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Research paper

Development, analytical validation, and initial clinical evaluation of a radioimmunoassay for the measurement of soluble CD25 concentrations in canine serum

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ABSTRACT

During immune activation, CD25 is expressed by T cells, and its soluble form (sCD25) is released into the extracellular matrix and the bloodstream. In humans, serum sCD25 concentrations are used as a surrogate marker for autoimmune diseases, malignancies, and transplant rejection. However, a canine-specific assay for the measurement of sCD25 in dog serum has not previously been described. Therefore, the aims of this study were to develop and analytically validate a radioimmunoassay to measure sCD25 in canine serum, to establish a reference interval for canine sCD25, and to test the clinical utility of this assay with serum samples for dogs with various diseases. A competitive radioimmunoassay (RIA) was developed and analytically validated. Analytical validation consisted of lower limit of detection (LLOD), dilutional parallelism, spiking recovery, and intra- and inter-assay variability using pooled surplus canine serum samples. A reference interval was established in healthy dogs and serum samples from dogs with various types of neoplasia, IBD, liver disease, suspected pancreatitis, or suspected small intestinal disease and serum samples with an increased C-reactive protein concentration (CRP) were analyzed to test the clinical utility of the assay. LLOD was calculated to be 0.5 ng/mL. The mean (± SD) observed-to-expected ratio (O/E) for serial dilutions was 101.7 ± 14.0%, and the mean (± SD) O/E for spiking recovery was 93.2 ± 4.2%. Coefficients of variation (CVs) for intra-assay variability were ≤12.5% (mean ± SD: 7.5 ± 4.2%), and inter-assay CVs were ≤15.7% (mean ± SD: 11 ± 4.4%). A reference interval (RI) for canine sCD25 of 1.2–4.2 ng/mL was established from a population of 112 clinically healthy dogs. Dogs with neoplasia and dogs with suspected small intestinal disease had decreased concentrations of serum sCD25 when compared to healthy dogs ($p < 0.0001$, respectively). However, the majority of clinical samples used in this study were within the reference interval. Median concentrations of serum sCD25 were 1.9 ng/mL for healthy dogs. Dogs with cancer, IBD, liver disease, suspected pancreatitis, or suspected small intestinal disease, as well as sera with an increased serum CRP concentration, had median serum sCD25 concentrations of 1.6 ng/mL, 2.1 ng/mL, 2.2 ng/mL, 1.7 ng/mL, 1.5 ng/mL, and 1.8 ng/mL, respectively.

Thus, the RIA described here is linear, accurate, precise, and reproducible for measuring sCD25 in canine serum. However, this assay shows little clinical utility of sCD25 as a biomarker for dogs with inflammatory, autoimmune, and/or neoplastic conditions.

1. Introduction

Interleukin-2 (IL-2) was discovered in 1976 because of its *in vitro* capacity to induce T cell activation and expansion (Morgan et al., 1976; Smith, 1988). This cytokine, produced mainly by activated CD4 + T cells (Boyman and Sprent, 2012), acts in a paracrine manner and exerts its downstream effect *via* receptor binding. The IL-2 receptor consists of three subunits: an alpha subunit (CD25; molecular weight [MW]

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55 kDa), a beta subunit (CD122; MW 75 kDa), and a gamma subunit (CD132; MW 64 kDa). Various combinations of the IL-2 receptor subunits can be arranged on the cell surface, with each having a different binding affinity for IL-2 (Taniguchi and Minami, 1993).

The binding between IL-2 and the receptor subunit CD25 alone does not result in activation of a downstream pathway (Taniguchi and Minami, 1993). However, during T cell activation, CD25 is recruited into the cytokine-receptor complex, and the affinity for IL-2 reaches maximum strength, with an increase in binding affinity between 10 and 100 fold (Caruso et al., 1993). The subunits CD122 and CD132 are commonly shared between receptors used for sensing several other cytokines (*e.g.*, IL-15) by different immune cells, whereas CD25 is exclusive to the IL-2 receptor, highlighting the importance of CD25 in T cell activation and maintenance of the immune response.

Once CD25 is expressed on the T cell membrane, it can be cleaved at the cell surface, producing a smaller molecule (45 kDa) that retains biological binding activity towards IL-2 (Rubin et al., 1986, 1985). This soluble CD25 (sCD25) is positively correlated with the expression of the cellularly bound CD25 subunit (Lai et al., 1991), and therefore, sCD25 has been used as a surrogate marker to assess T cell activation (Caruso et al., 1993).

