Thai Army) provided informed consent for gastroscopy, blood collection, and the proteomic study.

Optimisation of TCA-protein precipitation protocols for removal of albumin

The protocol optimisation included varying the concentration (1, 5, 10, or 20%) of TCA (AppliChem GmbH, Darmstadt, Germany) in several different organic solvents including ethanol, methanol, isopropanol, and acetone (V.S. Chem House, Bangkok, Thailand). Two dilution ratios (1:5 and 1:10) of serum to protein precipitation solution (TCA in an organic solvent) and two incubation times (45 and 90 min) were also compared.

Pooled serum (20 μl) was mixed with either 80 μl (1:5) or 180 μl (1:10) of ice-cold TCA-organic solvents and incubated at −20°C for either 45 or 90 min for precipitation. After centrifugation at 15,000 × g for 10 min, the pellets (albumin-depleted fraction) and supernatant (albumin-enriched fraction) were separately collected. Both pellets and supernatants were added with 1 ml of cold solvents and then centrifuged at 15,000 g for 10 min. At this step, albumin-enriched proteins in the supernatant were precipitated, and the supernatants were removed. Then both pellets were evaporated and resuspended with 50 μl of buffer containing 8 M urea (Research Organics, Cleveland, OH, U.S.A.), 4% W/V CHAPS (AppliChem GmbH), 15 mM DTT (Vivantis Technologies, Selangor Darul Ehsan, Malaysia), 0.15% IPG buffer (GE Healthcare, Uppsala, Sweden), and 0.001% bromophenol blue (VWR International, Radnor, PA, U.S.A.). Protein concentration was quantified using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

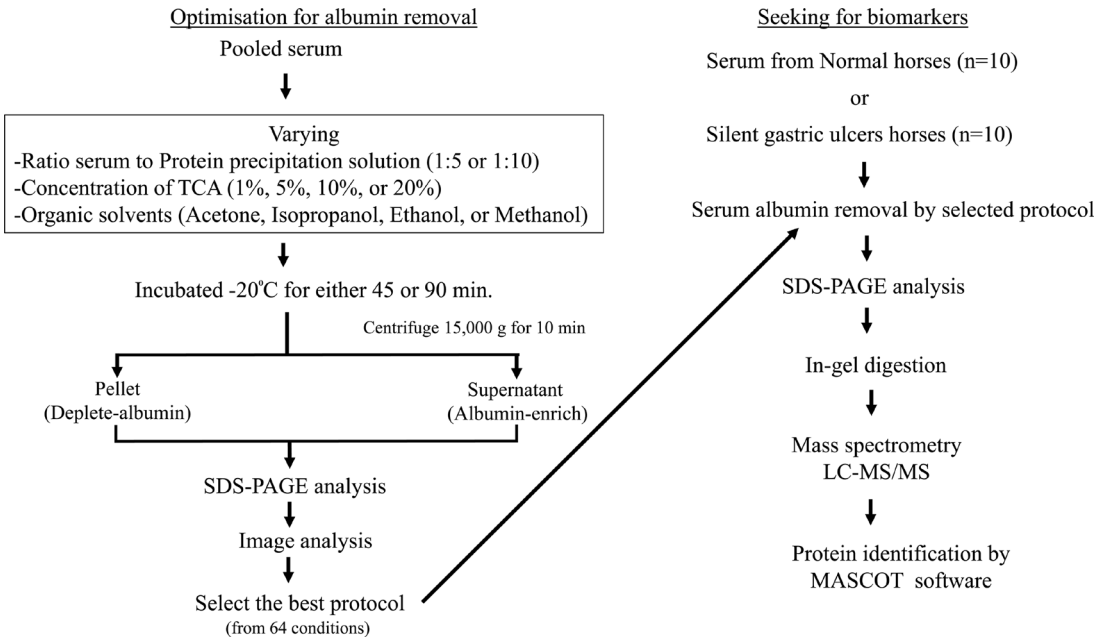

Fig. 1. Diagram of the experimental study.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Two micrograms of sample from the crude serum, the albumin-enriched fraction, or albumin-depleted fraction were mixed with 15 μl of loading buffer containing 0.2 M Tris (Bio Basic, Markham, ON, Canada; pH 6.8), 20% glycerol (Bio Basic), 10% SDS (Bio-Rad, Hercules, CA, U.S.A.), and 5% β-mercaptoethanol (EuroClone, Pero, MI, Italy) and then boiled at 95°C for 10 min. The mixtures were loaded on 8% SDS gels in a Mini-PROTEAN system (Bio-Rad) at 90 V for 90 min or until the dye front reached the bottom at the end of the gels. The gels were washed with distilled water and subsequently stained with 1% Coomassie brilliant blue R250 (Thermo Fisher Scientific) for 2 hr. Excess dye was washed with destaining solution (10% methanol and 7% glacial acetic acid) for at least 2 hr.

