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Fibrinogen heterogeneity in horses

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Abstract

Background: Fibrinogen heterogeneity has been observed in humans and can influence fibrinogen measurements when using the modified Clauss assay. We hypothesized that fibrinogen heterogeneity also exists in horses.

Objectives: To determine whether fibrinogen heterogeneity exists in horses.

Animals: Five clinically healthy horses from the university equine teaching herd.

Methods: Presumed fibrinogen was purified from pooled citrated plasma and electrophoresis performed. The purified protein was subjected to Western blotting using sheep antiserum against human fibrinogen, and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results: Gel electrophoresis of nonreduced equine purified protein yielded 2 protein bands (approximately 377 and 318 kDa) that corresponded with the molecular weights of human high molecular weight fibrinogen and low molecular weight fibrinogen fractions, respectively. Electrophoretograms of reduced purified protein, Western blots, and LC-MS/MS supported that the purified nonreduced protein bands were fibrinogen.

Conclusion: Fibrinogen heterogeneity exists in horses.

KEYWORDS
citrate, EDTA, equine, fibrinogen variants, high molecular weight fibrinogen, low molecular weight fibrinogen, protein purification, Vila method

1 | INTRODUCTION

Fibrinogen plays a critical role in coagulation and wound healing in humans and other animals. In equine medicine, measurement of plasma fibrinogen concentration is an inexpensive and widely available tool used for the detection of inflammation. Fibrinogen is a dimer with each half-molecule composed of Aα, Bβ, and γ polypeptide subunits.[1] Human fibrinogen exists in 3 forms: high molecular weight fibrinogen (HMW-Fb), low molecular weight fibrinogen (LMW-Fb), and very low molecular weight fibrinogen (LMW′-Fb), with molecular weights of approximately 340 kDa, 305 kDa, and 270 kDa, respectively.[2,3] The LMW- and LMW′-Fb are derived from HMW-Fb by proteolytic cleavage of the C-terminus of the Aα polypeptide subunit,[4,5] but the exact mechanism of this degradation remains unknown.[6] In healthy humans, the mean proportions of the fibrinogen fractions are 70% HMW-Fb, 26% LMW-Fb, and 4% LMW′-Fb.[2,3] These proportions are altered in certain disease states,[2,4,7-9] and can have prognostic utility.[10]

In veterinary medicine, total fibrinogen measurement now is commonly performed using the modified Clauss method,[11] which has largely superseded heat precipitation tests such as the Millar method.[12] In the modified Clauss method, highly concentrated thrombin is added to diluted patient plasma, and the time to clot formation is compared

Abbreviations: ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; Fb, fibrinogen; HMW, high molecular weight; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LMW, low molecular weight; LMW′, very low molecular weight; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PVDF, polyvinylidene difluoride; RI, reference interval; SAA, serum amyloid A; SDS, sodium dodecyl sulfate; TBS, tris-buffered saline.

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to a standard to curve to obtain the total fibrinogen concentration.[13] In human medicine, total fibrinogen concentrations measured by the Clauss method are higher in citrated samples than in ethylenediaminetetraacetic acid (EDTA) samples.[9] This discrepancy has been hypothesized to be a result of magnesium ion chelation in EDTA samples. Magnesium ions are thought to be required for optimal clot formation from LMW-Fb and LMW′-Fb and in EDTA samples, these fractions might be unable to contribute to clot formation, prolonging clotting times and lowering the total measured fibrinogen concentration.[9] Similar discrepancies in measured fibrinogen concentrations between citrated and EDTA plasma samples have been observed in our laboratory in equine samples, prompting us to question whether lower molecular weight fibrinogen fractions might also exist in horses.

We aimed to determine whether fibrinogen heterogeneity exists in equine plasma.

2 | MATERIALS AND METHODS

2.1 | Samples

The use of blood samples collected from our equine teaching herd was approved by the institutional animal ethics committee (ID 1714380.2).

Whole blood was collected by jugular venipuncture from 5 healthy adult horses (2 Standardbred mares, 1 Thoroughbred mare, and 2 Thoroughbred geldings) from the university's teaching herd. Horses were considered healthy and suitable for study enrollment if they had normal physical examination findings; total fibrinogen concentration (measured by the modified Clauss method in citrated plasma) within the reference interval (RI) established in our laboratory using 71 healthy horses; serum amyloid A concentration (LZ test SAA, Eiken Chemical Co, Tokyo, Japan run on ADVIA 1800 chemistry system, Siemens Healthcare Diagnostics, Erlangen, Germany) within established RIs; and normal CBC (Sysmex XN-1000, Sysmex Corporation, Kobe, Japan) and blood film cytology results (assessed by E.B. Russell; a third year clinical pathology resident).

Samples were collected into two 3.2% sodium citrate blood tubes and 2 gel-activated clot tubes (Vacuette tubes, Greiner Bio-One GmbH, Kremsmünster, Austria) from each horse. Samples were immediately chilled, centrifuged at 1500*g* at 4°C for 15 minutes within 30 minutes of collection, with the obtained serum and plasma separated and pooled.