Serum concentrations of soluble CD25 have been shown to be increased in human patients with infections (Kurane et al., 1991; Makis et al., 2005; Novikov et al., 2007), autoimmune (Buhelt et al., 2017; Dekkema et al., 2019; Downes et al., 2014; Nielsen et al., 1998; Vanmaris and Rijkers, 2017; Witkowska, 2005), and other chronic inflammatory processes (*e.g.*, chronic hepatitis) (Seidler et al., 2012). Moreover, an increase in sCD25 serum concentrations has also been described in patients with several types of malignancies, including leukemia (Moon et al., 2004; Wang et al., 2015), lymphoma (Toji et al., 2015), esophageal squamous cell carcinoma (Wang et al., 2000), hepatocellular carcinoma (Cabrera et al., 2010), breast cancer (Tesarova et al., 2000), and others (Bien and Balcerska, 2008; Murakami, 2004; Sobjanek et al., 2016). The concentration of sCD25 is correlated with disease activity, disease progression, and response to treatment (Bien and Balcerska, 2008; Dlouhy et al., 2017; Murakami, 2004; Yang et al., 2011). An increase in serum sCD25 concentrations has been reported in human patients after organ transplantation, where it has been used as a marker of organ rejection (Hagras et al., 2018; Rasool et al., 2015).

Moreover, CD25 has also been therapeutically targeted to treat some types of cancer (Flynn and Hartley, 2017) and to avoid transplant rejection (Webster et al., 2010).

Given the utility of sCD25 as a diagnostic and monitoring biomarker and as a therapeutic target in human medicine, we hypothesize that sCD25 could be a valuable marker for T cell activation in canine patients with various autoimmune, inflammatory, and/or neoplastic conditions.

To our knowledge, the development and analytical validation of a canine-specific immunoassay has not previously been reported. However, it has been reported that a human-specific assay was able to cross-react with sCD25 in canine serum (Prachar et al., 2013). It should be noted that the human CD25 protein only shares 61% sequence identity with the canine protein. In our laboratory, we tested two commercially available human ELISAs (Cusabio® and R&D Systems®), including the one reported by Prachar et al., in order to investigate the degree of cross-reactivity between human antibodies (provided with these kits) and recombinant canine CD25 (used here to develop our sCD25 RIA) or the naturally occurring sCD25 (present in canine serum). We were unable to detect any cross-reactivity between the human CD25 antibodies and canine CD25, recombinant or endogenous, using these commercially available ELISAs. Therefore, the aim of this study was to develop and analytically validate a radioimmunoassay (RIA) to detect and reliably measure concentrations of sCD25 in canine serum.

2. Materials and methods

2.1. Canine CD25 aligned against human CD25 and protein identification

Considered a previous report using a human-specific, commercially available ELISA to measure serum concentrations of soluble CD25, and the absence of cross-reactivity between the human antibodies and canine protein, endogenous or exogenous, an initial comparison and alignment of canine and human CD25 was performed using BLAST to evaluate the sequence identity of CD25 between dogs and humans.

In order to build a canine specific assay, a recombinant canine CD25 protein, manufactured by R&D Systems®, was also partially characterized, using two methods. First, the CD25 was submitted to the Protein Chemistry Laboratory at Texas A&M University, College Station, Texas, for peptide mass fingerprinting, following a method previously described (Shevchenko et al., 2006). Briefly, the recombinant canine CD25 was alkylated with iodoacetamide and digested with recombinant porcine trypsin. The sample was then analyzed with a Bruker ultrafleXtreme MALDI-TOF/TOF mass spectrometer in reflector mode. The cCD25 protein was mixed with alpha-cyano-4-hydroxycinnamic acid matrix. Samples were calibrated internally with known tryptic autocatalytic fragments ($m/z = 842$ and 2211). The mass accuracy at 1000 m/z is approximately 10–50 ppm. Finally, data were used to search the SwissProt database using Mascot (www.MatrixScience.com).

Next, nanospray liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) was performed to directly identify the protein in the commercially available recombinant canine CD25. The protein was digested using trypsin, and the resulting peptides were separated by capillary reversed phase-high performance liquid chromatography prior to in-line analysis of their masses and fragmentation patterns. Tandem mass spectra were extracted by Scaffold (Proteome Software, Portland, Oregon; version 7.2). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.6.2) and X! Tandem (The GPM, thegpm.org; version CYCLONE [2010.12.01.1]). Mascot was set up to search the SwissProt_2017_02 database (553,655 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search a reverse concatenated subset of the SwissProt_2017_02 database (2086 entries) also assuming trypsin as the digesting enzyme. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 20 PPM. Carbamidomethyl of cysteine was specified in Mascot and X! Tandem as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine, and acetylation of the n-terminus were specified in Mascot as variable modifications. Glu- > pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, Gln- > pyro-Glu of the n-terminus, deamidation of asparagine and glutamine, oxidation of methionine, and acetylation of the n-terminus were specified in X! Tandem as variable modifications.