Image analysis

The protein bands were imaged using a scanner (Docu-Print CM215b, Fuji Xerox, Bangkok, Thailand). The percentage of albumin and IgG removal was calculated by measuring the density of protein bands and comparing between pre- and post-protein precipitation. The density of protein bands was measured by using the ImageJ software [28].

In-gel protein digestion and mass spectrometry analysis

The specific protein bands were excised, and digestion with trypsin was performed according to previous reports [10]. The analysis of extract peptides was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Briefly, the extracted peptides were injected into the LC-MS/MS device with the ESI-QUAD-TOF technique. An UltiMate 3000 Nano and Capillary LC system (Dionex, Sunnyvale, CA, U.S.A.) and a maXis mass spectrometer detector (Bruker, Billerica, MA, U.S.A.) were used. Protein identification was performed using the Mascot 2.3 software (Matrix Science, Boston, MA, U.S.A.) connected to the *Equus caballus* NCBI database.

Statistics

Percentages of protein removal are shown as the mean ± standard error of the mean (SEM). Statistical differences were calculated using the IBM SPSS 21 software (IBM, Armonk, NY, U.S.A.). One-way ANOVA and multiple comparisons with LSD testing were used for comparison of more than 2 subjects. Student's *t*-test was used for comparison between two samples.

Results

The optimal protocols for equine albumin removal

After screening 64 protocols, the best five protocols were identified. These were found to be protocols 1, 2, 3, 21, and 22 (Fig. 2). These five conditions efficiently removed albumin (>90%) and did not degrade proteins. Protocols 1–3 used acetone, while protocols 21 and 22 used isopropanol as an organic solvent. Ethanol and methanol were unable

to efficiently remove albumin compared with acetone and isopropanol (Fig. 2).

We then repeated these five protocols in triplicate and calculated the efficiency of albumin and IgG depletion (Table 1). In this study, the molecular weights of albumin, IgG heavy chain, and IgG light chain were approximately 65, 45, and 25 kDa, respectively. All five optimised protocols removed albumin efficiently with depletion rates of more than 90%. However, the ability to remove IgG heavy and light chains differed among the protocols. Protocols 2, 21, and 22 were comparable with respect to removing IgG heavy chain (about 80% removal efficiency) and were significantly better (P<0.05) than protocols 1 and 3. For IgG light chain, protocol 2 was significantly superior to the other protocols (P<0.05), as it was able to remove up to 98% of IgG light chain protein. Taking these together, protocol 2 was superior to the other protocols due to its ability to remove both albumin and IgG efficiently.

The physical appearance of silent gastric ulcer horses compared to normal horses

From the records of our previous study [35], the physical appearances of healthy and silent gastric ulcer horses were similar, and both appeared normal. The gastric epithelium of normal horses had no lesions of ulceration or hyperkeratosis, and the gastric epithelium was intact (Grade 0). All horses with silent gastric ulceration had multiple small ulcers but less than five lesions which presented at the superficial layer of the non-glandular mucosa near the margo plicatus (Grade I–II).