2.2 | Purification of fibrinogen from equine plasma

Purification of fibrinogen from equine plasma was performed using a modified Vila method.[14] Precipitation of 400 μL of plasma was achieved by adding an equal volume of 80 g/L polyethylene glycol (PEG), M_n = 6000. The plasma-PEG mixture was placed in an ice bath, gently agitated for 10 minutes and then centrifuged at 1200*g* at 4°C for 7 minutes. The resulting precipitate was re-dissolved in 400 μL of 0.01 M phosphate-buffered saline (PBS), pH 7.4 (P3813, Sigma-Aldrich Pty. Ltd, Sydney, Australia) and then precipitated again by the addition of 800 μL of 2 M acetic acid-acetate buffer, pH 4.6. This mixture was placed back into an ice bath, gently agitated for 30 minutes and then centrifuged at 1200*g* at 4°C for 7 minutes. The resultant precipitate was once again dissolved in 400 μL of 0.036 M PBS, pH 7.8 with 200 000 kallikrein inhibitor units (KIU)/L aprotinin. The sample then was precipitated by the addition of 133 μL of 4 M ammonium sulfate followed by centrifugation at 1200*g* at 4°C for 7 minutes. The final precipitate was re-dissolved in 400 μL of 0.018 M PBS, pH 7.8 with 200 000 KIU/L aprotinin. To create negative control samples for Western blotting, the purification procedure was performed simultaneously on 400 μL of pooled serum.

2.3 | Gel electrophoresis of purified horse protein

Protein concentrations in purified plasma and serum samples were estimated using a commercial colorimetric assay[15] (Pierce BCA Protein Assay Kit, Thermo Scientific, Scoresby, Australia), in accordance with the manufacturer's directions.

Purified (presumptive) fibrinogen was subjected to electrophoresis in a nonreduced form to evaluate for the presence of fibrinogen heterogeneity (ie, the existence of high and low molecular weight fractions). Purified fibrinogen also was subjected to electrophoresis after reduction to ensure that the protein was comprised of the expected fibrinogen subunits (Aα, Bβ, and γ). For the nonreduced sample, 4 μg/μL purified pooled horse fibrinogen in 1× Laemmli sample buffer (Bio-Rad, Gladesville, Australia) was heated to 70°C for 10 minutes, then subjected to electrophoresis using tris-acetate sodium dodecyl sulfate (SDS) running buffer (NuPAGE Tris-Acetate SDS Running Buffer, Thermo Scientific) on a 3% to 8% tris-acetate polyacrylamide gel electrophoresis (PAGE) gel (NuPAGE Tris-Acetate Protein Gels, Thermo Scientific) for 55 minutes at 150 V (constant). For the reduced sample, 4 μg/μL purified pooled equine fibrinogen in 1× Laemmli sample buffer with 355 mM 2-mercaptoethanol (Bio-Rad) was heated to 100°C for 5 minutes, then subjected to electrophoresis using tris-glycine running buffer (Bio-Rad) on an 8% to 16% tris-glycine PAGE gel (Mini-Protean TGX Precast Protein Gels, Bio-Rad) for 45 minutes at 150 V (constant). For both reduced and nonreduced samples, prestained protein standards (HiMark Pre-stained protein standard, Thermo Scientific and Precision Plus Protein Dual Color protein standard, Bio-Rad) were included for molecular weight estimation, 3 μg of human fibrinogen (Sigma-Aldrich Pty. Ltd) was included as a positive control, and purified pooled equine serum (7.5 μL) and nonpurified pooled equine serum (3 μg) were included as negative controls. Purified pooled equine serum (treated identically to the purified plasma samples) was included as a negative control because it should not contain coagulation proteins (eg, fibrinogen). Nonpurified pooled equine serum also was included to detect nonspecific protein binding by antifibrinogen antibodies during Western blotting.

Gels were rinsed with deionized water to remove remaining SDS and then stained using Coomassie Blue (Bio-Safe Coomassie Stain, Bio-Rad), according to the manufacturer's protocol. Densitometry scanning was performed (ChemiDoc XRS+ system, Bio-Rad) to convert the optical densities of the obtained gel bands into an electrophoretogram. Molecular

