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Research paper

Use of Fourier-transform infrared spectroscopy to quantify immunoglobulin G concentration and an analysis of the effect of signalment on levels in canine serum

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ABSTRACT

Deficiency in immunoglobulin G (IgG) is associated with an increased susceptibility to infections in humans and animals, and changes in IgG levels occur in many disease states. In companion animals, failure of transfer of passive immunity is uncommonly diagnosed but mortality rates in puppies are high and more than 30% of these deaths are secondary to septicemia. Currently, radial immunodiffusion (RID) and enzyme-linked immunosorbent assays are the most commonly used methods for quantitative measurement of IgG in dogs. In this study, a Fourier-transform infrared spectroscopy (FTIR) assay for canine serum IgG was developed and compared to the RID assay as the reference standard. Basic signalment data and health status of the dogs were also analyzed to determine if they correlated with serum IgG concentrations based on RID results.

Serum samples were collected from 207 dogs during routine hematological evaluation, and IgG concentrations determined by RID. The FTIR assay was developed using partial least squares regression analysis and its performance evaluated using RID assay as the reference test. The concordance correlation coefficient was 0.91 for the calibration model data set and 0.85 for the prediction set. A Bland–Altman plot showed a mean difference of −89 mg/dL and no systematic bias. The modified mean coefficient of variation (CV) for RID was 6.67%, and for FTIR was 18.76%.

The mean serum IgG concentration using RID was 1943 ± 880 mg/dL based on the 193 dogs with complete signalment and health data. When age class, gender, breed size and disease status were analyzed by multivariable ANOVA, dogs <2 years of age ($p = 0.0004$) and those classified as diseased ($p = 0.03$) were found to have significantly lower IgG concentrations than older and healthy dogs, respectively.

Abbreviations: ATR, attenuated total reflectance; CV, coefficient of variation; FTIR, Fourier-transform infrared spectroscopy; FTPI, failure of transfer of passive immunity; IgG, immunoglobulin G; MCCV, Monte Carlo cross validation; PLS, partial least squares; RMMCCV, root mean squared error Monte Carlo cross validation; RMSEC, root mean squared error of calibration; RMSEP, root mean squared error of prediction; RID, radial immunodiffusion.

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1. Introduction

Immunoglobulin G (IgG) is the predominant immunoglobulin isotype in mammals, comprising approximately 75% of the antibody pool (Rehman et al., 2003). Changes in IgG concentrations occur in many disease states, and deficiencies may be congenital or acquired. Deficiency in IgG has been associated with an increased susceptibility to bacterial, viral, or fungal infections (Chappuis, 1998; McClure et al., 2001; Whelan et al., 2006).

Many newborn mammals are immunologically naïve and rely upon the passive transfer of maternal antibodies via the placenta or the colostrum. In the dog, endotheliochorial placentation results in a 5–10% in-utero transfer of IgG to the neonate, with the remainder being provided in the colostrum. Failure of transfer of passive immunity (FTPI) occurs when insufficient maternal antibody is absorbed by the neonate. Neonates with FTPI or other forms of primary immunodeficiency have a greatly increased risk of morbidity and mortality that is best prevented by early diagnosis and therapeutic intervention (Weaver et al., 2000). Although this is a common phenomenon in horses and domestic ruminants, the prevalence and significance of FTPI in puppies has not been extensively examined (Crawford et al., 2006). However, mortality rates in puppies range from 20 to 40%, with more than 30% of these deaths being secondary to septicemia, and >50% of the reported deaths in puppies occurring within 3 weeks of birth (Nielen et al., 1998; van der Beek et al., 1999; Chastant-Maillard et al., 2012). The similarity of this pattern of disease to that observed in other species suggests that FPTI may be a significant, yet unrecognized, problem in puppies. Development of an economical and rapid assay that measures serum immunoglobulin levels in dogs may facilitate further investigation in the area of early neonatal immunity and contribute to the understanding of early mortality and morbidity (Day, 2007).