2.2. Canine serum samples

Surplus serum samples, submitted to the Gastrointestinal Laboratory (GI Lab) at Texas A&M University for routine testing, were collected over a period of two months and used for the development and analytical validation of the assay. All samples were kept at −80 °C for up to 2 months until analysis.

Surplus serum from 112 clinically healthy dogs, collected for unrelated studies and frozen at −80 °C until analysis, were used to establish a reference interval for soluble CD25 in canine serum. Animals were considered clinically healthy based on clinical history, owner questionnaire, and a complete physical examination. Dogs with abnormal physical examination findings were excluded from this group. Sample collection was approved by ethical committees at Texas A&M University (IACUC 2016-0177 CA, IACUC 2014-0109). Blood was collected by venipuncture of a peripheral vein using a 6 mL syringe and

22 G needle. Serum was collected in a glass serum tube. Clinical utility of the assay was tested by using surplus serum samples from three unrelated studies (*i.e.*, studies investigating chronic enteropathies (CE), liver disease, and neoplasia) and surplus samples submitted to the GI Lab for diagnostic purposes.

The dogs in the CE group (n = 15) had clinical signs of GI disease for > 3 weeks, other causes of these signs were excluded, and enteritis was histologically diagnosed from endoscopically collected biopsy specimens. Dogs with CE receiving immunosuppressive medication at the time of enrollment were excluded from the study.

The dogs with liver disease (n = 7) underwent laparoscopic liver biopsy for diagnostic purposes. Four dogs were diagnosed with chronic hepatitis and three were diagnosed with nodular hyperplasia and/or vacuolar hepatopathy.

The dogs with neoplasia (n = 60) were diagnosed based on histopathological assessment of biopsy or cytology specimens with imaging or a combination of these. The type of neoplasia is documented in a Supplemental table.

Surplus serum samples identified in the GI Lab database were categorized into 4 groups: [1] dogs with suspected pancreatitis (*i.e.*, serum samples with a canine pancreatic lipase immunoreactivity [cPLI] ≥ 2000 μg/L, the upper limit of detection for this assay, n = 50); [2] dogs with suspected small intestinal diseases (*i.e.*, serum samples with a decreased serum concentration of cobalamin and folate when compared to the respective reference intervals, n = 50); [3] dogs with suspected liver diseases (*i.e.*, serum samples with increased pre- or post-prandial serum bile acids concentrations, n = 27); and [4] dogs with increased serum C-reactive protein (CRP) concentrations (*i.e.*, serum samples with an increased concentration of CRP when compared to the respective reference intervals, n = 52).

2.3. Iodination of canine CD25

A commercially available canine CD25 protein (R&D Systems) was used to develop this radioimmunoassay. All buffers used during this procedure were prepared freshly, and the iodination procedure was performed under a laminar fume hood. Tracer was produced by iodination of canine CD25 with ^{125}I, using the chloramine T method described elsewhere (Hunter and Greenwood, 1962). A partially modified procedure was then carried out as previously described by our laboratory (Steiner et al., 1996). Briefly, 10 μg of canine CD25 was dissolved in 0.25 M sodium phosphate (pH 7.5), then 10 μL of ^{125}I (approximately 0.1 mCi/μL at the time of production) and 10 μL of chloramine-T solution were added. After 60 s of incubation, 100 μL of sodium metabisulfite solution (0.04 mg of $Na_2S_20_5$/mL) and 870 μL of potassium iodide solution (2 mg of KI/mL) were added for a final volume of 1 mL. The final solution was applied to a dextran gel column in order to separate ^{125}I-CD25 from the unbound ^{125}I by size exclusion and buffer exchange. The column was washed with RIA buffer (RIAB: 0.05 M sodium phosphate, 0.2 g NaN_3, 5 g BSA). The eluent from the column was collected in 1-mL fractions, and the radioactivity was measured using a gamma counter. The fraction with the highest concentration of ^{125}I-labeled CD25 was retained. Tracer was produced, as needed, by diluting the above-mentioned fraction to about 44,000 counts per minute (cpm)/100 μL. Because ^{125}I has a half-life of 60 days, this iodination procedure was performed monthly to ensure consistent radioactivity of the tracer. The final tracer solution was maintained at 4 °C, and the initial fraction with high ^{125}I-labeled CD25 concentration was aliquoted and kept frozen at −20 °C for the remaining 30 days.