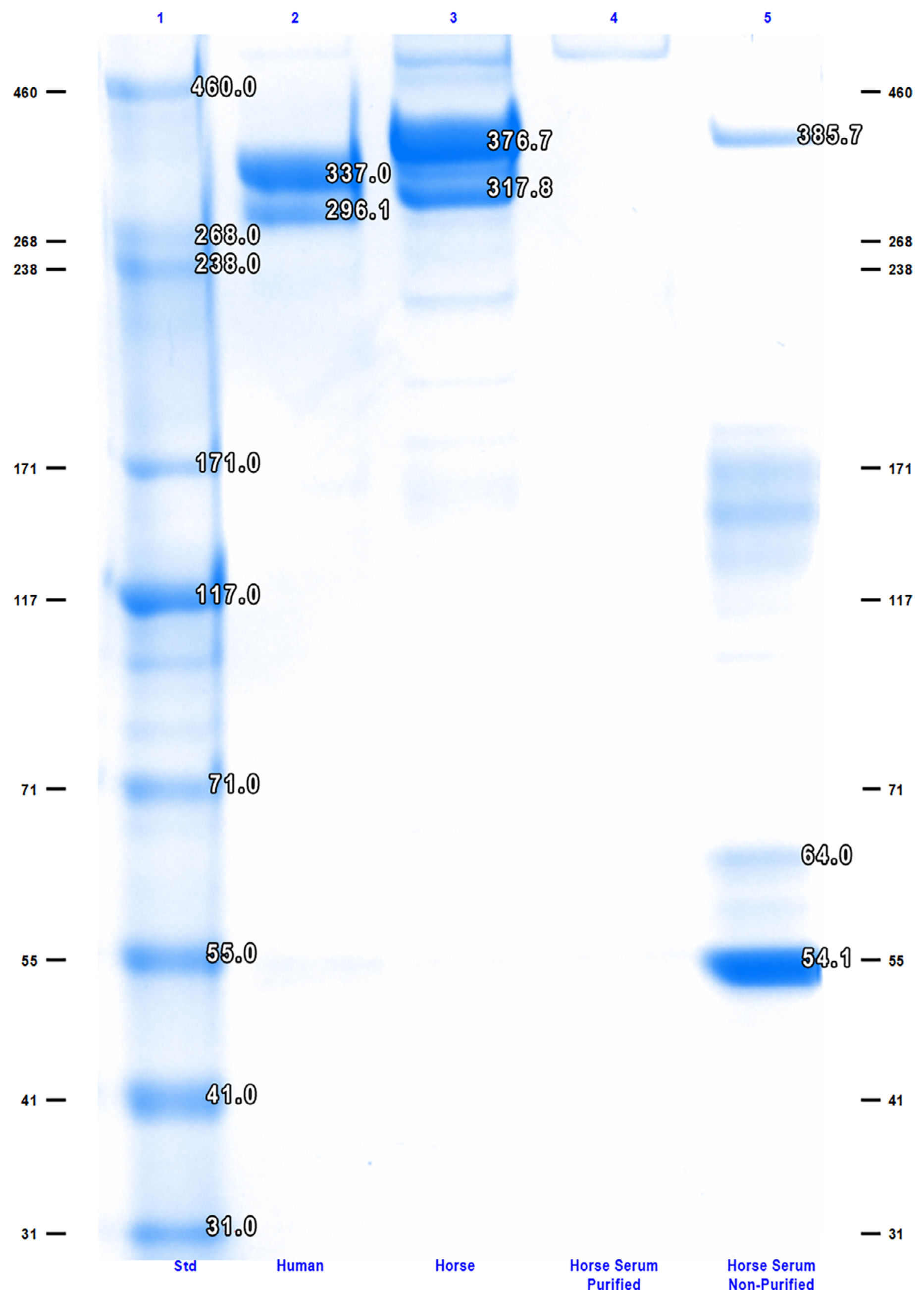


FIGURE 1 SDS-PAGE of nonreduced purified horse protein demonstrating the existence of two protein fractions, Coomassie Blue. Two distinct bands were observed, with molecular weights of 379 and 318 kDa. Lane 1: protein standard 15 μL, lane 2: human fibrinogen 3 μg, lane 3: purified pooled horse protein (presumed fibrinogen) 3 μg, lane 4: purified pooled horse serum 7.5 μL and lane 5: nonpurified pooled horse serum 3 μg. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

weight estimation of protein bands was performed using a point-to-point semilog regression method (ImageLab 6.0.1 software, Bio-Rad).

The proportion of each fibrinogen fraction was calculated from densitometric scans. The LMW- and LMW′-Fb were grouped together as LMW/LMW′-Fb, as described in previous studies,[16,17] because of poor separation of these bands and low proportions of LMW′-Fb.

2.4 | Western blotting

Western blotting was performed as previously described[18,19] to confirm that the purified, nonreduced proteins were fibrinogen and that the purified, reduced proteins were fibrinogen polypeptide subunits. Quantities of loaded protein were adjusted based on preliminary Western blots to optimize visibility. Proteins were transferred from the SDS-PAGE gel to a polyvinylidene difluoride (PVDF) membrane using a rapid transfer system (Trans-Blot Turbo Transfer System, Bio-Rad) and ready-to-assemble transfer kits (Trans-Blot Turbo Midi PVDF Transfer packs, Bio-Rad). Protein transfer was performed at 1.3 A, up to 25 V, for 15 minutes for nonreduced samples or for 7 minutes for reduced samples. After protein transfer, membranes were rinsed, and then soaked in washing buffer (tris-buffered saline [TBS] with 0.1% Tween 20, Chem-supply, Port Adelaide, Australia). Nonspecific antibody binding was minimized by blocking membranes with TBS and 5% skim milk for 1 hour at room temperature. Membranes then were incubated with 1 mg/mL sheep anti-human fibrinogen IgG antibodies conjugated to horseradish peroxidase (Affinity Biologicals, Ancaster, Canada) at a 1 : 10 000 dilution for 1 hour at room temperature. After removal of

