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Received: 11 December 2023 | Revised: 7 March 2024 | Accepted: 2 April 2024

DOI: 10.1111/vcp.13352

ORIGINAL ARTICLE

Development and validation of an ELISA to measure regenerating island-derived protein 3E in canine blood

Laureen M. Peters[1] | Theresia Reding Graf[2] | Luca Giori[3] | Meike Mevissen[4] | Rolf Graf[2] | Judith Howard[1]

[1]Clinical Diagnostic Laboratory, Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, Bern, Switzerland

[2]Pancreas Research Laboratory, Department of Surgery and Transplantation, University Hospital Zürich, University of Zürich, Zürich, Switzerland

[3]Endocrinology Service, Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee, USA

[4]Division of Veterinary Pharmacology and Toxicology, Department of Clinical Research and Veterinary Public Health, Vetsuisse Faculty, University of Bern, Bern, Switzerland

Correspondence
Laureen M. Peters, Clinical Diagnostic Laboratory, Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, Länggasstrasse 124, Bern 3012, Switzerland.
Email: laureen.peters@unibe.ch

Funding information
Stiftung Tierspital, Vetsuisse Faculty Bern, Switzerland

Abstract

Background: Regenerating island-derived proteins (REG) are upregulated in people with sepsis, pancreatitis, and gastrointestinal diseases. One member of the REG family, namely REG3E, was recently identified in pancreatic tissue and plasma of dogs, with high expression in pancreatitis and sepsis.

Objectives: We aimed to develop and validate an ELISA to measure REG3E concentrations in canine blood.

Methods: An indirect sandwich ELISA was developed using recombinant canine REG3E protein and polyclonal anti-canine REG3E antibodies raised in guinea pigs and rabbits. Antibody specificity was assessed using western blot and mass spectrometric analysis of protein purified from canine plasma. Assay validation included evaluation of dilutional linearity, parallelism, spiking recovery, repeatability and reproducibility, stability, interferences, and comparison of serum and heparinized plasma.

Results: Antibodies bound specifically to REG3E with no evidence of cross-reactivity with other proteins. The limit of detection of the ELISA was 15 ng/mL, and the lower limit of quantification was 30 ng/mL. The assay demonstrated good to excellent linearity, dilutional and mixing parallelism, and recovery, with mean observed-to-expected ratios of 104%, 107%, 102%, and 92%, respectively, and no evidence of a hook effect. Coefficients of variation were ≤8.5% for repeatability and ≤14.3% for reproducibility at three different levels. Measurements of REG3E in plasma were not significantly influenced by different storage conditions, freeze-thawing cycles, hemolysis, lipemia, or icterus. There was no significant difference between REG3E concentrations in heparinized plasma and serum samples.

Conclusions: The canine REG3E ELISA has acceptable precision, accuracy, linearity, and reproducibility for the measurement of REG3E in canine plasma and serum.

KEYWORDS
analytical validation, biomarker, pancreas, pancreatitis-associated protein

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1 | INTRODUCTION

Regenerating island- (or islet-) derived proteins (REG) are small (approximately 16–17 kDa) proteins with a C-type lectin domain,[1] expressed primarily in the pancreas and small intestine.[2] They have been attributed to a multitude of biological functions, including the promotion of cell proliferation, differentiation, and, as their name suggests, regeneration.[3–5] Furthermore, these proteins have been shown to have antibacterial[6–8] and immunomodulatory effects.[9–11] Owing to their increased expression in response to both acute and chronic inflammation, these proteins have also been termed pancreatic secretory stress proteins.[2,12,13]

In people, the most widely studied member of the REG protein family is REG1A, also known as pancreatic stone protein, due to its discovery in pancreatic stones in patients with chronic pancreatitis.[14] More recently, REG1A has gained significance as a promising novel marker for sepsis,[15] both for the early detection of sepsis development[16] and for the prediction of survival.[17] Serum concentrations of both REG1A and REG3A, also termed pancreatitis-associated protein, are increased in pancreatitis,[18,19] and upregulation of REG1A and REG3A genes and proteins has been demonstrated in people with inflammatory bowel disease, including Crohn's disease and ulcerative colitis.[20–22] Furthermore, REG3A has been shown to be overexpressed in cystic fibrosis, making it a valuable novel marker for newborn screening.[23,24]

While REG proteins have been studied in laboratory animal models, very little is known about this protein family in dogs. There is genomic and proteomic evidence for the expression of a REG3 subfamily member, namely REG3E, in the pancreas of dogs, which appears to be increased in pancreatitis.[25] Furthermore, the presence of REG3E was demonstrated in plasma from dogs with sepsis and gastrointestinal disease.[25] Additionally, in both dogs and cats, fecal concentrations of a member of the REG3 protein subfamily, identified as PAP-1/REG3α by the authors, were greater in animals with chronic enteropathies compared to healthy controls.[26,27]

Taken together, these findings suggest that canine REG3E could parallel the functions of REG3A and possibly REG1A in people, making it a potentially interesting biomarker for sepsis and gastrointestinal and pancreatic diseases in dogs. The goals of this study were therefore to develop an ELISA to measure REG3E concentrations in canine blood and to analytically validate this novel assay.

2 | MATERIALS AND METHODS

2.1 | Production of recombinant canine REG3E protein and anti-canine REG3E antibodies

Recombinant protein, spanning the amino acids 28–176 of the canine REG3E protein, was expressed in Chinese hamster ovary cells by a commercial production service (Biotem Custom Antibodies & Services, Apprieu, France). Briefly, the gene was synthesized and cloned into the company's expression vector with a human Fc recombinant protein tag and transfected in Chinese hamster ovary cells. The recombinant protein was harvested from the cell culture supernatant and purified by protein A affinity chromatography. The tag was cleaved using human Rhinovirus 3C protease and removed by protein A purification. Glycosylated protein was removed with reverse-phase high-performance liquid chromatography.