The effect of passively transferred maternal antibody on the efficacy and timing of vaccination in puppies has also engendered concerns. Problems occur in puppies when maternal antibodies persist at the time of vaccination, dampening the response to vaccines, and resulting in either vaccination failure or a need to repeat vaccination (Chappuis, 1998; Jolles et al., 2005; Whelan et al., 2006). This means that the correct timing of vaccination is difficult to determine, and the problem may lead to reduced protection against infection (Day, 2007; Ling et al., 2012). While the half-life of antibodies against individual pathogens varies, measuring serum IgG concentrations in puppies using a rapid and economic method may enable clinicians to tailor an appropriate vaccination schedule, rather than relying upon universal recommendations (Carmichael et al., 1962; Chappuis, 1998; Horzinek, 2006).

The radial immunodiffusion assay (RID) is the accepted reference method for the quantitative measurement of IgG in many domestic species, including dogs (Ginel et al., 1997; Riley et al., 2007). While RID generates quantitative IgG data, the test kits have limited availability through a few manufacturers, take 18–24 h to obtain results, require appreciable technical care and skill to perform and interpret accurately, are not readily automated, utilize reagents with relatively short expiration dates, and are relatively expensive (Riley et al., 2007). Due to the limited availability of RID test kits, recent studies have used ELISA tests to quantify IgG levels, with their accompanying limitations (Davis and Giguere, 2005; Riley et al., 2007; Roerig et al., 2013). Consequently, there is need for a sensitive and specific, rapid, cost-effective test for the determination of canine IgG serum concentrations that can be used to identify potential disorders of humoral immunity and aid future studies exploring the question of optimal timing of vaccination in puppies.

Fourier transform infrared spectroscopy (FTIR) is a powerful technique for quantitative and qualitative characterization of biological specimens/molecules in human and veterinary medicine (Shaw and Mantsch, 2000). The use of FTIR for such applications generally flows from the chemometric analysis of the spectroscopic data, which are composed of overlapping absorption bands that arise from their constituent molecular species (Dubois and Shaw, 2004; Shaw et al., 2008). These absorption patterns for biological tissues and fluids provide the basis to quantify diagnostically relevant constituents in serum, urine and other biological fluid, and may also be used for the direct diagnosis of diseases (Petrich et al., 2002). The possibility of quantitative method development has been realized by the integration of FTIR techniques with chemometric tools such as partial least squares (PLS), principal component analysis, and other approaches. These tools enable the development of quantitative analytical methods that require no molecular separation techniques or reagents (Hou et al., 2014). In our laboratory, these techniques have been previously used as the basis to develop rapid, reagent-free methods for measuring serum or plasma IgG levels in horses and alpacas (Riley et al., 2007; Burns et al., 2014). If the same approach were to provide an accurate test in canines, FTIR spectroscopy may prove to be a desirable testing method for measurement of canine serum (or plasma) IgG concentrations and further investigations into neonatal immunity.

The primary objective of this study was to develop a FTIR-based assay for the measurement of IgG concentration in canine serum, and to compare its performance to a canine RID assay. Additional objectives were to determine the IgG serum concentrations in a large population of dogs presented to a veterinary teaching hospital and whether serum IgG concentrations were different among dogs based on demographic or health status data.

2. Materials and methods

2.1. Experimental animals

This study was conducted in accordance with an animal utilization protocol approved by the University of Prince Edward Island Animal Care Committee. Sera were collected from a convenience sample of privately owned dogs ($n = 207$) that were presented to the Atlantic Veterinary College Veterinary Teaching Hospital between December

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2011 and July 2013. The population sampled was comprised of diseased and healthy animals. Each dog's disease status (clinically normal, focal disease, metabolic disease or systemically ill) was determined based on physical examination and further diagnostics as dictated by their presenting complaints. Age, gender, breed, and disease status were available for 193 dogs.

2.2. Serum sampling protocol

Blood specimens were sampled via venipuncture from dogs for which blood was already being collected for routine preventative health evaluation, elective surgeries, and examination for illness. Blood samples were allowed to clot, centrifuged at 2500 rpm for 10 min, and 0.5 mL aliquots of the serum were placed into cryovials and stored at −80 °C for batch laboratory analysis at a later date.