2.4. Radioimmunoassay

Commercially available canine-specific polyclonal anti-CD25 antibodies produced in sheep were used to develop this radioimmunoassay (R&D Systems®). A competitive radioimmunoassay was established. The assay was set up in duplicates. Tubes were labeled: total count (TC); non-specific binding (NB); reference blank (B0); and standards labeled 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.4 ng/mL. The tubes were filled accordingly: TC received 100 μL of tracer only; NB received 100 μL of tracer and 300 μL of RIA buffer (RIAB); B0 received 100 μL tracer, 100 μL antibody solution against sCD25 (100 ng/mL), 100 μL of antibody solution against sheep IgG (500 ng/mL), and 100 μL of RIAB. The standards received 100 μL tracer; 100 μL antibody solution against sCD25; 100 μL of antibody solution against sheep IgG; and 100 μL of standard solution of 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, and 0.4 ng/mL sCD25 in RIAB, respectively. Serum samples for testing received 100 μL tracer, 100 μL antibody solution against sCD25, 100 μL of antibody solution against sheep IgG, and 100 μL of the unknown sample. All tubes were vortexed and then incubated for 24 h at 4 °C. At the end of the incubation period, 100 μL of rabbit carrier solution (1 mL normal rabbit serum mixed with 99 mL RIAB) and 1 mL of a commercially available precipitation solution (BP Biomedicals) were added to all tubes, except TC. All tubes were then vortexed and centrifuged at 3000 × *g* and 4 °C for 20 min. The supernatant of all tubes except for TC was decanted and all tubes were counted for one minute on a gamma counter. A standard curve was calculated using a 5-parameter curve-fit equation. Canine sCD25 concentrations were extrapolated from the standard curve.

2.5. Validation of the assay

The canine sCD25 radioimmunoassay was analytically validated by determining assay sensitivity, working range, linearity, accuracy, precision, and reproducibility by testing the lower limit of assay detection, dilutional parallelism, spiking recovery, and intra- and inter-assay variability. Samples used to validate the assay were generated from a pool of several surplus serum samples. Serum left from samples received at the Gastrointestinal Laboratory for other purposes was used in the validation process. Samples were initially analyzed individually, and because leftover samples generally had volumes less than 0.5 mL, those with similar sCD25 concentrations were pooled to generate samples with sufficient volume for the entire validation process.

To cover the entire working range of the assay (*i.e.*, 0.4–50 ng/mL), a sample was spiked with three different amounts of CD25, and then these three prepared samples were treated as natural unknown samples. The analytical sensitivity was determined by analyzing 10 blank samples (duplicates of B0) and calculating the standard deviation of the raw counts of these 10 duplicates. Three standard deviations were then subtracted from the mean count, and the resulting value estimated on the standard curve. The sensitivity was also used as the lower limit of quantitation (LLOD) of the assay. The upper limit of detection was considered as the highest standard (50 ng/mL).

Linearity of the assay was determined by the use of six samples (natural and spiked) tested in two-fold dilutions until the LLOD was reached. Observed to expected ratios were then calculated using the following equation: (observed value/expected value) × 100.

Accuracy of the assay was tested by spiking three samples with different concentrations of CD25 (5, 10, 25, 35, and 40 ng/mL), thus covering the whole working range of the assay. For linearity and accuracy (measured as % canine sCD25), recovery was calculated using the following equation: (observed value/expected value) × 100.

Precision (*i.e.*, intra-assay variability) of the assay was evaluated by assaying eight samples (of which three were initially spiked) 12 times within the same assay run, followed by calculating the coefficient of variation: CV% = (SD/mean) × 100.