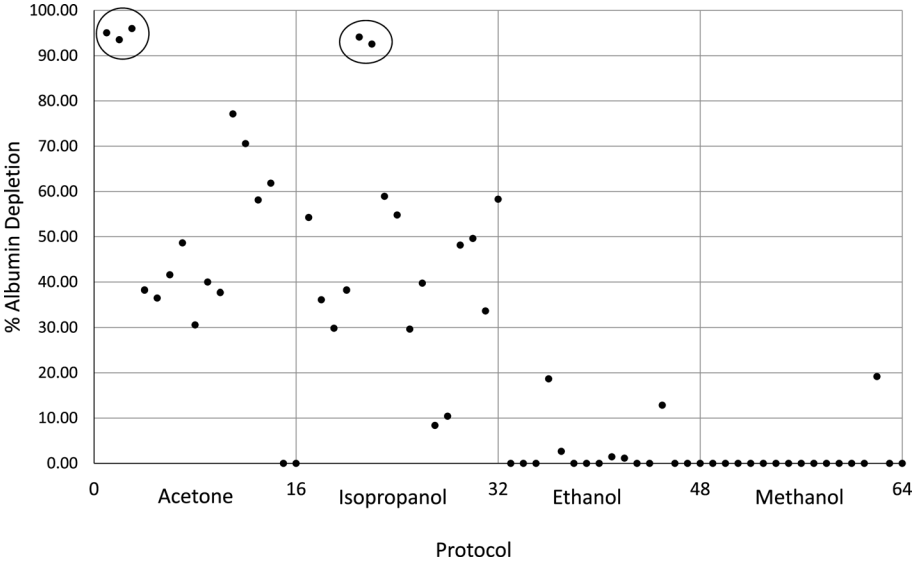

Fig. 2. Screening of 64 protocols for removing serum albumin. Optimisation was performed by varying the organic solvents, percentage of TCA, serum per protein precipitation solutions, and incubation times. The five best protocols are marked with black circles. Acetone was used in protocols 1–16, isopropanol was used in protocols 17–32, ethanol was used in protocols 33–48, and methanol was used in protocols 48–64.

Table 1. The five optimal conditions of the albumin removal protocol

Protocol	Serum: solution	TCA (%)	Solvents	Incubation (min)	Depletion (%)		
					Albumin	IgG (heavy)	IgG (light)
1	1:5	20	Acetone	90	96.94 ± 0.02	67.66 ± 0.87	88.92 ± 2.52
2	1:5	10	Acetone	90	97.85 ± 0.07	79.54 ± 0.29*	98.12 ± 0.18*
3	1:5	5	Acetone	90	92.13 ± 0.13	21.20 ± 5.47	55.60 ± 1.67
21	1:10	20	Isopropanol	90	94.41 ± 0.09	80.15 ± 0.06*	75.37 ± 2.37
22	1:10	10	Isopropanol	90	95.03 ± 0.17	79.19 ± 0.97*	72.46 ± 3.23

The percentage of albumin and IgG removal is presented as the mean ± SEM. *Represents significant difference (P<0.05).

The differential protein expression in horse serum between normal gastric mucosa and silent gastric ulceration groups

Protocol 2 was used to remove albumin, and then gel electrophoresis was performed to compare the protein patterns between normal gastric mucosa and silent gastric ulceration groups. There was clearly a difference in density of protein bands at a molecular weight of approximately 75 kDa (Fig. 3) when comparing normal and silent gastric ulceration horses. These protein bands from protocol 2 were excised for mass spectrometry analysis, which identified four proteins from silent gastric ulceration horses. These proteins were keratin type II cytoskeletal 1 (KRT1), keratin type II cytoskeletal 6A isoform X1 (KRT6A), keratin type I cytoskeletal 18 (KRT18), and transferrin (TF) (Table 2). The proteins from normal horses were identified as TF, splicing factor, arginine/serine-rich 19 (SCAF1), and prolow-density lipoprotein receptor-related protein 1 (LRP1) (Table 3).

Discussion

The present study showed that the most efficient protocol to remove equine albumin and IgG from serum samples used 10% TCA in acetone as the protein precipitation solution. The optimal ratio of serum to protein precipitation solution was 1:10, and the optimal incubation time for precipitation was 90 min. This is consistent with the results of Chen *et al.* [3], who reported that 5–10% TCA in acetone is the optimal condition for removing human albumin and IgG, but Liu *et al.* [14] reported that 1% TCA in isopropanol is the optimal solution to remove albumin from human and monkey serum for a proteomic study. Notably, our protocol is simple and inexpensive, and it is more efficient than using human commercial kits to remove equine serum albumin and IgG [22].