2.3. Radial immunodiffusion assay for IgG antibodies

A commercial RID assay (Canine IgG test kit, Triple J Farms; Bellingham, WA) was used as the reference method for determining IgG serum concentrations. The RID assay was performed according to the manufacturer's instructions. The diameter of each zone of precipitation was read at 18–22 h by the same individual to the nearest 0.1 mm using a handheld caliper. Values for samples that surpassed the manufacturer's stated upper limit (3000 mg/dL) were diluted by 50% with 0.85% saline and rerun (25 of 207 samples). Each sample and standard was tested in replicates of five, with one replicate per RID plate. The average of the five replicates of the assay standards were fitted by linear regression and that regression equation was used to determine the concentration of IgG for the unknown serum samples. Regression equations based on standards with a coefficient of determination R^2 value >0.9 were accepted for analysis. The average of the five replicate results for each sample was used to determine the RID-based IgG concentration of that sample.

2.4. Attenuated total reflectance FTIR spectroscopy measurements

Serum samples were thawed to 20 °C, vortexed, and a 40 μL aliquot was then diluted 1:1 with distilled water, and vortexed again. Following dilution, a 4 μL aliquot of each sample was placed individually on the sample stage of a 3-bounce attenuated total reflectance (ATR) element accessory interfaced with a bench top FTIR spectrometer (Cary 630 FTIR spectrometer, 3 B Diamond ATR Module ZnSe element, Agilent Technologies, Dansbury, Connecticut) by evenly spreading diluted sample onto surface of the ATR element, creating a circular spot 3 mm in diameter. Each sample was then air dried by for 4 min. Once the film was thoroughly dried, the single beam FTIR intensity I_s was acquired, ratioed against the counterpart intensity I_0 for a clean ATR element, and the (pseudo) absorbance spectrum determined as $-\log(I_s/I_0)$. Five replicates of each sample were tested. Absorbance spectra within the range of 650–4000 cm^{-1} were recorded for each replicate. Between each application of serum the sample stage was cleaned using alcohol and a laboratory wipe, and a fresh background spectrum I_0 was recorded before the next sample was applied.

2.5. FTIR spectrum preprocessing

Absorbance spectra were converted into printable format using the GRAMS software package (GRAMS software ver. 7.02, Thermo Fisher Scientific Inc, Waltham, MA, USA), then imported into MatLab® R2011b (v. 7.13.0.564) (MathWorks, Natick, MA, USA). Further analyses were carried out using programs written by one of the authors in MatLab® (Hou et al., 2014). Each spectrum was smoothed using Savitzky Golay method (2nd degree polynomial functions with 9-point smoothing) (Savitzky and Golay, 1964) and then processed by standard normal variate transformation to correct for light scattering (Barnes et al., 1989). The spectral regions 1300–1800 and 2600–3700 cm^{-1} were selected to develop the multivariate calibration model. Spectroscopic outliers within each group of replicate spectra were detected using Dixon's *Q*-test at each wavenumber with a confidence level set at 95% (Dean and Dixon, 1951; Rorabacher, 1991). Any spectrum that encompassed outliers for over 50% of the wavenumbers within a spectrum was excluded from further analysis. The average spectrum for each sample was then used for subsequent analysis.

2.6. Calibration model development and assay validation

The 207 samples were sorted based on the IgG concentrations acquired by the RID method. Eight samples with an RID IgG higher than 4000 mg/dL were excluded to reduce the leverage effects of this small number of samples, leaving behind spectra from 199 samples for building and validating the analytical method.

A subset of samples designed to encompass the range of IgG concentrations reflected in the population of 199 samples was assigned into a prediction set ($N = 40$) to later test the performance of the new analytical method. The remaining spectra samples ($N = 159$) were randomly further split into a training set ($N = 111$) and a validation set ($N = 48$). Partial least squares (PLS) regression was applied to the training set to develop 50 trial calibration models with the number of PLS factors ranging from 1 to 50. Each of the models was employed to calculate the IgG concentrations of each sample in the independent validation set, and an error estimate (sum of the squares of the differences between RID IgG values and the FTIR predicted IgG values) was calculated. The process was then repeated 10,000 times, with new random splitting of the samples into training set and validation set (and the subsequent PLS regression) for each of the 10,000 trials.