Reproducibility (*i.e.*, inter-assay variability) of the assay was determined by analysis of six samples that were analyzed in seven assays performed on seven consecutive days, followed by the calculation of inter-assay coefficient of variation: CV% = (SD/mean) × 100.

```
Query  1    MDSYLLMWGLLTFIMVPGCQAELCDDDPPEIPHATFKAMAYKEGTMLNCECKRGFRRIKS  60
            M+  LLMWG+LTFI V G   +LCDDDPP + HATFKA+ YK GT+LNC+C+RGFRRI S
Sbjct  1    MEPCLLMWGILTFITVSGYTTDLCDDDPPNLKHATFKALTYKTGTVLNCDCERGFRRISS  60

Query  61   GSLYMLCTGNSSHSSWDNQCQCTSSATRNTTKQVTPQPEEQKERKTTEMQSPMQPVDQAS  120
               YM CTGNSSH+SW+N+C+C S +  N   +VT +PEEQK     TEMQS   P+D+
Sbjct  61   ---YMHCTGNSSHASWENKCRCKSVSPENRKGKVTTKPEEQKGENPTEMQSQTPPMDEVD  117

Query  121  LPGHCREPPPWENEATERIYHFVVGQMVYYQCVQGYRALHRGPAESVCKMTHGKTRWTQP  180
            L GHCREPPPWE+E ++RIYHFVVGQ ++YQC+QG+ ALHRGPA+S+CK   GKTRWTQP
Sbjct  118  LVGHCREPPPWEHENSKRIYHFVVGQTLHYQCMQGFTALHRGPAKSICKTIFGKTRWTQP  177

Query  181  QLICTGEMETSQFPGEEKPQAS---PEGRPESETSCLVTTTDFQIQTEMAATMETSIFTT  237
             L C  E   SQFP +E+ QAS   P GR  S      +T DF   TE+A TME+ IFTT
Sbjct  178  PLKCISE---SQFPDDEELQASTDAPAGRDTSSPFITTSTPDFHKHTEVATTMESFIFTT  234

Query  238  EYQVAVAGCVFllisvlllsglTWQRRQRKSR  269
            EYQ+AVA CV LLIS++LLSGLTWQRR+RKSR
Sbjct  235  EYQIAVASCVLLLISIVLLSGLTWQRRRRKSR  266
```

Fig. 1. Alignment of human CD25 (top line – query) against canine CD25 (bottom line – Subject).
Note: Intermediate line shows the identical amino acids shared between the two proteins. + signs are indicating similar amino acids shared between human and canine CD25.

2.6. Reference interval

Serum samples from 112 clinically healthy dogs were used to establish a reference interval for canine serum sCD25 concentration using the robust method with a free software package (Reference Value Advisor) (Friedrichs et al., 2012; Geffre et al., 2011).

2.7. Statistical analysis

Differences in serum CD25 concentrations between groups were analyzed using Mann-Whitney U or Kruskal Wallis, as appropriate. A commercially available software was used for the statistical analysis (GraphPad Prism 7). Significance was set at p value < 0.05.

3. Results

Canine and human CD25 share a 61.8% sequence identity, and homology was calculated to be 74.6% (Fig. 1). The commercially available recombinant canine CD25 used for this study was analyzed by mass spectrometry. The sequences highlighted by the mass spectrometry analysis reached a coverage of the canine protein of 43% and allowed identification as canine CD25. The commercially available polyclonal antibodies (R&D AF6227) were able to bind the denatured recombinant canine CD25, showing a molecular weight of approximately 55 kDa for CD25. In contrast, monoclonal anti-canine CD25 antibodies (Biorad) were not able to bind the denatured protein used in this experiment (data of the western-blot are not shown).

3.1. Radioimmunoassay

The analytical sensitivity of the assay was calculated to be 0.5 ng/mL. Linearity of the assay was tested by dilutional parallelism; the observed to expected ratios (O/E) for serial dilutions ranged from 89.4% to 120.6% (mean 101.7% ± 14.0%) (Table 1). The accuracy of the assay was tested by spiking recovery; the O/E ratios for spiking recovery ranged from 91.0% to 97.6% (mean 93.2% ± 4.2%) (Table 2). To test the precision of the assay, intra-assay coefficient of variations (CVs) were calculated; they ranged from 2.9% to 12.5% (mean 7.5% ± 4.2%) (Table 3). Reproducibility was evaluated by inter-assay variability; inter-assay CVs ranged from 5.9% to 15.7% (mean 11.1% ± 4.4%) (Table 4).

Table 1
Dilutional parallelism for the radioimmunoassay (RIA) for canine soluble CD25 (sCD25) shown for six serum samples. Samples S1, S2, and S3 were natural serum samples, while samples S4, S5, and S6 were serum samples with a low serum sCD25 concentration that had been spiked with canine recombinant CD25 in order to analytically validate the assay for the entire working range.

Sample ID	Dilution	Observed (ng/mL)	Expected (ng/mL)	O/E (%)	Average O/E %
S1	neat	1.2	N/A	N/A	102.5
	1:2	0.6	0.6	102.5	
S2	neat	1.77	N/A	N/A	116.6
	1:2	1.0	0.9	116.6	
S3	neat	3	N/A	N/A	120.6
	1:2	1.7	1.5	113.2	
	1:4	0.9	0.8	127.9	
S4	neat	6.10	N/A	N/A	89.4
	1:2	2.8	3.1	90.2	
	1:4	1.4	1.5	93.6	
	1:8	0.6	0.8	84.5	
S5	neat	21.2	N/A	N/A	90.6
	1:2	10.5	10.6	99.3	
	1:4	5.1	5.3	96.1	
	1:8	2.5	2.6	92.4	
	1:16	1.3	1.3	100.1	
	1:32	0.4	0.7	64.9	
S6	neat	41.2	N/A	N/A	90.6
	1:2	20.0	20.6	97.2	
	1:4	10.0	10.3	97.5	
	1:8	4.8	5.2	92.6	
	1:16	2.2	2.6	82.1	
	1:32	1.0	1.3	74.6	
	1:64	0.62	0.64	95.7	