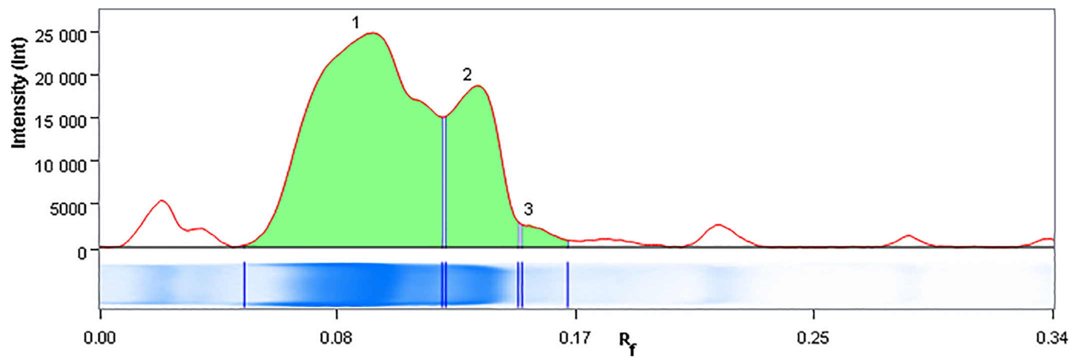


FIGURE 2 Nonreduced purified horse protein lane profile. Two distinct peaks are observed in the region expected of HMW (peak 1) and LMW Fb (peak 2). An indistinct shoulder (peak 3) is observed at 289 kDa. Fb, fibrinogen; HMW, high molecular weight; LMW, low molecular weight