Once all 10,000 trials had been completed, the root-mean square error of predicted validation set concentrations encompassing all 10,000 trials (Monte Carlo cross validation error estimate; "RMMCCV") was evaluated for each of the provisional PLS calibration models; i.e. for models ranging from 1 to 50 factors (Picard and Cook, 1984; Xu and Liang, 2001). This procedure (not including the assignment of the test set) was repeated 10,000 times and the

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Table 1
Serum immunoglobulin G concentrations as measured by RID and population signalment.

Signalment	*n*	IgG concentration mg/dL (mean + s.d.)	Range mg/dL[a] (min–max)
Clinical status			
Normal dogs	46	2260 ± 820	522–4207a[b]
Focal disease	46	1885 ± 901	482–4979b
Systemic or metabolic disease	101	1825 ± 870	432–4694b
Gender			
Female	86	1899 ± 841	432–3766
Male	107	1979 ± 912	482–4979
Age (years)			
<2	28	1283 ± 604	482–2677c[c]
≥2 to <4	23	2123 ± 595	1112–3131d
≥4 to <6	38	2184 ± 1037	671–4979d
≥6 to <8	31	1933 ± 809	432–3766d
≥8 to <10	25	2210 ± 919	440–4207d
≥10	48	1920 ± 854	447–3768d
All ages	193	1943 ± 880	432–4979
Breeds			
Labrador retriever	32	1745 ± 693	482–3253
Mixed breed	32	1991 ± 887	440–4207
Golden retriever	16	2109 ± 706	671–3252
German shepherd	11	1870 ± 1284	522–3766
Boxer	8	1803 ± 1233	709–4694
Shih-tzu	8	2188 ± 513	1273–2866
Bernese mountain dog	5	2478 ± 799	1694–3759
Border collie	4	1839 ± 1213	447–3396
Bulldog	4	1782 ± 987	945–3152
Newfoundland	4	2779 ± 1585	1282–4979
Rottweiler	4	2013 ± 847	914–2852
Other pure breeds[d]	65	–	–
All samples	193	1943 ± 880	432–4979
Reference range for normal canines[e]	46	–	522–4207

Complete signalment data was available for 193/210 dogs.
[a] Letter denotes different and common statistical groupings.
[b] $p = 0.03$.
[c] $p < 0.0004$.
[d] Large, medium and small sized breeds with 1–3 animals per breed.
[e] Using Reference Value Advisor.

Monte Carlo cross validation value (MCCV) was calculated by the equation

$$\mathrm{MCCV} = \frac{1}{Nn_v}\sum_{i=1}^{N}\left\|y_i - \hat{y}_i\right\|^2$$

where N denotes the number of repeated procedure ($N = 10{,}000$), n_v is the number of samples in the validation set ($n_v = 48$), and y_i and $\hat{y}_i$ represent the IgG concentrations for the samples, in the validation set, obtained from RID experiments and predicted from the calibration models, respectively. The number of PLS factors was then chosen as the one giving the lowest RMMCCV value.

After the number of PLS factors had been determined, the training set and validation set were merged to build the final PLS calibration model. The samples in the prediction set were then used to test the predictive performance of the new calibration model; i.e. to test the accuracy of the new analytical method on blinded samples.

2.7. Statistical analysis

The model's predictive performance was evaluated by comparing the predicted IgG concentrations as determined by the FTIR-based analytical method, with concentrations obtained independently by RID. The level of agreement was first assessed by a scatter plot and the concordance correlation coefficient, followed by the Bland–Altman plot, and by the normal probability plot (Altman and Bland, 1983). Finally, the precision of both the FTIR and RID methods were investigated. The mean and standard deviation were calculated for the five replicates of each serum sample, and from these a modified coefficient of variation, accounting for the small number of replicates per sample tested ($\mathrm{CV}^* = \mathrm{CV}(1 + (1/4n))$; $n = 5$) was determined for each sample tested (five replicates) and plotted against the mean concentration for IgG.