3.2. Clinical samples

The reference interval for canine sCD25 was determined to be in the range of 1.2–4.2 ng/mL. Reference intervals were tested for outliers, but outliers were not excluded from analysis. The 90% CI at lower limit was determined to be in the range of 1.1–1.26 ng/mL and the 90% CI at the upper limit was determined to be in the range of 3.6–5.1 ng/mL.

Oncological patients and dogs with suspected small intestinal disease, but not dogs with IBD, had significantly decreased concentrations of serum sCD25 when compared to healthy dogs ($p < 0.0001$, respectively). The median concentration of serum sCD25 was 1.9 ng/mL for healthy dogs. Dogs with different types of neoplasia, IBD, liver

Table 2
Spiking recovery for the RIA for canine soluble sCD25 shown for three serum samples and five spiking concentrations.

Sample ID	Amount added (ng/mL)	Observed (ng/mL)	Expected (ng/mL)	O/E (%)	Average O/E (%)
S1	0	1.1	N/A	N/A	91.0
	5	5.4	6.1	89.0	
	10	9.9	11.1	89.3	
	25	23.6	26.1	90.2	
	35	33.5	36.1	92.6	
	40	38.6	41.1	93.8	
S2	0	1.4	N/A	N/A	91.1
	5	6.2	6.4	96.2	
	10	10.3	11.4	89.8	
	25	23.9	26.4	90.2	
	35	34.0	36.4	93.2	
	40	35.7	41.4	86.0	
S3	0	3.0	N/A	N/A	97.6
	5	8.0	8.0	100.6	
	10	12.2	13.0	94.2	
	25	27.2	28.0	97.3	
	35	37.0	38.0	97.5	
	40	42.4	43.0	98.7	

disease, suspected pancreatitis, suspected small intestinal disease, and dogs with increased serum CRP concentrations had median serum sCD25 concentrations of 1.6 ng/mL, 2.1 ng/mL, 2.1 ng/mL, 1.7 ng/mL, 1.5 ng/mL, and 1.8 ng/mL, respectively. However, the majority of clinical samples used in this study were within the reference interval (Fig. 2).

4. Discussion

We successfully established a radioimmunoassay for the measurement of sCD25 in canine serum. The detection limit of the assay (LLOD), based on the standard deviation of the blank, was 0.5 ng/mL. The reference interval for serum canine sCD25 concentrations was determined to be between 1.2 and 4.2 ng/mL and most healthy dogs had a measurable serum concentration of sCD25.

The validation data showed that the assay complies with the parameters stated in the FDA guidelines for analytical validation of an immunoassay (Booth et al., 2015). No general cut-off values for O/E for dilutional parallelism, for spiking recovery, or for CVs for intra- and inter-assay variability have been established, but it is widely accepted that O/Es between 80% and 120% are acceptable. Moreover, CVs should generally be less than 10%, with values of less than 20% being considered acceptable for results towards the edges of the working range of the assay.

Linearity of the canine sCD25 assay was tested by dilutional parallelism with an average O/E of 101.7% (min-max: 89.4%–120.6%). These values show that the assay is linear. Eighteen of the 20 measurements were within the 20% guidelines, and the remaining two

Table 4
Reproducibility of the radioimmunoassay for serum soluble CD25 for six serum samples. Duplicates of the samples were analyzed daily over one week.

Sample ID	Canine soluble CD25 concentration (ng/mL)								CV %
	I	II	III	IV	V	VI	VII	Mean	
S1	1.3	1.6	1.8	1.5	1.2	1.3	1.3	1.4	**15.0**
S2	1.7	2.0	2.0	1.8	1.6	1.7	1.8	1.8	**7.3**
S3	3.1	2.9	3.4	3.3	2.8	2.8	2.8	3.0	**8.0**
S4	6.3	6.0	6.1	6.7	5.8	5.7	5.8	6.0	**5.9**
S5	18.8	21.5	13.6	21.9	20.5	19.4	20.9	19.5	**14.4**
S6	27.9	44.0	47.1	46.1	41.3	40.0	40.1	40.9	**15.7**