The epithelium of the non-glandular part of the equine stomach is a stratified squamous epithelium, which is similar to the epithelial type found in the oesophagus and skin [2,

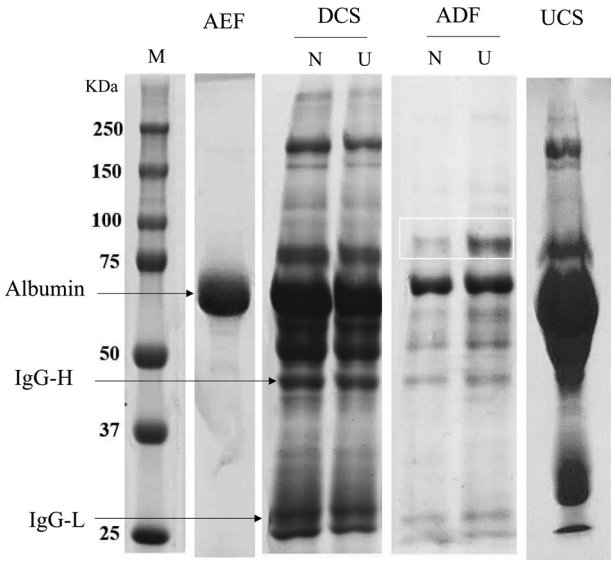

Fig. 3. Gel electrophoresis. The white rectangle represents 75 kDa proteins with densities that differ between normal and silent gastric ulceration horses. The protein bands for albumin, IgG heavy chain (IgG-H), and IgG light chain (IgG-L) are indicated with black arrows. UCS, undiluted crude serum; DCS, diluted crude serum (1:40 dilution); ADF, albumin-depleted protein fractions; AEF, albumin-enriched fractions (1:40 dilution); M, protein marker; N, pooled serum from normal gastric mucosa horses; U, pooled serum from silent gastric ulceration horses.

Table 2. Proteins identified by mass spectrometry in serum from silent gastric ulceration horse

Accession No.	Protein name	Protein score	Protein mass (Da)	Peptide sequences
gi338726100	Keratin 1	67	64,976	TLLEGEESR
gi149714759	Keratin type II cytoskeletal 6A isoform X1	39	61,223	AQYEEIAQR
gi545220094	Keratin type I cytoskeletal 18	39	48,343	AQYEELARK
gi136190	Transferrin	37	80,268	KNSNFQLNQLQGK
gi335773196	Septin-7-like protein	19	50,673	SPLAQMEEERREHVAKMK

Table 3. Proteins identified by mass spectrometry in horse with a normal mucosa

Accession No.	Protein name	Protein score	Protein mass (Da)	Peptide sequences
gi136190	Transferrin	217	80,268	DSALGFLR
gi953864613	Splicing factor, arginine/serine-rich 19	25	103,446	MEEEDESRGKTEESGEDR
gi545220722	Prolow-density lipoprotein receptor-related protein 1	19	516,445	GGRILQEDFTCRAVNSSCR

8]. Most squamous epithelia express a variety of keratins which provide structural integrity between the cells [37]. The present study found 3 types of keratins (KRT1, KRT6A, and KRT18) in serum of horses which had silent gastric ulceration. KRT18 is the most commonly found member of these keratins and is found in many tissues, while KRT1 and KRT6A are specifically expressed in the normal stratified squamous epithelium of the oesophagus [37]. KRT1 is associated with chronic disease such as inflammatory bowel disease in humans [7]. KRT6A expression is important and high during wound healing in the stratified squamous epithelium [6, 40] and associated with mucosal change, ulceration, and hyperkeratosis in the oesophagus and pars oesophagea of pigs [13, 25], where it has a similar structure to the non-glandular part of the equine stomach [2, 8].

TF is an iron transportation protein, and it may be associated with gastric ulceration when gastric haemorrhage occurs, resulting in a reduction of serum TF [4, 42]. The leaked TF in the gastrointestinal tract can be detected and used to diagnose peptic ulcer with haemorrhage [4].

The other three proteins identified are not specifically expressed in the GI tract and/or are unrelated with gastric ulceration in the non-glandular stomach. Septin-7-like protein may have similar function to septin 7 protein which is expressed in many tissues [37] and involved in cytoskeleton organization, cytokinesis, and membrane dynamics [19]. SCAF1 can be found in almost all tissues in the body [37] and is involved in pre-mRNA splicing [36]. LRP1 is a receptor found in many tissues, and it is involved in several cellular processes, including intracellular signalling, and apoptosis [36, 37]. LRP1 modulates the inflammatory response, autophagy, and apoptosis in human gastric ulcers caused by *Helicobacter pylori* infection [17, 41].