Descriptive statistics were performed on the available signalment information of the study population. The distribution of the RID determined IgG and age (in months) values were evaluated for normality by a probability plot of the data and the Kolmogorov–Smirnov test. Univariate analysis of variance (ANOVA) or Kruskal–Wallis ANOVA was performed to examine the effects of age (transformed to categorical variables: <2; ≥2 to <4; ≥4 to <6; ≥6 to <8; ≥8 to <10; and ≥10 years), gender (male; female; spay/neuter status), disease status (clinically normal; focal disease; metabolic or systemic disease), and breed, with

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significant associations recognized at $p \leq 0.05$. Breed comparisons were limited to the six breeds represented with $n \geq 7$. Dogs were also compared on the basis of breed size (large, medium and small); mixed breeds were excluded from this comparison. Following univariate analyses, age class, gender, breed size and disease status were analyzed by multivariable ANOVA. The Reference Value Advisor was used to calculate the reference range for IgG values in this population of clinically normal dogs (Geffré et al., 2011).

3. Results

Of the 193 canines for which a complete signalment was available, 10 were intact females, 76 were spayed females, 24 were intact males, and 83 were neutered males. The mean age of the study population was 6.8 ± 3.9 years (Table 1). The study population encompassed 49 recognized pure breeds of small, medium and large size, as well as a large number of dogs of mixed breeds (Table 1). Of the 193 dogs for which disease status was available, 46 had no clinical illness at the time of sample collection and 147 had disease at sample collection including: 25 with orthopedic disease, 25 with neoplasia, 21 with non-specific gastrointestinal signs, 12 with suspected immune-mediated disease, 11 with renal disease, 10 with diagnosed endocrine disease, 6 with skin allergies, 5 with neurologic signs, 5 with elevated serum biochemistry values consistent with hepatic disease, and 5 with urinary tract infections. The remaining 22 ill animals included individual diseases affecting the integumentary (1), reproductive (2), cardiovascular (4), ophthalmic (1), and respiratory systems (6), as well as urinary sphincter incompetence (2), vehicular trauma (2) and nonspecific clinical signs where no specific diagnosis was obtained (4). The RID IgG concentrations for the 193 serum samples with available signalment data ranged from 432 mg/dL to 4979 mg/dL. The laboratory reference range for IgG for clinically healthy dogs was 522–4207 mg/dL based on the non-parametric option in the Reference Value Advisor (Geffré et al., 2011).

For the FTIR based assay, the 207 samples were sorted based on the IgG concentrations acquired from the RID method. Eight samples with an RID IgG higher than 4000 mg/dL were excluded to reduce the leverage effects of this small number of samples, leaving behind spectra from 199 samples for building and validating the analytical method (Fig. 1). The optimal number of PLS factors was determined to be 17 based on the lowest Monte Carlo cross validation value (RMMCCV = 529 mg/dL). A scatter plot (Fig. 2) exhibits the correlation between IgG concentrations obtained via RID and FTIR (RMSEC = 326 mg/dL, RMSEP = 404 mg/dL). The concordance correlation coefficients for the calibration model data set and the prediction set were 0.91 and 0.85, respectively.

The Bland–Altman plot (Fig. 3) shows the mean value of the difference (FTIR–RID) as −89 mg/dL. When compared to high IgG concentrations, this value was small and indicated

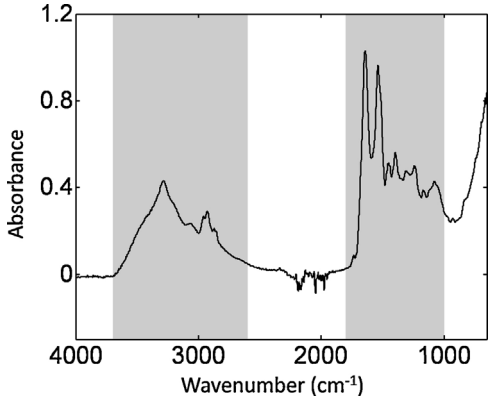

Fig. 1. A representative raw infrared spectrum of canine serum. The shaded spectral regions were selected to build the multivariate calibration model.