Polyclonal antibodies against canine REG3E were produced by a commercial laboratory (Biotem Custom Antibodies & Services, Apprieu, France). In short, two New Zealand White rabbits were injected four times with purified recombinant canine REG3E protein emulsified in incomplete Freund's adjuvant. In parallel, three guinea pigs were initially inoculated with purified recombinant canine REG3E protein emulsified in complete Freund's adjuvant, followed by four boosters of recombinant protein in incomplete Freund's adjuvant. Antibody titers were monitored by indirect ELISA on plates coated with recombinant canine REG3E protein starting 14 days after the third inoculation, and when sufficient antibodies were raised against the target protein, serum was harvested by exsanguination. For each species, the pooled polyclonal serum was subsequently purified by affinity chromatography against the recombinant canine REG3E protein using a chromatography column (HiTrap NHS-activated HP chromatography column, GE Healthcare, Tremblay-en-France, France) prepared in accordance with the manufacturer's instructions. Purity was verified by polyacrylamide gel electrophoresis.

2.2 | Specificity testing of anti-canine REG3E antibodies

Western blotting was performed, as previously described.[25] In short, 12 μL aliquots of canine plasma with suspected high REG3E concentrations were loaded onto acrylamide gels (Bolt 10% Bis-Tris Plus, Invitrogen, Reinach, Switzerland), separated by an electrophoretic gradient and blotted onto polyvinylidene difluoride membranes (Trans-Blot Turbo Mini 0.2 μm PVDF Transfer Packs, BioRad, Feldkirchen, Germany). The membranes were blocked for 3 h in 5% milk (Rapilait, Migros, Switzerland), incubated overnight at 4°C with guinea pig or rabbit anti-canine REG3E antibody at a 1:1000 dilution, followed by 2 h incubation at room temperature with a secondary anti-guinea pig or anti-rabbit IgG horseradish peroxidase-coupled antibody, respectively, at a 1:2000 dilution, and 5 min incubation with the chemiluminescent substrate (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, Reinach, Switzerland).

Additionally, REG3E protein was purified from canine plasma using Protein A Sepharose High Performance SpinTrap columns and buffer kit (both Cytavia, Marlborough, MA, USA), following the manufacturer's instructions for crosslink protocol. Briefly, naive columns were incubated for 1 h with 300 μL of pooled canine plasma with high REG3E concentration (as measured by the ELISA described herein) to reduce non-specifically binding proteins from the sample; the plasma was subsequently collected for further use, and columns

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were discarded. New columns were incubated for 30 min at room temperature with 200 μL antibody solution (1 mg/mL guinea pig or rabbit anti-canine REG3E antibodies, respectively), subsequently crosslinked for 60 min at room temperature using dimethyl pimelimidate dihydrochloride (Sigma-Aldrich, Buchs, Switzerland) in triethanolamine (Cytavia), and blocked with ethanolamine (Cytavia). After several wash steps to eliminate unbound antibody, columns were incubated for 1 h at room temperature with 250 μL pre-purified canine plasma and subsequently washed to eliminate unbound protein. Bound protein was thereafter eluted using 2 M urea (Sigma-Aldrich) in 0.1 M glycine-HCl at pH 2.9 (Cytavia), and sample pH was neutralized with the addition of Tris–HCl buffer (Sigma-Aldrich). One aliquot of protein eluted using the guinea pig antibody was additionally subjected to the entire procedure using a column bound with rabbit antibody to evaluate binding to both antibodies in series. Furthermore, one plasma aliquot underwent the whole protocol using a blank column without antibody to assess non-specific binding to the protein A Sepharose matrix. Western blot was performed on eluted protein samples, as described above.

Eluted protein samples were submitted to the Proteomics and Mass Spectrometry Core Facility at the Department for Biomedical Research of the University of Bern, Switzerland, for protein analysis. Briefly, protein eluates underwent in-gel digestion, as described earlier,[28] and were subsequently analyzed by liquid chromatography (Dionex Ultimate 3000, ThermoFisher Scientific, Reinach, Switzerland) coupled to a mass spectrometer (QExactive HF, ThermoFisher Scientific, Reinach, Switzerland) using a top15 acquisition method. Mass spectrometry data were subsequently searched with FragPipe[29,30] (version 20.0) against the Canis Lupus UniprotKB protein database downloaded in September 2023 and concatenated 230 common contaminant protein sequences. The mass spectrometry proteomics data, including detailed sample and data processing protocols, have been deposited to the ProteomeXchange Consortium via the PRIDE[31] partner repository with the dataset identifier PXD046936 (https://www.proteomexchange.org/).

2.3 | Development of a sandwich ELISA for measurement of canine REG3E

This assay was designed based on the previously published protocol for the anti-human REG3A ELISA[12] with optimization of different parameters, including antibody concentrations, blocking solutions, sample and washing buffers, and incubation times during several trial runs. In the final protocol, flat-bottom, 96-well enhanced binding ELISA plates (Nunc-Immuno Plate, MaxiSorp Surface, Thermo Fisher Scientific) were coated with polyclonal guinea pig anti-canine REG3E antibodies at a 1:1000 dilution in TRIS-buffered saline (TBS; Thermo Fisher Scientific), corresponding to 1.8 ng/μL or 180 ng antibody per well, and incubated overnight at room temperature on a shaker. Optimal working concentrations for primary and secondary antibodies were previously determined using a matrix of different combinations of concentrations ranging from 0.9 to 3.6 μg/mL (1:2000–1:500 dilution) for the capture antibody and 0.2 to 2.0 μg/mL (1:5000–1:500 dilution) for the detection antibody. The plate was subsequently washed in 3 cycles with washing buffer (TBS with 0.05% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20, Sigma-Aldrich)) using an automated plate washer (Crocodile 5-in-one ELISA miniWorkstation, Berthold Technologies GmbH & Co. KG, Bad Wildbach, Germany) and blocked for 1 h with 100 μL 1% bovine serum albumin (BSA; Sigma-Aldrich) in TBS per well with shaking motion to avoid non-specific protein binding, followed by 3 wash cycles as described above.