In the present study, horses with silent gastric ulceration had no clinical signs, but they had multiple small ulcers at the superficial layer of the non-glandular stomach close to the margo plicatus. This area is commonly affected by gastric ulcers due to its exposure to acid and pepsin from the glandular stomach [27, 38]. From our observations, silent gastric ulceration can be found in approximately 33% of non-working Thoroughbred horses and in about 77% of horses with gastric ulceration [35]. This is consistent with the results of Vatistas *et al.* [38], who found that 80% of Thoroughbred racehorses with gastric ulcer have no relevant clinical signs.

It may be concluded from our study that the best conditions for equine serum albumin and IgG removal by the TCA protein precipitation method are use of 10% TCA in acetone as the precipitation solution and precipitation by dilution of serum in the precipitation solution at a ratio 1:5 and incubation at −20°C for 90 min. The candidate protein biomarkers for equine silent gastric ulceration in the non-glandular stomach are KRT1, KRT6A, and KRT18. Notably, KRT6A may be the most relevant biomarker, as it is expressed in a tissue-specific manner in the non-glandular stomach and its expression is high during gastric ulcer development. Although proteomics is a powerful tool to screen protein biomarkers, confirmation of candidate markers with specific antibodies in a larger study cohort is required before they can be applied at veterinary clinics or horse farms.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgment

This project was funded by the Faculty of Veterinary Science, Mahidol University (to T. Wongtawan).

References

1. Andrews, F.M., and Nadeau, J.A. 1999. Clinical syndromes of gastric ulceration in foals and mature horses. *Equine Vet. J. Suppl.* **29**: 30–33. [Medline]
2. Burdas, K.D., Sack, W.O., Rock, S., Horowitz, A., and Berg, R. 2009. Anatomy of the Horse, 5th ed. Schlütersche, Hannover.
3. Chen, Y.Y., Lin, S.Y., Yeh, Y.Y., Hsiao, H.H., Wu, C.Y., Chen, S.T., and Wang, A.H. 2005. A modified protein precipitation procedure for efficient removal of albumin from serum. *Electrophoresis* **26**: 2117–2127. [Medline] [CrossRef]
4. Chiang, C.H., Jeng, J.E., Wang, W.M., Jheng, B.H., Hsu, W.T., and Chen, B.H. 2006. A comparative study of three fecal occult blood tests in upper gastrointestinal bleeding. *Kaohsiung J. Med. Sci.* **22**: 223–228. [Medline] [CrossRef]
5. Chiaradia, E., Pepe, M., Tartaglia, M., Scoppetta, F., D'Ambrosio, C., Renzone, G., Avellini, L., Moriconi, F., Gaiti, A., Bertuglia, A., Beccati, F., and Scaloni, A. 2012. Gambling on putative biomarkers of osteoarthritis and osteochondrosis by equine synovial fluid proteomics. *J. Proteomics* **75**: 4478–4493. [Medline] [CrossRef]
6. Cooper, L., Johnson, C., Burslem, F., and Martin, P. 2005. Wound healing and inflammation genes revealed by array analysis of 'macrophageless' PU.1 null mice. *Genome Biol.* **6**: R5. [Medline] [CrossRef]
7. Dong, X., Liu, Z., Lan, D., Niu, J., Miao, J., Yang, G., Zhang, F., Sun, Y., Wang, K., and Miao, Y. 2017. Critical role of Keratin 1 in maintaining epithelial barrier and correlation of its down-regulation with the progression of inflammatory bowel disease. *Gene* **608**: 13–19. [Medline] [CrossRef]
8. Eurell, J.A., and Frappier, B.L. 2006. Dellmann's Textbook of Veterinary Histology, 6th ed. Blackwell Publishing,