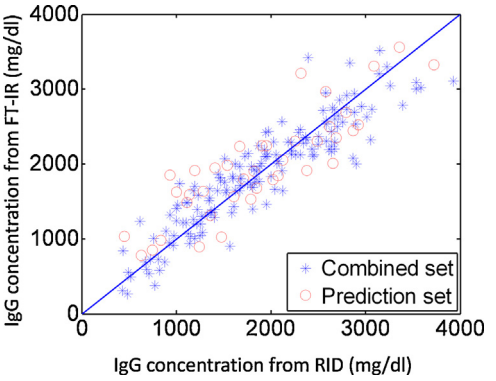

Fig. 2. A scatter plot comparing the immunoglobulin G (IgG) concentrations obtained from radial immunodiffusion (RID) and Fourier transform infrared spectroscopy (FTIR) methods. The *R* values for the concordance coefficient were 0.91 and 0.85, respectively for the combined set and test set. The asterisks denote samples used in building the calibration model and the circles indicate the samples in the prediction set. Seventeen partial least squares (PLS) factors were retained. If RID and FTIR give comparable results, the data points should distribute closely around the reference line.

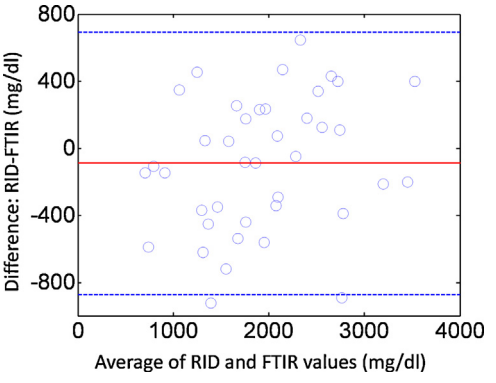

Fig. 3. A Bland–Altman plot of the differences in the IgG concentrations in the prediction set as obtained by radial immunodiffusion (RID) and Fourier transform infrared spectroscopy (FTIR) methods. The solid horizontal line represents the mean difference between RID and FTIR assays (−89 mg/dL). The dashed lines represent the 95% confidence interval. If there is no systemic bias between RID and FTIR methods, the mean value of the differences should be close to zero. If the model assumption is that the errors are independent and identically distributed and follow a normal distribution, the data points should randomly distribute around the mean of the difference.

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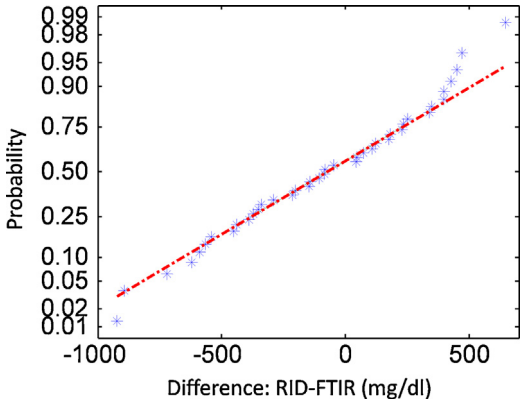

Fig. 4. Normal probability plot of the differences of the immunoglobulin G (IgG) concentrations in the prediction set obtained from radial immunodiffusion (RID) and Fourier transform infrared spectroscopy (FTIR) methods. The data points should be closely located around the reference line if the measurement errors follow a normal distribution.

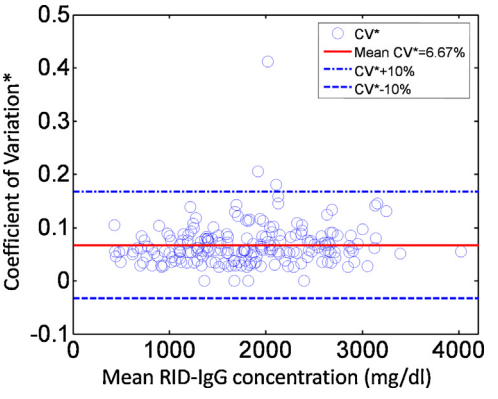

Fig. 6. Coefficient of variance (CV*) plots for the radial immunodiffusion (RID) method. Lower values of CV* are interpreted as higher precision of the test method.

no significant systematic bias between the two methods. A normal probability plot for the differences (Fig. 4) shows the majority of data points scattered closely around the reference line. Precision of the FTIR test and RID analysis is graphically demonstrated in Figs. 5 and 6. The mean coefficient of variation (CV) for RID was 6.67% and for FTIR it was 18.76%.