For standard solutions, 16, 8, 4, 2, 1, 0.5, and 0.25 ng/mL recombinant REG3E protein were dissolved in sample diluent (1% BSA in 1× TBS); no protein was added to the blank. Plasma or serum samples were diluted 1:25, 1:250, 1:1000, or 1:2000 in sample diluent, depending on anticipated protein concentration based on prior measurements using this assay and clinical presentation with diseases potentially consistent with high concentrations when extrapolated from human medicine (i.e., sepsis, pancreatitis, gastrointestinal diseases). A total of 100 μL of all standards and samples was loaded onto each plate in duplicates and incubated on the shaker for 1 h at room temperature. Following another three wash cycles, polyclonal rabbit anti-canine REG3E antibody was added to the plates at a 1:1000 dilution in sample diluent, corresponding to 1.04 ng/μL or 104 ng antibody per well, and incubated with shaking motion for 1 h at room temperature. After three wash cycles, alkaline phosphatase coupled monoclonal mouse anti-rabbit IgG γ-chain specific antibody (Sigma-Aldrich) was added at a 1:6000 dilution in sample diluent and incubated for 45 min, shaking, at room temperature. After three wash cycles, wells were incubated for 20 min at room temperature with 100 μg/well 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich) dissolved in diethanolamine substrate (Thermo Fisher Scientific). The reaction was stopped with 50 μL/well 2 M NaOH (Thermo Fisher Scientific) and read at a wavelength of 405 nm after 5 min using an automated plate reader (Crocodile 5-in-one ELISA miniWorkstation, Berthold Technologies GmbH & Co. KG). Standard curve and sample REG3E concentrations were calculated with commercial software (MicroWin 2010, Labsis Laborsysteme GmbH, Neunkirchen-Seelscheid, Germany), using a four-parameter logistic curve fit algorithm.

2.4 | Analytical validation

Analytical validation was performed following quality assessment and control guidelines and recommendations of the American Society of Veterinary Clinical Pathology, Food and Drug Administration, and European Medicines Agency.[32–36]

The limit of blank (LoB) was determined by calculating the protein concentration corresponding to the mean + 1.65 standard deviation (SD) of optical density of 20 replicates of blank sample diluent (100 μL 1% BSA/1× TBS) based on the standard curve. The limit of detection (LoD) was calculated by adding 1.65 SD of 20 replicates of sample diluent spiked with different low concentrations

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of recombinant REG3E protein (1 ng/mL, 1.5 ng/mL, 2 ng/mL) to the previously determined LoB. The lower and upper limits of quantification (LLoQ and ULoQ) were determined as the lowest and highest concentrations, respectively, from the different validation experiments described below, which demonstrated acceptable imprecision of below 20% for the majority of samples.[34–36]

Dilutional linearity was evaluated by spiking five plasma samples with low endogenous REG3E concentrations with recombinant REG3E protein to achieve concentrations of 1000, 2500, 5000, 7500, and 10 000 ng/mL and diluting back to serial concentrations within the measuring range of the assay (2, 4, 8 ng/mL). Before dilution, REG3E concentrations were measured in spiked samples to rule out a hook effect. To assess dilutional parallelism, five plasma samples with moderate REG concentrations were diluted in serial twofold dilutions within the measuring range (1:2, 1:4, 1:8). Additionally, parallelism was evaluated by mixing plasma samples with high and low REG3E concentrations within the linear range of the respective sample dilutions of 1:25 and 1:250 (three pairs each) at ratios of 100:0%, 75:25%, 50:50%, 25:75% and 0:100%. To study spiking recovery, five plasma samples with very low REG3E concentrations were spiked with 90, 180, and 300 ng/mL recombinant REG3E. The sample diluent was added at the same volume as the recombinant protein to the neat sample. Observed-to-expected ratios (O/E; [observed concentration (ng/mL)/expected value (ng/mL)] × 100), expressed in %, were calculated for all three above experiments and assessed with scatter plots and linear regression. Statistical evidence of linearity was accepted when the 95% confidence intervals (CI) for the slope included 1.[32]

Repeatability, or intra-assay variability, was determined by running three levels of pooled plasma samples corresponding to low, mid, and high REG3E concentrations 10× in duplicates on the same plate. Reproducibility, or inter-assay variability, was calculated using the same three pools, measured 4× in duplicates on 5 different days, with two different operators. The SD and coefficients of variation (CV), were calculated (%CV = [SD/mean] × 100).[32,33] Imprecision was considered to be acceptable if it was <20%.[34,35]

To assess sample stability, plasma with four different REG3E concentrations was divided into 20 aliquots of 10 μL each, one of which was immediately placed at −80°C to serve as reference value. Storage conditions of the remaining aliquots were as follows: Six samples each were stored at room temperature and 4°C for 1, 2, 4, 24, 48, and 168 h, respectively, before being frozen at −80°C. Two samples were kept at −20°C for 2 and 4 weeks, respectively, before storage at −80°C. Another five samples were immediately frozen at −80° and underwent 1, 2, 3, 4, or 5 freeze–thaw cycles, with a minimum thawing time of 2 h and a minimal freezing time of 24 h between cycles. All aliquots from the same sample were run in duplicates on the same plate. The effect of these storage conditions was assessed by calculating the % deviation of REG3E concentrations from the reference sample. Additionally, a repeated measures ANOVA was performed for samples with complete datasets, and a mixed effect model was used for the samples where measurements had to be excluded from the analysis.

To study the influence of icterus, lipemia, and hemolysis, plasma samples of three different REG3E concentrations were spiked with different levels of interferent, diluted in the same volume of sample diluent, with blank sample diluent added for the reference measurement. All measurements of the same sample were run on the same plate. Icterus was mimicked by the addition of bilirubin (Sigma-Aldrich), dissolved in sample diluent with the addition of one drop of 2 M NaOH per mL. Final bilirubin concentrations in the samples were 1.7, 6.6, 16, and 30 mg/dL, corresponding to + to ++++ icterus as defined by the Clinical and Laboratory Standards Institute (CLSI).[37] Lipemia was simulated by dissolving a triglyceride mix including C2-C10 (Sigma-Aldrich) in sample diluent and emulsifying by sonication. Samples were spiked with triglycerides to obtain concentrations of 125, 250, 500, and 1000 mg/dL, corresponding to + to ++++ lipemia.[37] To generate hemolyzed samples, leftover cell pellets from canine lithium-heparinized blood were washed for three cycles in an automated cell washer (Rotolavit II, Hettich AG, Bäch, Switzerland) and frozen overnight at −20°C after the addition of an equal volume of distilled water. Subsequently, the samples were centrifuged for 10 min at 2500*g* (Universal 320, Hettich AG, Bäch, Switzerland) to eliminate solid cellular components, and the hemolyzed supernatant was harvested. Final sample concentrations were 50, 150, 250, 500 (+ to ++++ hemolysis), and 1000 mg/dL, as per CLSI recommendations.[37] Differences between spiked and neat samples were assessed with a repeated measured ANOVA, calculation of O/E, and visual assessment of line charts.