Ames.
9. Haudenschild, D.R., Eldridge, A., Lein, P.J., and Chromy, B.A. 2014. High abundant protein removal from rodent blood for biomarker discovery. *Biochem. Biophys. Res. Commun.* **455**: 84–89. [Medline] [CrossRef]
10. Havlis, J., Thomas, H., Sebela, M., and Shevchenko, A. 2003. Fast-response proteomics by accelerated in-gel digestion of proteins. *Anal. Chem.* **75**: 1300–1306. [Medline] [CrossRef]
11. Hewetson, M., Sykes, B.W., Hallowell, G.D., and Tulamo, R.M. 2017. Diagnostic accuracy of blood sucrose as a screening test for equine gastric ulcer syndrome (EGUS) in adult horses. *Acta Vet. Scand.* **59**: 15. [Medline] [CrossRef]
12. Jonsson, H., and Egenvall, A. 2006. Prevalence of gastric ulceration in Swedish Standardbreds in race training. *Equine Vet. J.* **38**: 209–213. [Medline] [CrossRef]
13. Knezević, M., Aleksić-Kovacević, S., and Aleksić, Z. 2007. Cell proliferation in pathogenesis of esophagogastric lesions in pigs. *Int. Rev. Cytol.* **260**: 1–34. [Medline] [CrossRef]
14. Liu, G., Zhao, Y., Angeles, A., Hamuro, L.L., Arnold, M.E., and Shen, J.X. 2014a. A novel and cost effective method of removing excess albumin from plasma/serum samples and its impacts on LC-MS/MS bioanalysis of therapeutic proteins. *Anal. Chem.* **86**: 8336–8343. [Medline] [CrossRef]
15. Loftin, P., Cartmill, J.A., and Andrews, F.M. 2017. Gastroscopy and Esophagoscopy. pp. 195–200. *In*: Manual of Clinical Procedures in the Horse. (Costa, L.R.R., and Paradis, M.R. eds.), John Wiley & Sons, Inc., Hoboken.
16. Luthersson, N., Nielsen, K.H., Harris, P., and Parkin, T.D. 2009. The prevalence and anatomical distribution of equine gastric ulceration syndrome (EGUS) in 201 horses in Denmark. *Equine Vet. J.* **41**: 619–624. [Medline] [CrossRef]
17. May, P. 2013. The low-density lipoprotein receptor-related protein 1 in inflammation. *Curr. Opin. Lipidol.* **24**: 134–137. [Medline] [CrossRef]
18. Meng, R., Gormley, M., Bhat, V.B., Rosenberg, A., and Quong, A.A. 2011. Low abundance protein enrichment for discovery of candidate plasma protein biomarkers for early detection of breast cancer. *J. Proteomics* **75**: 366–374. [Medline] [CrossRef]
19. Neubauer, K., and Zieger, B. 2017. The mammalian septin interactome. *Front. Cell Dev. Biol.* **5**: 3. [Medline] [CrossRef]
20. Ngo, A.N., Ezoulin, M.J., Youm, I., and Youan, B.B. 2014. Optimal concentration of 2,2,2-Trichloroacetic acid for protein precipitation based on response surface methodology. *J. Anal. Bioanal. Tech.* **5**: 198. [Medline] [CrossRef]
21. Nowatzke, W., and Bowsher, R.R. 2016. The role of commercial biomarker assay kits in preclinical and clinical trials. pp. 77–86. *In*: Translating Molecular Biomarkers into Clinical Assays: Techniques and Applications. (Weiner, R., and Kelley, M. ed.), Springer International Publishing, Cham.
22. Olver, C.S., Webb, T.L., Long, L.J., Scherman, H., and Prenni, J.E. 2010. Comparison of methods for depletion of albumin and IgG from equine serum. *Vet. Clin. Pathol.* **39**: 337–345. [Medline] [CrossRef]
23. Raoufinia, R., Mota, A., Keyhanvar, N., Safari, F., Shamekhi, S., and Abdolalizadeh, J. 2016. Overview of albumin and its purification methods. *Adv. Pharm. Bull.* **6**: 495–507. [Medline] [CrossRef]
24. Riond, B., Wenger-Riggenbach, B., Hofmann-Lehmann, R., and Lutz, H. 2009. Serum protein concentrations from clinically healthy horses determined by agarose gel electrophoresis. *Vet. Clin. Pathol.* **38**: 73–77. [Medline] [CrossRef]
25. Roels, S., Ducatelle, R., and Broekaert, D. 1997. Keratin pattern in hyperkeratotic and ulcerated gastric pars oesophagea in pigs. *Res. Vet. Sci.* **62**: 165–169. [Medline] [CrossRef]
26. Rothstein, F. 1994. Differential precipitation of proteins: science and technology. pp. 115–208. *In*: Protein Process Engineering. (Harrison, R. ed.), Marcel Dekker Inc., New York.
27. Roy, M.A., Vrins, A., Beauchamp, G., and Doucet, M.Y. 2005. Prevalence of ulcers of the squamous gastric mucosa in standardbred horses. *J. Vet. Intern. Med.* **19**: 744–750. [Medline] [CrossRef]
28. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**: 671–675. [Medline] [CrossRef]
29. Shiota, S., and Yamaoka, Y. 2014. Biomarkers for Helicobacter pylori infection and gastroduodenal diseases. *Biomarkers Med.* **8**: 1127–1137. [Medline] [CrossRef]
30. Steel, L.F., Trotter, M.G., Nakajima, P.B., Mattu, T.S., Gonye, G., and Block, T. 2003. Efficient and specific removal of albumin from human serum samples. *Mol. Cell. Proteomics* **2**: 262–270. [Medline] [CrossRef]
31. Steelman, S.M., and Chowdhary, B.P. 2012. Plasma proteomics shows an elevation of the anti-inflammatory protein APOA-IV in chronic equine laminitis. *BMC Vet. Res.* **8**: 179. [Medline] [CrossRef]
32. Sykes, B.W., Hewetson, M., Hepburn, R.J., Luthersson, N., and Tamzali, Y. 2015. European college of equine internal medicine consensus statement—equine gastric ulcer syndrome in adult horses. *J. Vet. Intern. Med.* **29**: 1288–1299. [Medline] [CrossRef]
33. Taharaguchi, S., Nagano, A., Okai, K., Miyasho, T., Kuwano, M., Taniyama, H., and Yokota, H. 2007. Detection of an isoform of alpha(1)-antitrypsin in serum samples from foals with gastric ulcers. *Vet. Rec.* **161**: 338–342. [Medline] [CrossRef]
34. Tamzali, Y., Marguet, C., Priymenko, N., and Lyazrhi, F. 2011. Prevalence of gastric ulcer syndrome in high-level endurance horses. *Equine Vet. J.* **43**: 141–144. [Medline]