On univariate analysis, dogs that were assessed as clinically normal had significantly ($p = 0.03$) greater IgG concentrations than ill patients, but there was no significant difference between those with a focal complaint and those that were classified as systemically ill (Table 1). Dogs less than 2 years of age had significantly ($p = 0.0004$) lower values than all other age groups (Table 1), but the overall relationship between age and IgG status was not linear. There were no significant differences in IgG values associated with gender, spay/neuter status, breed or pure breed size. When age class, gender (forced), breed size (forced) and disease status were analyzed by multivariable ANOVA, only age class ($p = 0.0004$) and disease status ($p = 0.03$) persisted as significant effects.

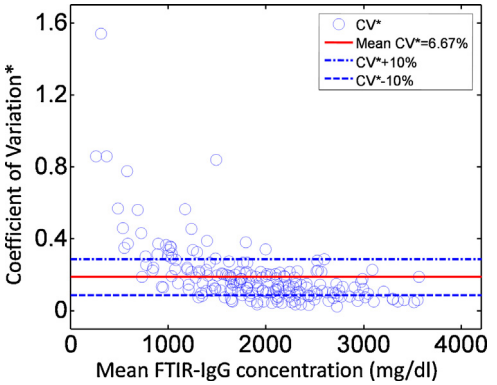

Fig. 5. Coefficient of variance (CV*) plots for the Fourier transform infrared spectroscopy (FTIR) method. Lower values of CV* are interpreted as higher precision of the test method.

4. Discussion

This study showed that FTIR, in combination with chemometric tools, is a promising technique for the measurement of IgG concentrations in canine serum. This method was validated by its ability to accurately quantify IgG concentrations in an independent prediction set of samples. Other findings of the study indicate that disease and young age (<2 years) results in lower serum IgG levels in a mixed population of dogs.

It is important for FTIR-based assay development (and for any assay validation) to recruit a broad representation of the target population, and obtain a large number of samples where possible. The population sampled had a wide age distribution, contained almost 50 dog breeds, and was thus characteristic of the population that would be seen at many referral veterinary practices. To the authors' knowledge, this is the first study of serum IgG concentrations in dogs to characterize such a wide range of clinical presentations and diagnoses, and this resulted in significant new findings. In particular, the finding of lower concentrations of serum IgG in ill patients suggests that further investigation of IgG concentrations in specific disease states may ultimately lead to the use of this protein assay as a screening tool for patient evaluation of disease. However, the overlap of IgG concentrations for healthy and diseased indicates these differences may not be distinct enough to be clinically useful as a stand-alone parameter. The heterogeneity of the caseload presented for sampling as described in our study did not provide significant power to evaluate the diagnostic utility of IgG concentrations for any specific disease state. In contrast, several previous reports have found significant associations between IgG concentrations and disease status, including stray dogs with leishmaniasis, and Weimaraner, Shar-Pei, and Rottweiler breeds with various inflammatory diseases (Rivas et al., 1995; Day et al., 1997; Day, 1999; Maden et al., 2013). Given our findings, the recruitment of cases with specific syndromes and an investigation of associated IgG concentrations, including those of neonates, are warranted.

Previous studies of IgG concentrations as determined by RID have either sampled smaller populations or large

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Table 2
Comparative data from the literature for immunoglobulin G (IgG) concentrations in healthy and diseased populations quantified by radial immunodiffusion (RID).

Author	Breed	Disease status	*n*	Mean IgG concentration (mg/dL)	Range of IgG concentration (mg/dL)
Present study	MP	Healthy	46	2260 ± 820	522–4207
Reynolds and Johnson (1970)	Mongrel	Healthy	20–35	1445[a]	–
Watson et al. (2006)	CKCS	Healthy	16	850	480–1700
Heddle and Rowley (1975)	Mongrel	Healthy	14	980	520–1730
Poffenbarger et al. (1991)	Beagle	Healthy	6	2030	600–5000
Provost et al. (2003)	Beagle	Healthy	N/A	–	772–992
Present study	MP	Ill	147[b]	1885 ± 901	432–4979
Watson et al. (2006)	CKCS	Ill	9	320	120–800