To compare REG3E concentrations between different matrices, 20 paired canine plasma and serum samples, each pair taken in the same blood draw and handled identically, spanning a wide range of REG3E concentrations, were measured in parallel and in duplicates on the same plate. Differences between REG3E concentrations in plasma and serum samples were evaluated with a paired samples t-test after logarithmic transformation.

Data analysis was performed with Excel, version 2305 (Microsoft Schweiz GmbH, Zürich-Flughafen, Switzerland), MedCalc Statistical Software version 20.218 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org), and GraphPad Prism version 10.0.1 (218) (GraphPad Software, Mosten MA, USA; https://www.graphpad.com/). For all experiments, O/E and variations were considered acceptable when they were between 80%–120% and <20%, respectively.[34–36] Statistical significance was set at $P < .05$.

2.5 | Animal samples

All samples used in this study were surplus heparinized plasma or serum samples from routine diagnostic submissions to the Clinical Diagnostic Laboratory of the Vetsuisse Faculty, University of Bern, Switzerland, from client-owned dogs. Signed owner consent for the use of leftover biological material was available, and ethical approval was waived by the local ethics committee. Blood was submitted either in lithium-heparin or serum tubes with clot activator; samples were centrifuged at 6080*g* for 2 min (Mikro 200, Hettich AG, Bäch,

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Switzerland) or at 1500*g* for 10 min (Universal 320, Hettich AG, Bäch, Switzerland), before pipetting off the plasma and serum, respectively. After routine biochemical analysis, leftover samples were stored at room temperature or at 4°C for up to 5 days, aliquoted and stored for up to 1 month at −20°C and for up to 15 months at −80°C, unless stated otherwise for the stability study.

3 | RESULTS

3.1 | Antibody specificity

Western blot using guinea pig and rabbit anti-canine REG3E antibodies on canine plasma and plasma purified using Protein A Sepharose High-Performance SpinTrap columns coated with the same antibodies gave a distinct band at the expected size of approximately 16 kDa (Figure 1). Mass spectrometric analysis of the purified protein fractions confirmed the presence of REG3E in the sample purified in series with both antibodies (43% coverage of amino acid sequence), alongside immunoglobulin light and heavy chains, canine albumin, and complement. Proteomic analysis of plasma purified using the SpinTrap columns without prior coating with a specific antibody contained high amounts of different immunoglobulin chains with lower amounts of albumin and complement, amongst other proteins, but only a single peptide spectrum match for REG3E, and no positive signal was obtained using western blot analysis of this sample. The full proteomic dataset is accessible at https://www.proteomexchange.org/ with the dataset identifier PXD046936.

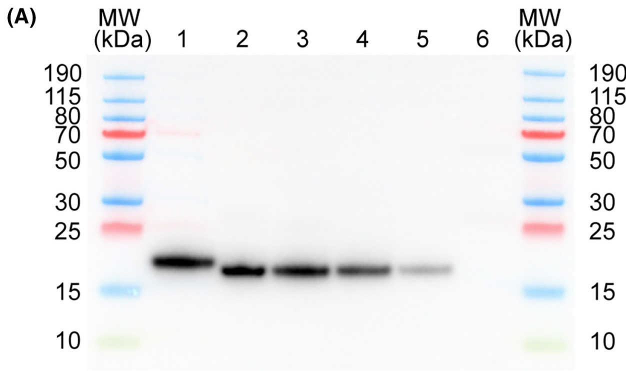

FIGURE 1 Western blot using polyclonal guinea pig (A) and rabbit (B) anti-canine REG3E antibodies yields a positive protein band of approximately 16 kDa. MW – Molecular weight marker in kilodalton; 1 – recombinant canine REG3E protein (including cleavage site); 2 – untreated canine plasma; 3 – canine plasma purified with SpinTrap columns coated with guinea pig anti-REG3E antibodies; 4 – canine plasma purified with columns coated with rabbit anti-REG3E antibodies; 5 – canine plasma purified first with columns coated with guinea pig anti-REG3E antibodies followed by columns coated with rabbit anti-REG3E antibodies; 6 – canine plasma purified with blank columns (no antibody coating).

3.2 | ELISA development and validation

An ELISA for the measurement of REG3E concentration in canine blood was successfully developed. Standard curves were reproducible in the range of 1–16 ng/mL (Figure 2), with less than 10% of measurements deviating >20% from the expected value, mainly at a concentration of 1 ng/mL. However, standards of 0.25 and 0.5 ng/mL could not be measured in a reproducible fashion, with the former often being unmeasurably low.

The LoB, back-calculated from an optical density of 0.25, was 0.3 ng/mL, and the LoD was approximately 0.6 ng/mL, which translates to a sample concentration of 15 ng/mL when considering the 1:25 sample dilution factor. The LLoQ was 1.2 ng/mL or 30 ng/mL in diluted samples. The ULoQ of the assay was set at 12 ng/mL, corresponding to 300 ng/mL in diluted sample. When this threshold was exceeded, samples were diluted at 1:250; if the subsequent reading gave >3000 ng/mL, a dilution of 1:1000 or 1:2000 was performed, yielding acceptable results for concentrations exceeding 10 000 ng/mL.

Criteria for dilutional linearity, parallelism, and spiking recovery were fulfilled, with no evidence of hook effect. Results are summarized in Table 1 and shown in Figure 3A–E, respectively.

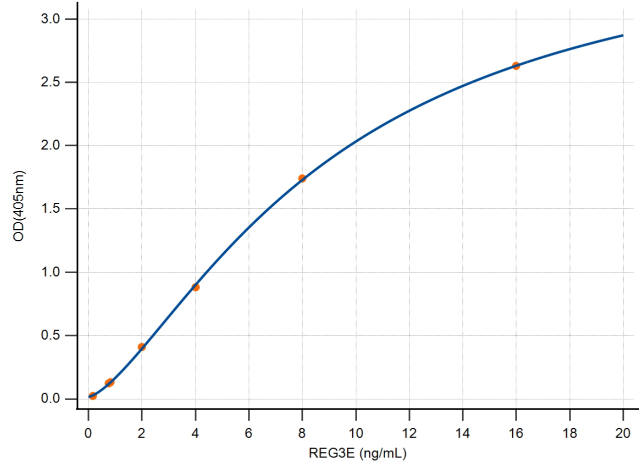

FIGURE 2 Representative standard curve for the canine REG3E ELISA (4-parameter logistic regression). OD, optical density.