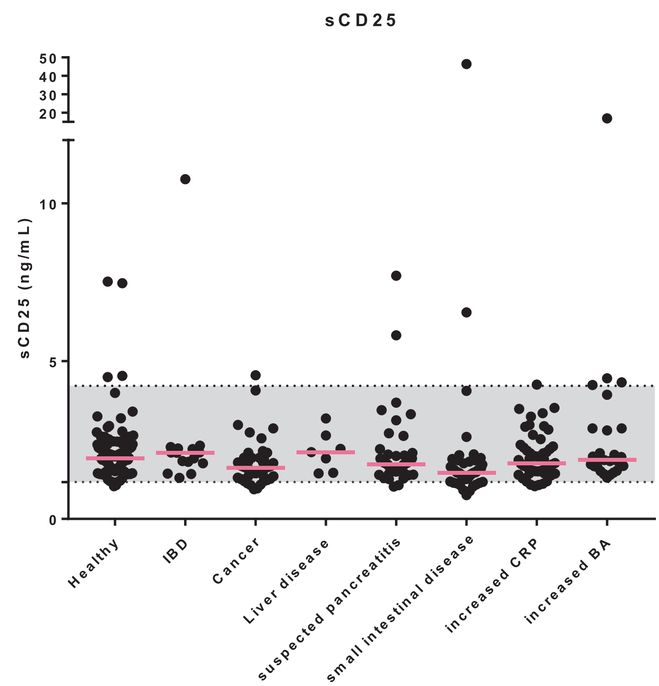

Fig. 2. Soluble CD25 concentrations measured in serum from healthy dogs and dogs with IBD, cancer, liver disease, suspected pancreatitis, suspected small intestinal disease, suspected increased inflammation with increased CRP, and suspected liver disease. The gray area represents the reference interval calculated with a clinically healthy canine population (n = 112). Purple lines represents median values (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

values were at the lower limit of the working range of the assay. Linearity was determined using pooled, natural and spiked sera until the results reached the LLOD of the assay. Considering the results obtained here, no further testing for linearity is necessary on clinical samples since the majority of the samples were within the working range of the assay, and therefore the need to dilute clinical samples is

Table 3
Precision data for the RIA for sCD25 for eight serum samples. Duplicates of the samples were analyzed 12 times in the same assay run.

Sample ID	Canine soluble CD25 concentration (ng/mL)													CV %
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Mean	
S1	1.5	1.4	1.5	1.6	1.5	1.6	1.7	1.6	1.5	1.5	1.8	2.0	1.6	**10.1**
S2	1.9	1.9	1.8	1.9	1.9	1.9	2.0	1.9	2.0	2.0	2.0	1.3	1.9	**10.5**
S3	2.1	2.1	1.7	1.8	2.0	2.2	2.2	2.1	2.1	2.3	2.3	1.4	2.0	**12.5**
S4	2.3	2.3	2.2	2.3	2.1	2.4	2.4	2.3	2.3	2.4	2.5	2.5	2.3	**4.6**
S5	3.1	3.1	3.1	2.9	3.2	3.2	3.0	3.2	3.1	3.2	3.4	3.1	3.1	**3.7**
S6	6.2	6.3	6.5	6.6	6.3	6.3	6.6	6.4	6.7	6.5	6.1	6.7	6.4	**2.9**
S7	22.1	20.8	22.3	20.5	22.2	21.0	22.5	22.3	22.6	22.7	21.9	21.3	21.8	**3.4**
S8	42.0	39.8	43.7	39.9	41.2	45.1	37.8	28.0	38.2	49.2	41.1	41.5	40.6	**12.4**

likely small. O/E ratios for spiking recovery averaged 93.2%, indicating that the assay is accurate. Intra- and inter-assay % CVs averaged 7.5% and 11.1%, respectively, suggesting that the assay is precise and reproducible. Inter-assay CVs for samples at both ends of the working range of the assay reached maximum values of 15.0% and 15.7%, respectively. Higher %CVs at both ends of the working range of the assay are to be expected and will not change the interpretation of the results. Thus, the radioimmunoassay for the measurement of sCD25 in dogs developed here is linear, accurate, precise, and reproducible.

To test the assay over its entire working range (concentrations: 0.5–50 ng/mL), we initially screened many serum samples to identify sera with different concentrations of sCD25, such as low-, mid-, and high-concentrations. Unfortunately, the majority of samples screened for this preliminary study fell into the low end of the working range of the assay. To complete the analytical validation of the assay, we decided to pool samples with the same range of sCD25, to spike a few pooled serum samples with purified recombinant canine CD25, and then to treat them as samples with naturally occurring mid- and high-concentrations of sCD25.