Complete signalment data was available for 193/199 dogs in the present study.
[a] Reynolds and Johnson gave IgG values for individual subclasses. For comparison with our own and other data these have been summed; N/A, the number of patients sampled was not available; MP, mixed population of breeds represented; CKCS, Cavalier King Charles spaniel.
[b] The dogs in our study were initially classified into focal or systemic disease. However, no significant difference between these two groups was noted, therefore the values listed are from the group with the largest standard deviation reported and the range is from a combination of both groups.

numbers within a specific breed (Table 2). Published RID-based serum IgG concentrations in healthy dogs vary widely, ranging from 120 to 5000 mg/dL, with sample sizes ranging from 6 to 35 dogs (Reynolds and Johnson, 1970; Poffenbarger et al., 1991; Watson et al., 2006). The mean IgG concentrations in our study were higher than many of the previously reported RID-IgG studies, as was the range of values in our study, with the exception of the study by Poffenbarger et al. (Table 2). The may reflect the diversity of the population in the current study, as the previously reported ranges are based on studies performed in specific breeds and diseases, or describe findings in small sample sizes.

Newborn puppies are reported to have a serum IgG concentration of 123 ± 7 mg/dL increasing to 2300 mg/dL 12 h after ingestion of colostrum. Serum IgG concentrations in older puppies have not be reported to the authors' knowledge and there are limited studies regarding immune system development in dogs under 6 months of age (Day, 2007; Tvarijonaviciute et al., 2013). Differences have been previously observed in IgG concentrations in dogs 8–10 months old (Schreiber et al., 1992), but to the authors' knowledge a difference between dogs <2 years of age and older adults has not been described, highlighting the need for further study of antibody levels in adolescent dogs. One limitation of this study, in common with previous studies, is that no samples were available for collection from dogs <6 months of age. Several other studies using other assay methods have failed to define age, or sampled dogs that were greater than 6 months of age (Ginel et al., 1997; Provost et al., 2003; Tvarijonaviciute et al., 2013). The need to define immunoglobulin levels in dogs less than 6 months of age is warranted, as these are the dogs most likely to be affected by septic disease and related increased mortality (Nielen et al., 1998; van der Beek et al., 1999; Day, 2007; Chastant-Maillard et al., 2012).

In this study, FTIR based IgG concentrations were comparable to those in previous studies using RID (Heddle and Rowley, 1975; Provost et al., 2003). The Bland–Altman plot, a measure of equivalence of the two methods, showed strong agreement when the large IgG values were taken into consideration. However, the values were not equivalent and the 95% confidence intervals on this plot indicated a high variability between the two methods. Therefore, when using the FTIR method a correction factor equivalent to the mean difference demonstrated by the Bland–Altman plot may be considered. Other reports have found that CV values for RID vary between 4 and 10%, and in one instance was reported to be up to 41% in some laboratories (Ginel et al., 1997). The CV reported in this study was a modified calculation and may be a conservative estimate. Laser nephelometry reportedly has better day-to-day precision (CV of 3.9%) than RID. However, the cost of the nephelometer has limited its use in veterinary medicine (Ginel et al., 1997). Although good correlation is generally seen between RID and nephelometric procedures, variation in correlation results have been reported to be poor by a few investigators, therefore further investigation into this method is warranted. A critical consideration with all methods is the overall lack of uniformity in the units expressing concentration in the calibration standards and variation in kit performance. This contributes to poor standardization of serum IgG measurements in canines (Narayanan, 1982).

The accuracy and precision of the FTIR-based analytical method for canine serum IgG were comparable to those developed for horses and alpacas using research laboratory FTIR spectrometers (Riley et al., 2007; Burns et al., 2014), however the current study was performed on more economically accessible bench-top equipment developed to perform point-of-care testing (its footprint is 13 × 28 cm). The level of precision and accuracy obtained with this bench top equipment will facilitate the translation of this new knowledge to practical testing at the veterinary laboratory or practice level.

5. Conclusion

Fourier-transform infrared spectroscopy, in conjunction with chemometric methods, is an accurate technique for quantitating IgG concentrations in canine serum. This method is a quick, low cost, bench-top, and reagent-free method that requires very small sample volumes. In this study, age (<2 years) and positive disease status were

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associated with significantly lower serum IgG concentrations and thus, further studies investigating these findings are warranted.

Conflict of interest

The authors disclose no conflict of interest.

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