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TABLE 1 Summary of dilutional linearity, dilutional parallelism, mixing parallelism, and spiking recovery of the canine REG3E ELISA. Samples were diluted to three levels or spiked with three different concentrations of recombinant REG3E, respectively.

	Sample	Mean O/E (%) ± SD	R^2	Regression equation	95% CI of slope	*P*
Dilutional linearity	All	104 ± 9	.95	y = −0.25 + 1.1x	0.96–1.25	<.001
	1	110 ± 14[a]	.99	y = −0.97 + 1.37x	0.12–2.62	.046
	2	99 ± 6	1	y = 0.01 + 98x	0.27–1.7	.036
	3	113 ± 5	1	y = −0.37 + 1.23x	0.63–1.83	.024
	4	97 ± 3	1	y = 0.16 + 0.92x	0.79–1.06	.007
	5	100 ± 3	1	y = −0.07 + 1.02x	0.59–1.44	.021
Dilutional parallelism	All	107 ± 7	1	y = 3.9 + 0.98x	0.97–0.99	<.001
	1	109 ± 4	1	y = 5 + 0.98x	0.9–1.05	<.001
	2	103 ± 1	1	y = 1.8 + 0.99 x	0.95–1.04	<.001
	3	114 ± 11[a]	1	y = 7.41 + 0.96x	0.93–0.99	<.001
	4	105 ± 4	1	y = 2.37 + 0.99x	0.97–1	<.001
	5	105 ± 7	1	y = 3.2 + 0.99x	0.95–1.02	<.001
Mixing parallelism	All	102 ± 4	1	y = 2.3 + 1.01x	0.99–1.02	<.001
	1	100 ± 4		y = −5.3 + 1.03x	0.91–1.16	<.001
	2	108 ± 4		y = 6.8 + 1.01x	0.78–1.23	<.001
	3	98 ± 1		y = −2.74 + 1.01x	0.93–1.09	<.001
	4	101 ± 1		y = −1.35 + 1.01x	0.97–1.04	<.001
	5	103 ± 5		y = 16.96 + 1.01x	0.86–1.15	<.001
	6	101 ± 0		y = 6.5 + 1x	0.98–1.03	<.001
Spiking recovery	All	92 ± 11	.97	y = −11 + 1.01x	0.93–1.1	<.001
	1	90 ± 6	1	y = −5.08 + 0.95x	0.81–1.1	.001
	2	96 ± 15	.98	y = −22.73 + 1.14x	0.67–1.62	.010
	3	81 ± 14[a]	.98	y = −6.73 + 0.89x	0.49–1.28	.011
	4	96 ± 8	.99	y = −12.98 + 1.06x	0.72–1.4	.005
	5	96 ± 8	.99	y = −9.53 + 1.04 x	0.75–1.33	.004

Abbreviations: CI, confidence interval; O/E, observed:expected ratio; R^2, coefficient of determination; SD, standard deviation.

[a]One sample with O/E > 120%.

Coefficients of variation for repeatability were 8.5%, 7.4%, and 8.1% for low (33 ng/mL), mid (193 ng/mL), and high (3382 ng/mL) concentration samples, respectively, and CVs for reproducibility were 14.3%, 9.8%, and 10%, respectively (Table 2).

Concentrations of REG3E stored under different conditions were not significantly different from baseline values for up to 5 freeze–thaw cycles ($P = .36$), storage at room temperature ($P = .34$), storage at 4°C ($P = .35$), and storage at −20°C ($P = .36$). The effect of different storage conditions on REG3E concentrations in four canine samples is shown in Table 3 and Figure 4A–D. Two samples, namely the time points 72 and 168 h of the high sample at room temperature, had to be eliminated due to fibrin clots in the aliquots.

No significant interference was observed at any level of icterus, lipemia, or hemolysis ($P > .42$ for all samples) (Table 4, Figure 5A–C).

There was no significant difference in REG3E measurements between 19 paired heparinized plasma and serum samples ($P = .47$; mean CV 5.6%) with concentrations ranging from 35 to 4473 ng/mL (Figure 6); one pair was excluded as the REG3E concentration was below the assay's LLoQ (<30 ng/mL).

The complete data set can be found in Table S1.

4 | DISCUSSION

We describe here the successful development and validation of a sandwich ELISA to measure concentrations of REG3E protein in canine blood, to our knowledge the first described validation of an assay to quantify this protein in plasma or serum specific for this species. To date, only one previous publication was found describing measurement of this protein in dogs, but assay validation has not been published and measurements were performed in fecal samples of dogs with enteropathies.[26]

Western blot and protein purification with the custom-made canine-specific anti-REG3E antibodies confirmed binding of our antibodies to REG3E, with other eluted proteins likely reflecting non-specific binding to the SpinTrap column, as they were also present in high amounts in the protein fractions eluted with blank columns. This suggests that the ELISA presented here using these antibodies