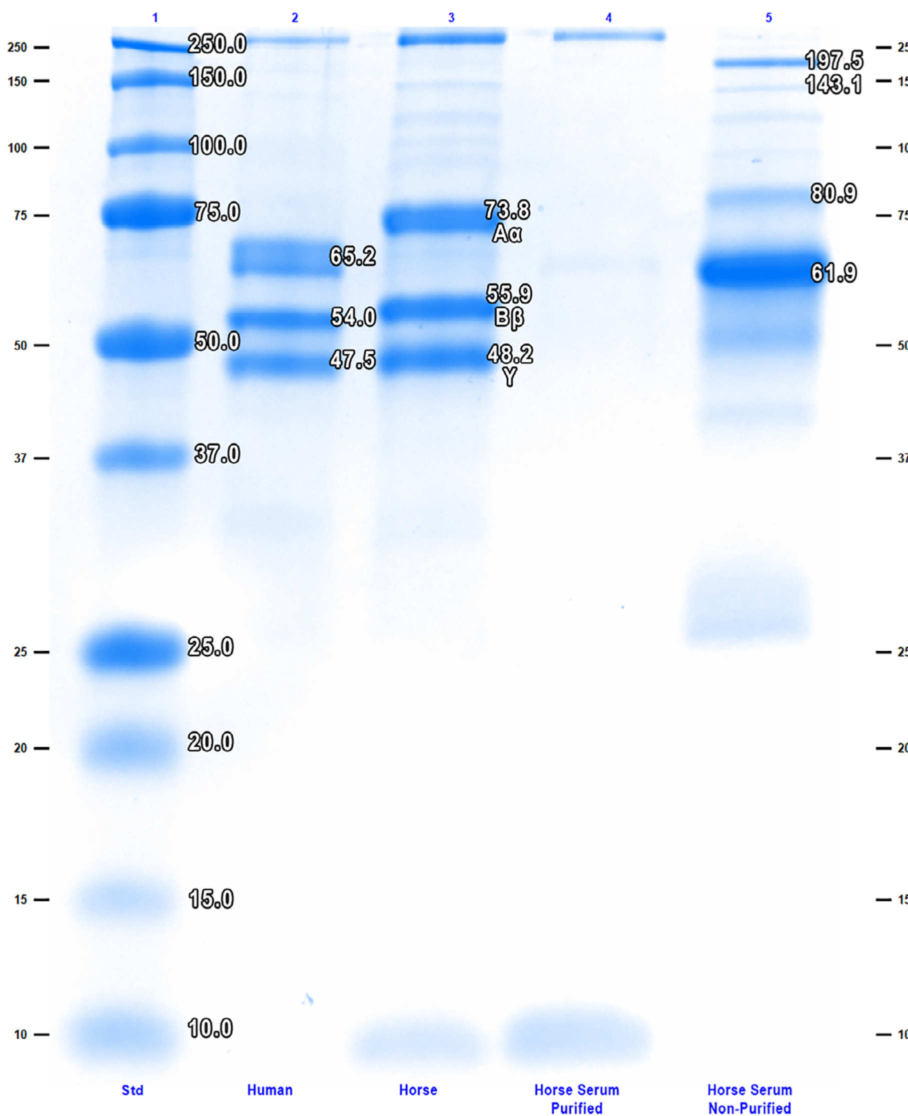
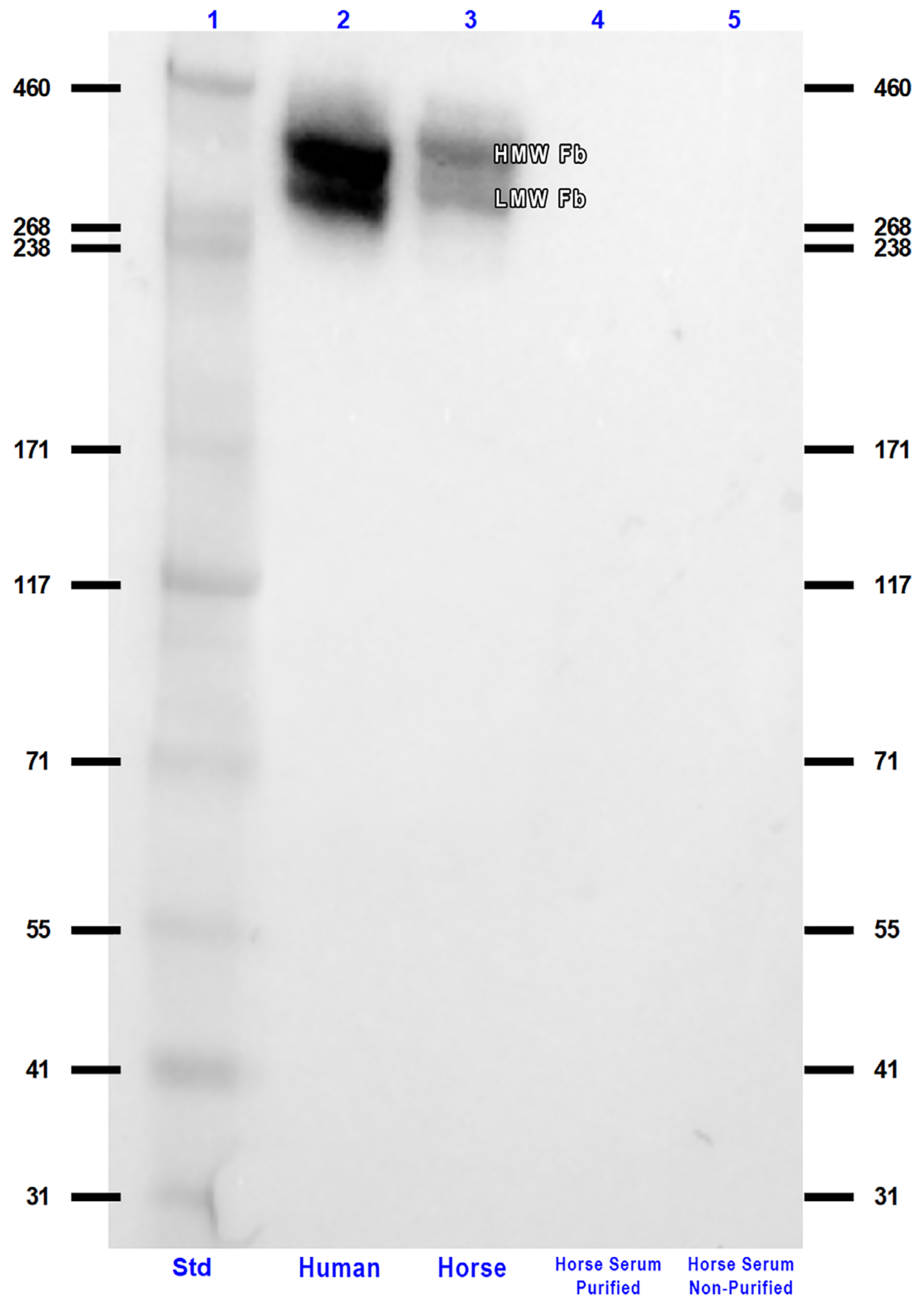


FIGURE 3 SDS-PAGE of reduced purified horse protein demonstrating three protein bands corresponding to Aα, Bß, and γ polypeptide chains, Coomassie Blue. Lane 1: protein standard 4 μL, lane 2: human fibrinogen 3 μg, lane 3: purified pooled horse protein (presumed fibrinogen) 3 μg, lane 4: purified pooled horse serum 7.5 μL, and lane 5: nonpurified pooled horse purified serum 3 μg. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

the antibody solution and washing, enhanced chemiluminescence (ECL) substrate (Clarity Western ECL Substrate, Bio-Rad) was added for generation of a chemiluminescent signal. Membranes were imaged using chemiluminescent and colorimetric settings to allow visualization of both samples and molecular mass markers in a single merged image (ChemiDoc XRS+ system and ImageLab 6.0.1 software, Bio-Rad).