[CrossRef]

35. Tesena, P., Yingchutraku, Y., Wongtawan, T., and Angkanaporn, K. 2016. Electrophoresis separation of serum protein in Thoroughbred horse with gastric ulcer syndrome. pp. 316–318. Proceeding of the 9th VRVC, July 24–27, Muang Thong Thani.
36. The UniProt Consortium 2017. UniProt: the universal protein knowledgebase. *Nucleic Acids Res.* **45**:(D1): D158–D169. [Medline] [CrossRef]
37. Uhlén, M., Fagerberg, L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C.A., Odeberg, J., Djureinovic, D., Takanen, J.O., Hober, S., Alm, T., Edqvist, P.H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J.M., Hamsten, M., von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., von Heijne, G., Nielsen, J., and Pontén, F. 2015. Proteomics. Tissue-based map of the human proteome. *Science* **347**: 1260419. [Medline] [CrossRef]
38. Vatistas, N.J., Snyder, J.R., Carlson, G., Johnson, B., Arthur, R.M., Thurmond, M., Zhou, H., and Lloyd, K.L. 1999. Cross-sectional study of gastric ulcers of the squamous mucosa in thoroughbred racehorses. *Equine Vet. J. Suppl.* **29**: 34–39. [Medline]
39. Videla, R., and Andrews, F.M. 2009. New perspectives in equine gastric ulcer syndrome. *Vet. Clin. North Am. Equine Pract.* **25**: 283–301. [Medline] [CrossRef]
40. Wojcik, S.M., Bundman, D.S., and Roop, D.R. 2000. Delayed wound healing in keratin 6a knockout mice. *Mol. Cell. Biol.* **20**: 5248–5255. [Medline] [CrossRef]
41. Yahiro, K., Satoh, M., Nakano, M., Hisatsune, J., Isomoto, H., Sap, J., Suzuki, H., Nomura, F., Noda, M., Moss, J., and Hirayama, T. 2012. Low-density lipoprotein receptor-related protein-1 (LRP1) mediates autophagy and apoptosis caused by Helicobacter pylori VacA. *J. Biol. Chem.* **287**: 31104–31115. [Medline] [CrossRef]
42. Zhu, A., Kaneshiro, M., and Kaunitz, J.D. 2010. Evaluation and treatment of iron deficiency anemia: a gastroenterological perspective. *Dig. Dis. Sci.* **55**: 548–559. [Medline] [CrossRef]