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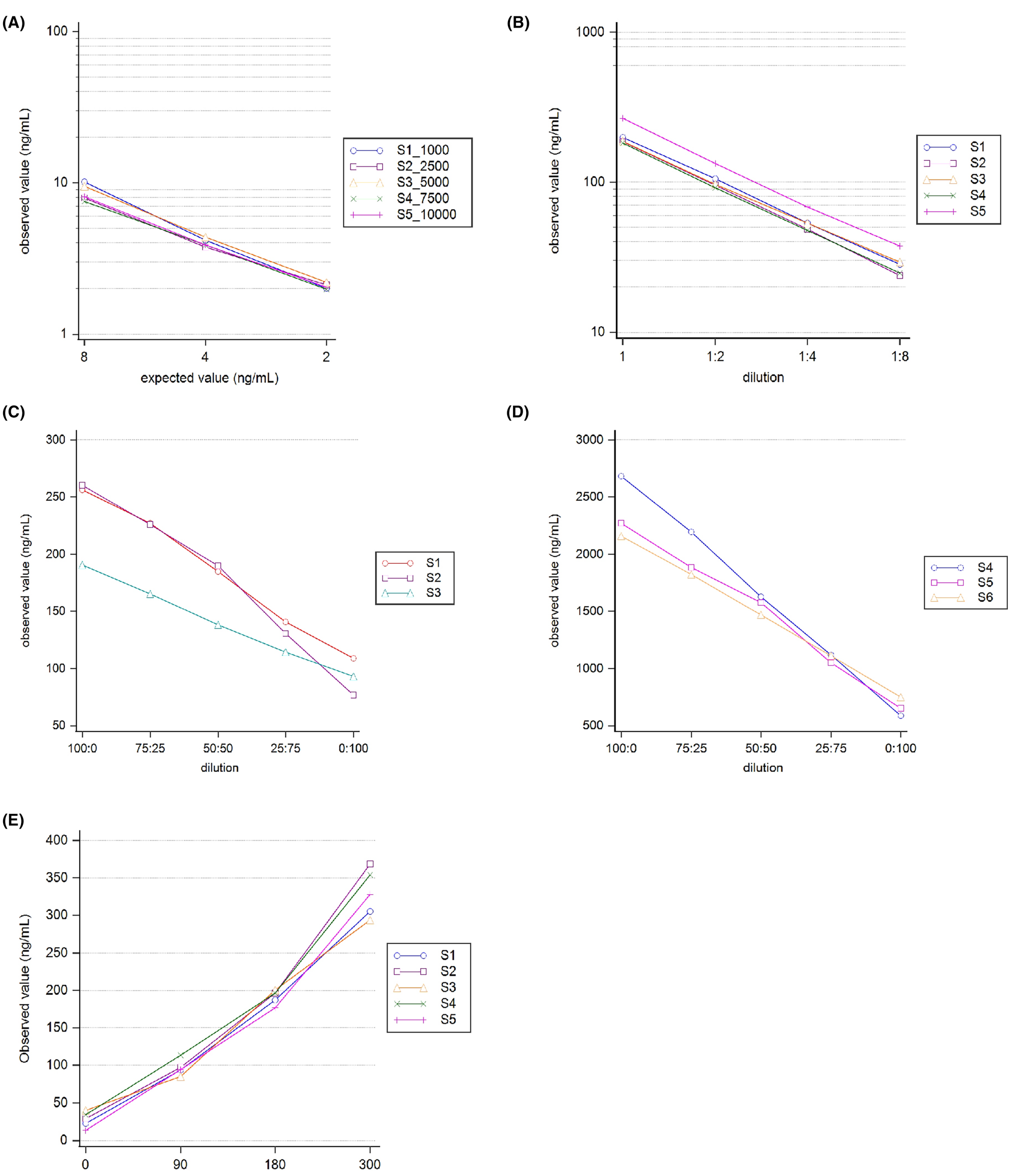

FIGURE 3 (A) Dilutional linearity of five plasma samples spiked with high levels of recombinant canine REG3E and diluted to serial measurements within the measuring range. (B) Dilutional parallelism of REG3E concentration in five serially diluted canine plasma samples measured by the REG3E ELISA. (C) Mixing parallelism of three serially mixed pairs of plasma samples at low and (D) at high REG3E concentrations. (E) Assessment of spiking recovery by adding three different concentrations (90, 180, and 300 ng/mL) of recombinant REG3E to five canine plasma samples with low endogenous REG3E concentrations measured by the canine REG3E ELISA.

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TABLE 2 Repeatability and reproducibility of the canine REG3E ELISA.

	Level	Mean ± SD (ng/mL)	CV (%)
Intra-assay variability	Low	33 ± 2.8	8.5
	Mid	194 ± 14.4	7.4
	High	3382 ± 273	8.1
Inter-assay variability	Low	32 ± 4.6	14.3
	Mid	202 ± 19.7	9.8
	High	3133 ± 313	10.0

Abbreviations: CV, coefficient of variation; SD, standard deviation.

TABLE 3 Effects of different storage conditions on REG3E concentrations in four canine plasma samples.

Sample [REG]	Conditions	*n*	Mean O/E (%) ± SD
Low (61 ng/mL)	Freeze-thawing (1–5 cycles)	5	112 ± 6[b]
	Room temperature (1–168 h)	6	119 ± 5[c]
	4°C (1–168 h)	6	113 ± 3
	−20°C (2–4 weeks)	2	114 ± 1
Mid (224 ng/mL)	Freeze-thawing (1–5 cycles)	5	105 ± 11[b]
	Room temperature (1–168 h)	6	115 ± 5
	4°C (1–168 h)	6	103 ± 5
	−20°C (2–4 weeks)	2	94 ± 3
High (982 ng/mL)	Freeze-thawing (1–5 cycles)	5	111 ± 7
	Room temperature (1–168 h)	4[a]	115 ± 5[b]
	4°C (1–168 h)	6	117 ± 20[b]
	−20°C (2–4 weeks)	2	116 ± 1
Extreme (13 269 ng/mL)	Freeze-thawing (1–5 cycles)	5	109 ± 7
	Room temperature (1–168 h)	6	116 ± 13[c]
	4°C (1–168 h)	6	115 ± 6[c]
	−20°C (2–4 weeks)	2	120 ± 5[b]

Abbreviations: O/E, observed:expected ratio; SD, standard deviation.
[a]Two aliquots contained clots and were excluded.
[b]One sample with O/E > 120%.
[c]Two samples with O/E > 120%.

is likely specific for REG3E. As it was previously demonstrated that REG3E is probably the only REG present in detectable quantities in canine plasma,[25] it is unlikely that other proteins of this family would cause cross-reactivity in our assay. However, REG3E is prone to glycosylation, and it is not clear whether the antibodies and, thus, the assay described here detects only the unglycosylated form or also the glycosylated protein.

Concentrations of recombinant REG3E to establish the standard curve were chosen to represent a logarithmic scale with an optical density of the highest standard lying between 2.5 and 3 O.D., which lies within the dynamic range of the plate reader used. However, during validation, it became apparent that the lowest value of 0.25 ng/mL was below the LoB, and the next standard of 0.5 ng/mL was just below the LoD of the assay. A standard curve omitting the lowest value and instead adding a standard of 12 ng/mL, which represents the ULoQ (before sample dilution), would have been favorable, and will be implemented for this assay going forward.