To intensify the Aα chain chemiluminescent signal, SDS-PAGE and Western blotting were repeated on the reduced sample under

FIGURE 4 Western blot of nonreduced purified horse fibrinogen. The two isolated protein bands bound sheep anti-human fibrinogen antibody, identifying them as fibrinogen. Lane 1: protein standard 15 μL, lane 2: human fibrinogen 0.2 μg, lane 3: purified pooled horse fibrinogen 0.5 μg, lane 4: purified pooled horse serum 7.5 μL, and lane 5: nonpurified pooled horse serum 1 μg



identical conditions to those described above but using a 3% to 8% tris-acetate gel and tris-acetate SDS running buffer (NuPAGE Tris-Acetate Protein Gels and SDS Running Buffer, Thermo Scientific). The horse protein lane was excised from the PDVF membrane before chemiluminescent signal detection.

2.5 | Confirmation of findings by centrifugal filtration

To confirm that the lower molecular weight bands corresponding to LMW/LMW′-Fb identified on SDS-PAGE were not an in vitro artifact of chemical manipulation associated with the fibrinogen purification procedure, 500 μL of citrated plasma from a single horse was filtered using a centrifugal device (Amicon Ultra 0.5 mL 100 kDa Centrifugal Filter, Merck, Darmstadt, Germany). The filtration device was centrifuged at 14 000*g* for 10 minutes, with mixing of the sample by gentle pipetting at 5 minutes. The concentrate obtained then was subjected to electrophoresis in a non-reduced form followed by Western blotting as described above.

2.6 | Liquid chromatography-tandem mass spectrometry

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed to further confirm that the 2 isolated nonreduced protein bands were fibrinogen. Protein bands corresponding to HMW-Fb and LMW-Fb were excised from the gel and prepared for LC-MS/MS based

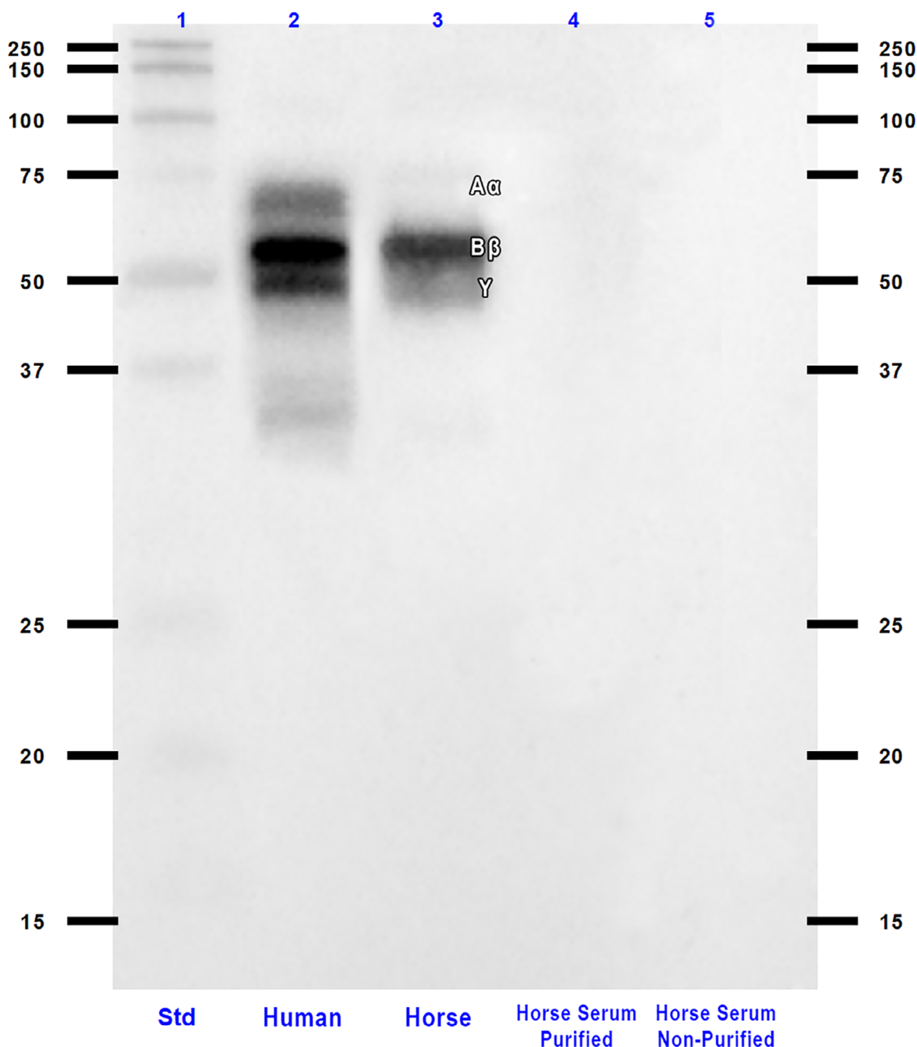


FIGURE 5 Western blot of reduced purified horse fibrinogen. There is a distinct chemiluminescent signal in the region of the Bß chain and an indistinct signal in the region of the γ chain, identifying these protein bands as fibrinogen. There is a very faint signal in the region of the Aα band. Lane 1: protein standard 4 μL, lane 2: human fibrinogen 0.75 μg, lane 3: purified pooled horse fibrinogen 3 μg, lane 4: purified pooled horse serum 7.5 μL, and lane 6: nonpurified pooled horse serum 3 μg

on previously described protocols.[20-23] Liquid chromatography-tandem mass spectrometry was performed using a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive Mass Spectrometer, Thermo Scientific) fitted with nanoflow reversed-phase-high performance liquid chromatography (HPLC; Dionex Ultimate 3000 RSLC, Thermo Scientific) for separation of peptides.