The use of spiked samples with recombinant canine CD25 might help to achieve better %CVs or O/Es, but the majority of the natural samples measured here fell in the low working-range of the assay where there was no need for spiked samples, and the values of %CVs and O/Es for samples in this concentration range were acceptable (< 15%).

Among the more than 350 samples analyzed for our study, several dogs were diagnosed with different types of cancers, inflammatory bowel disease, or liver disease. Other samples were from dogs suspected of having pancreatitis, liver disease, or small intestinal disease, while others were used to investigate the correlation between an acute phase reactant (*i.e.*, CRP) and sCD25. Soluble CD25 is increased in human patients with cancer, transplant rejection, and in patients with various autoimmune diseases (Bien and Balcerska, 2008). In contrast to the studies reported in humans, dogs with neoplasia in our study showed decreased concentrations of sCD25 when compared to the healthy controls. However, the difference of sCD25 concentrations among these groups did not affect the interpretation of the results as the majority of these patients had serum sCD25 concentrations within the reference interval. Based on the results obtained here, we believe that sCD25 has limited clinical utility as a diagnostic marker of T cell activation in dogs. sCD25 might be used as a marker for patient monitoring, as suggested by studies performed in human patients, but further longitudinal studies in dogs with various diseases would be needed to determine this. It should be noted that a reference interval might lose utility to judge individual patients when a high group inter-individual variability is present (high index of individuality) (Sikaris, 2014). Therefore, a baseline for each patient should be taken in order to determine the effectiveness of sCD25 as a monitoring marker.

At this time, we have no explanation for the lack of increase in serum sCD25 concentrations in dogs with various cancers, IBD, liver diseases, suspected pancreatitis, or small intestinal disease compared to humans. It should be noted that our cancer population was made exclusively of solid tumors, and it is possible that an increase in sCD25 serum concentration may be present only in dogs with liquid tumors, such as lymphoma or leukemia. The difference in results between studies reported in humans and our study when comparing healthy subjects and cancer patients may have been caused by different storage conditions among groups, although our groups of patients were collected over a similar timeframe and freeze-thaw cycles were avoided whenever possible. Further studies are needed to clarify the impact of these conditions on serum sCD25 concentrations in dogs. Also, healthy dogs were included in our study based on owner questionnaire, clinical history, and physical examination. Although dogs with recurrent medical issues (*e.g.*, otitis, dermatitis, diarrhea) or abnormal physical examination findings were excluded from this study, no laboratory blood work was performed in these dogs. It is therefore possible that some of these apparently healthy animals had some laboratory abnormalities. It is also reported that sCD25 is an early marker of T cell activation (it has been reported to increase within 24–48 hrs in humans) and once released into the extracellular milieu, its reported half-life in humans ranges from 2 to 6 h (Junghans and Waldmann, 1996). It is possible that increases in sCD25 concentrations would be able to be detected in canine serum during the early or acute phase of the disease. Future studies should be conducted to evaluate the ability of this assay to discriminate between acute *versus* chronic inflammation.

Since sCD25 retains the ability to bind IL-2 in circulation (Rubin et al., 1986), it is reasonable to hypothesize that the assay might not be able to measure sCD25 when sCD25 is bound to IL-2. It is thought that sCD25 may be used as a decoy receptor to attenuate an excessive immune response (Russell et al., 2012). It is also known that sCD25 bound to IL-2 can change the spatial configuration of IL-2, increasing the affinity of IL-2 for immature inactive T cells expressing only CD122 and CD132 (Yang et al., 2011). Therefore, the binding of sCD25 and IL-2 in circulation might explain why the concentrations of most clinical samples in this study were within the reference interval.

In veterinary medicine, only one study has been published on sCD25 in dogs (Prachar et al., 2013). That study reported that dogs with different diseases had increased serum sCD25 concentrations when compared to healthy dogs (using a human ELISA). However, when we evaluated the same assay, no binding was observed between the human antibodies and the canine recombinant and endogenous sCD25, although we cannot exclude that the serum samples that we used to test this ELISA had unmeasurably low sCD25 concentrations. In contrast, our healthy dogs had comparable concentrations of sCD25 when compared to other human studies (Buhelt et al., 2017). Additionally, the peer-reviewed literature does not appear to contain further work using a human-specific immunoassay to measure canine sCD25. This is not surprising as the reported sequence identity of CD25 between humans and dogs is only 62%.

5. Conclusion

The assay for the measurement of sCD25 in dogs described in this study is linear, accurate, precise, and reproducible. Initial studies suggested that, in contrast to humans, the measurement of sCD25 concentrations has little clinical utility in dogs with inflammatory, autoimmune, and/or neoplastic conditions. However, further studies are needed to confirm these findings.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109904.

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