Although the assay has a relatively narrow measuring range of 1.2–12 ng/mL, patient samples are routinely diluted 1:25 before measurement, translating to a de facto measuring range of 30–300 ng/mL at initial working dilution. As the assay demonstrated good linearity and parallelism under dilution, samples can be diluted at least up to 1:2000, giving the assay a very wide working range. In fact, the highest measured sample in this study had a concentration of 13 269 ng/mL. Given that precision was not evaluated at such high concentrations, no absolute ULoQ could be determined in this study. Importantly, samples spiked with very high concentrations of REG3E consistently gave OD readings exceeding the measuring range, which suggests no evidence of a hook effect, meaning that antigen excess will unlikely lead to false low REG3E concentrations. Measurements of REG3E concentrations in a large population of healthy and sick dogs will be needed to establish whether the analytical range is appropriate for covering the breadth of REG3E levels in canine samples.

Coefficients of variation were within acceptable limits proposed by the guidelines for bioanalytical method validation and study sample analysis published by the Food and Drug Administration and the European Medicines Agency,[34,35] but with a somewhat suboptimal inter-assay variability of 14% at the low measured level. It is, however, noteworthy that the mean concentration of this sample pool was 32 ng/mL, which is just above the LLoQ, where a higher variability, up to 25%, is tolerated.[34,35] Ideally, total error should be calculated to assess the analytical performance of an assay. However, in the absence of a gold standard test or quality control material, bias or systematic error of the assay could not be evaluated. Furthermore, as clinical decision limits and biological variation of the assay are to date unknown, no goals for total allowable errors could be set to evaluate the clinical usefulness of the test.[33]

The different tested storage conditions, including up to 1 week refrigerated or at room temperature, up to 4 weeks at −20°C, and up to 5 freeze–thaw cycles, did not significantly influence the measured REG3E concentrations. Most (85%) measurements were within 20% of baseline values, with only two measurements being outside 75%–125%, which has been previously deemed acceptable criteria by some authors.[38] The two values which deviated strongly from these recommendations were 156% O/E in the high-concentration sample after 4 h of storage at 4°C and 138% O/E in the extreme sample after 168 h at room temperature. In the context of all other results of the stability study, these were considered to be outliers. Unfortunately, the two last time points of the high sample at room temperature had to be eliminated due to clots in the samples, and therefore, the effect of prolonged storage at room temperature of the sample with the next lower concentration could not be assessed, and a systematic change in concentration may have potentially been missed. Nonetheless, the results of the stability study support the use of routinely handled and stored samples for retrospective REG3E

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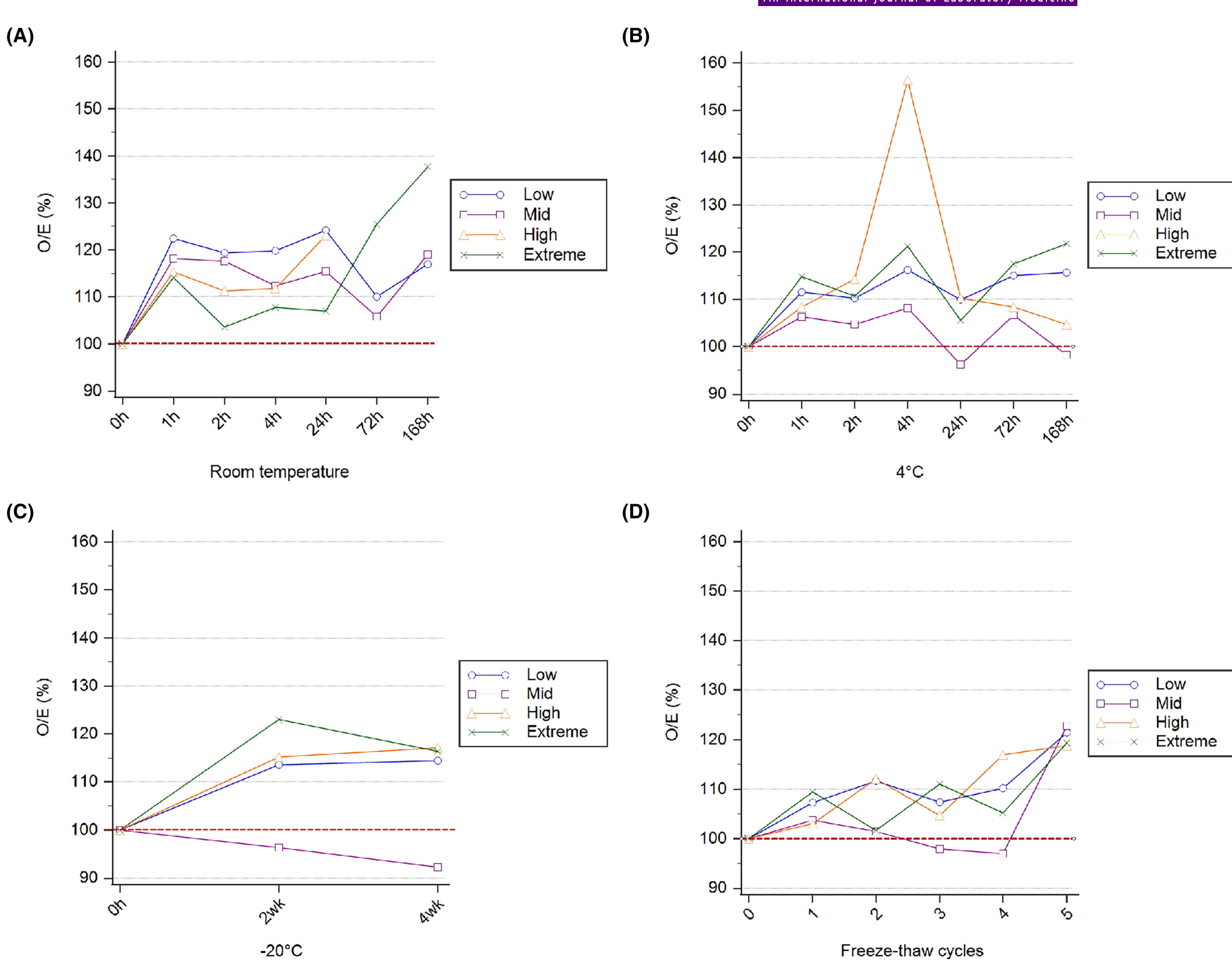

FIGURE 4 Stability of canine REG3E in plasma at 4 different concentrations (Low, 61 ng/mL; Mid, 224 ng/mL; High, 982 ng/mL; Extreme, 13 269 ng/mL) under different conditions. (A) Storage at room temperature for 1, 2, 4, 24, 72, and 168 h. (B) Storage at +4C° for 1, 2, 4, 24, 72, and 168 h. (C) Storage at −20°C for 2 and 4 weeks. (D) Samples undergoing 1–5 freeze–thaw cycles.