Raw data files from LC-MS/MS were searched against the *Equus caballus* protein reference proteomes (UniProt Proteome ID: UP000002281) using MaxQuant-Andromeda (version 1.6.7.0).[24] The false discovery rate (FDR) was set at 0.01 for both peptides and proteins.[24-26] Andromeda results were filtered to exclude potential contaminants, and include only proteins with scores >20 (based on our previously selected FDR of 0.01) to exclude low confidence identifications.[25]

3 | RESULTS

3.1 | Gel electrophoresis of purified horse protein

Gel electrophoresis of nonreduced samples showed separation of purified horse protein into 2 distinct bands of approximately 377 and 318 kDa (Figure 1), with a small shoulder at 289 kDa visible only on densitometry scanning (Figure 2). Approximate proportions were 74% HMW-Fb, and 26% LMW-Fb and LMW′-Fb.

Gel electrophoresis of reduced samples showed separation of the purified equine fibrinogen into 3 distinct bands of approximately 74, 56, and 48 kDa (Figure 3), similar to reported molecular weights of horse Aα, Bß, and γ chains, respectively.[27] For both nonreduced and reduced samples, no protein bands were visible in the lane containing purified pooled equine serum.

Gel electrophoresis of the nonreduced sample obtained by centrifugal plasma filtration showed 2 distinct bands of similar molecular weights as those obtained using the modified Vila method (Figure S1).

3.2 | Western blotting of purified horse protein

Western blot membranes obtained after electrophoresis of nonreduced purified equine protein showed 2 bands after incubation with labeled antifibrinogen antibodies, confirming their identity as fibrinogen (Figure 4).

Western blot membranes obtained after electrophoresis of reduced purified equine protein showed 1 distinct band corresponding to the Bß chain, an indistinct band corresponding to the γ chain and a very faint

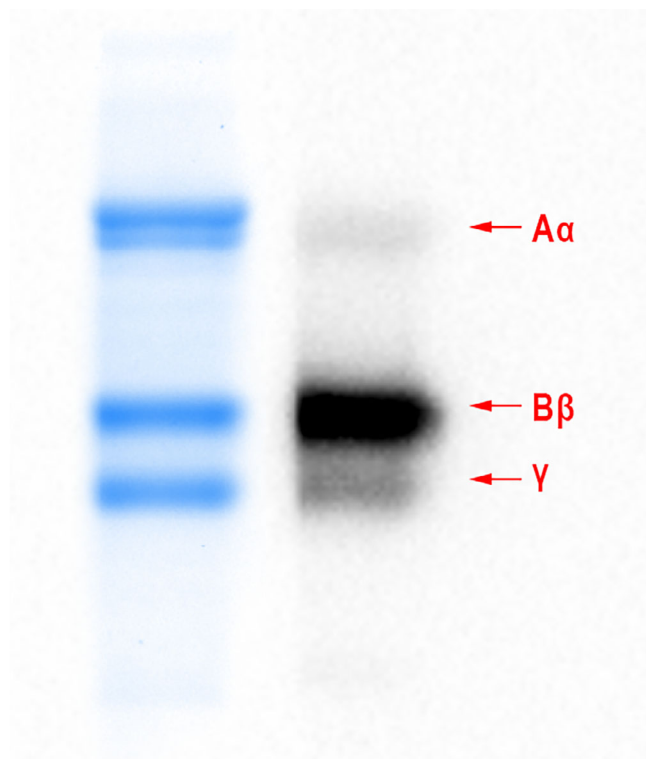


FIGURE 6 Western blot of excised reduced purified pooled horse fibrinogen lane (right) showing a more conspicuous signal in the region of the Aα chain. The corresponding SDS-PAGE lane has been provided for comparison (left). SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

band in the region of the Aα chain (Figure 5). When electrophoresis and Western blotting were repeated and the lane containing purified equine fibrinogen was separated and imaged using a prolonged exposure without the human fibrinogen control lane, the band in the region of the Aα chain was more conspicuous (Figure 6). For both reduced and nonreduced samples, no chemiluminescent signal was detected in the negative control lanes containing purified and nonpurified pooled equine serum.

Western blotting of the nonreduced sample obtained by centrifugal plasma filtration showed 2 distinct bands of similar molecular weights to those obtained using the modified Vila method (Figure S2).

3.3 | Liquid chromatography with tandem mass spectrometry of purified horse protein

Liquid chromatography with tandem mass spectrometry of proteins bands corresponding to HMW-Fb and LMW-Fb both generated spectra consistent with Aα, Bβ, and γ fibrinogen subunits, among several other proteins. Of all the suggested proteins, the Aα, Bβ, and γ fibrinogen subunits had the highest numbers of razor and unique peptides, highest sequence coverage, and highest protein scores and sequencing events (MS/MS counts; Table S1).