TABLE 4 Interfering effects of different levels of hemolysis, lipemia, and icterus on REG3E concentrations, measured by a novel ELISA, in three canine plasma samples.

Sample [REG]	Interferent	*n*	Mean O/E (%) ± SD
Low (32 ng/mL)	Bilirubin	4	106 ± 2
	Triglycerides	4	103 ± 3
	Hemoglobin	5	106 ± 4
Mid (191 ng/mL)	Bilirubin	4	100 ± 3
	Triglycerides	4	104 ± 5
	Hemoglobin	5	95 ± 6
High (3354 ng/mL)	Bilirubin	4	111 ± 2
	Triglycerides	4	105 ± 2
	Hemoglobin	5	105 ± 6

Abbreviations: O/E, observed:expected ratio; SD, standard deviation.

measurements, for example, for research purposes. However, by visual inspection of the line charts, a trend toward higher measurements, particularly in samples stored on the benchtop or undergoing repeated freezing and thawing, was observed. One possible explanation for this could be the evaporation of water from plasma at room temperature, which may lead to the concentration of solid components, including proteins such as REG3E. While this suspected effect is likely negligible in routine sampling size, including several 100 μL of sample volume, this may have had a considerable effect on the small aliquot size of 10 μL used for our stability study due to limited available sample volumes. Another potential reason for an increase in protein concentration after multiple freeze–thaw cycles could be that REG3E may be partially bound to other proteins in circulation, which are less stable and release any bound REG3E under certain storage conditions, making it more readily detectable by the antibodies in this assay. However, investigation of this hypothesis was beyond the scope of this study.

Our investigations furthermore demonstrate that lipemia, hemolysis, and icterus have no interfering effects with the REG3E assay, including at very high levels of interferents. This is not surprising, as the dilution of a sample of at least 1:25 minimizes the effects of such interferents. Nonetheless, this information is valuable as dogs with

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FIGURE 5 Effect of interference on canine REG3E measurements in plasma at three different concentrations (low, 32 ng/mL; mid, 191 ng/mL; high, 3354 ng/mL). (A) Four incrementing levels of bilirubin. (B) Four incrementing levels of triglycerides. (C) Five incrementing levels of hemoglobin.

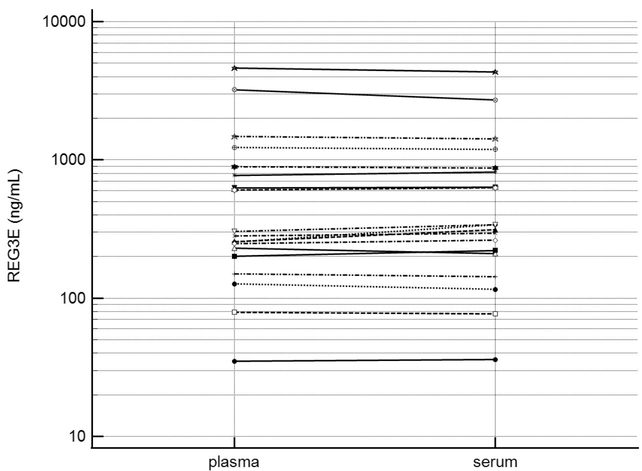

FIGURE 6 Comparison of canine REG3E concentrations in paired plasma and serum samples.

some diseases with potentially high REG3E levels, most notably pancreatitis,[25] can have significant icterus or lipemia due to direct effects of pancreatitis, such as posthepatic cholestasis, co-morbidities or predisposing factors, such as hepatobiliary diseases or hyperlipidemia,[39] which can in turn lead to hemolysis due to increased erythrocyte fragility.[40]

We found no significant difference in REG3E concentrations in lithium heparin plasma compared to serum, giving the assay a certain flexibility regarding the choice of matrix. Our study design did not include the comparison of different types of serum collection tubes, including tubes with separator gel, with or without different clot activating substances, nor of different types of plasma, most notably EDTA-plasma, as this was logistically not feasible. However, in most routine diagnostic laboratories, either heparinized plasma or serum is available for biochemical analyses. Thus, our investigations covered the most common scenarios encountered in clinical practice.

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5 | CONCLUSIONS

We have successfully developed an ELISA to measure REG3E concentrations in both canine plasma and serum, which is specific and demonstrates acceptable to excellent precision, accuracy, linearity, and parallelism. The assay appears to be unaffected by interferents, and the protein has proven stable under different tested storage conditions. These results set the groundwork for the measurement of REG3E in canine blood. Future studies comparing REG3E concentrations in blood from healthy dogs and dogs with a variety of diseases, such as sepsis, pancreatitis, and enteropathies, are needed to establish the diagnostic performance and clinical utility of this potential novel biomarker in this species.

ACKNOWLEDGMENTS

The authors would like to thank the following people and institutions: Manfred Heller and Anne-Christine Uldry from the Proteomics and Mass Spectrometry Core Facility (Department for BioMedical Research, University of Bern, Switzerland) for proteomic analysis and support with data interpretation; the team of clinical immunology (Division of Neurosciences, Vetsuisse Faculty Bern, Switzerland) for their advice on ELISA development and optimization; the laboratory technicians of the Clinical Diagnostic Laboratory (Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, Switzerland), particularly Fabienne Liechti, for their assistance with sample archiving and help running ELISAs. Open access funding provided by Universitat Bern.

FUNDING INFORMATION

This study was supported by the initiator grant of the Stiftung Tierspital of the Vetsuisse Faculty Bern, Switzerland.

CONFLICT OF INTEREST STATEMENT

Dr. Peters, Dr. Howard, Dr. Graf, and Dr. Reding Graf have filed a European patent application under the name "Detection of sepsis and pancreatitis by blood REG3 quantification in dogs" that covers the ELISA assay described in this article. The results of this study were presented as an oral abstract at the annual meeting of the American College of Veterinary Pathologists in Chicago, IL, USA (29–31 October 2023).

ORCID

Laureen M. Peters https://orcid.org/0000-0002-6302-4240
Luca Giori https://orcid.org/0000-0002-7329-683X

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