4 | DISCUSSION

We found fibrinogen heterogeneity in the plasma of a small group of healthy horses, with 2 major fractions identified. Ours is the first study to isolate lower molecular weight fibrinogen fractions in a nonhuman species and describes a technique for purification of these proteins from equine plasma. The molecular weights of HMW-Fb and LMW-Fb in horses were found to be slightly higher than those described in humans,[3] but fraction proportions were remarkably similar to those found in previous studies of healthy humans.[2,3] The small shoulder observed at 289 kDa on densitometry scanning of the nonreduced pooled equine fibrinogen gel is presumed to correspond to LMW′-Fb, given its similar molecular weight to human LMW′ fibrinogen. However, because of its low proportion, further analysis by Western blotting or mass spectrometry was not performed and thus we cannot be sure that this protein truly is fibrinogen.

Reduced purified equine fibrinogen had poor Aα chain antibody binding on the Western blot, but mass spectrometry identified the presence of fibrinogen Aα chain in purified samples, supporting the identity of this protein. The poor antibody binding likely reflects the use of anti-human, rather than anti-horse fibrinogen antibodies. The suboptimal avidity of anti-human fibrinogen antibody for the horse Aα chain may reflect the difference in molecular weight of this protein in horses (74 kDa) versus humans (66 kDa) on gel electrophoresis, which suggests differences in structure. When comparing the percentage identity of the various fibrinogen chains between humans and horses using a Needleman-Wunsch global alignment,[28-30] the percentage identity of Bß and γ chains was 82% (accession XP_003364583.1 and AAA18024.2, NCBI protein) and 84% (accession XP_001914833.2 and AAB59531.1, NCBI protein), respectively, but Aα chains showed only 44% identity (accession XP_005607860 and AAA17055.1, NCBI protein). Unfortunately, anti-horse fibrinogen antibodies are not currently commercially available.

As suggested earlier, it is uncertain if the lower molecular weight fibrinogen fractions truly exist in vivo, and one could speculate that the lower molecular weight fibrinogen fractions identified in our study are simply an artifact of poor sample preservation. We attempted to limit in vitro degradation by replicating methodology of studies of humans as closely as possible, ensuring timely processing (ie, cold centrifugation within 30 minutes of collection followed by immediate purification) and using freshly prepared aprotinin as an antiprotease. The electrophoretogram and Western blot obtained from centrifugal filtration of plasma indicate that the LMW/LMW′-Fb fractions are unlikely to be artifacts of chemical manipulation of fibrinogen using the modified Vila method. The third band (approximately 350 kDa) observed in the horse lane of the PAGE gel after centrifugal filtration also was present in unfiltered serum, and was not visible on Western blots, thus this band likely represents a nonfibrinogen protein. Regardless, whether lower molecular weight fractions are products of in vivo or in vitro degradation, they are likely to be present in samples presented to clinical pathology laboratories for processing, and thus could be the cause of differences in total fibrinogen between citrated and EDTA-anticoagulated equine plasma observed in some horses in our laboratory.

Mass spectrometry identified several nonfibrinogen proteins in both the 379 and 318 kDa protein bands, which is not surprising considering the method of protein purification (multistep precipitation rather than immunologic methods) and the high sensitivity of mass spectrometry. Although absolute quantification of proteins was not performed, fibrinogen proteins had much higher razor and unique peptide counts, and higher sequence coverage than did nonfibrinogen proteins, which suggests a higher likelihood that fibrinogen proteins were present. In addition, their markedly higher MS/MS counts suggest that they were probably the most abundant protein in these lanes.[31]

Our study was limited by the low number of healthy horses, samples from which were pooled together rather than run individually for the first part of this study. This approach was used to facilitate rapid sample processing and limit protein degradation. Considering that the aim of our study was to identify the existence of fibrinogen heterogeneity in horses, rather than to determine RIs for fraction proportions, pooling was considered most suitable. It remains possible that fibrinogen heterogeneity may not occur in all horses, because only a small group of pooled samples was evaluated.

Additional studies are needed to determine if the difference between EDTA and citrated plasma fibrinogen concentrations in horses is caused by the presence of lower molecular weight fibrinogen, and if this can be corrected with the addition of magnesium chloride to EDTA samples, as has been done in humans.[9] Although the proportions of fibrinogen fractions in our study are similar to those described in humans, ours was not a RI study and larger scale studies are required to determine the true prevalence of fibrinogen heterogeneity in horses and the relative fraction proportions in health.

The modified Vila method of fibrinogen purification is not commercially feasible, because it is time- and labor-intensive. Although not commercially feasible, the modified Vila method may be suitable for research purposes, and for development of a more time- and labor-efficient alternative, such as immunoassay.

In conclusion, we identified the existence of fibrinogen heterogeneity in horses, which, with additional studies, could be determined to have important implications for how fibrinogen concentrations are analyzed and interpreted in this species.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Ethics approval was granted for the collection of small volumes of blood for research purposes from the University of Melbourne equine teaching herd (1714380.2).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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