

## Use of Fourier-Transform Infrared Spectroscopy to Quantify Immunoglobulin G Concentrations in Alpaca Serum

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**Background:** Rapid, economical, and quantitative assays for measurement of camelid serum immunoglobulin G (IgG) are limited. In camelids, failure of transfer of maternal immunoglobulins has a reported prevalence of up to 20.5%. An accurate method for quantifying serum IgG concentrations is required.

**Objective:** To develop an infrared spectroscopy-based assay for measurement of alpaca serum IgG and compare its performance to the reference standard radial immunodiffusion (RID) assay.

**Animals:** One hundred and seventy-five privately owned, healthy alpacas.

**Methods:** Eighty-two serum samples were collected as convenience samples during routine herd visits whereas 93 samples were recruited from a separate study. Serum IgG concentrations were determined by RID assays and midinfrared spectra were collected for each sample. Fifty samples were set aside as the test set and the remaining 125 training samples were employed to build a calibration model using partial least squares (PLS) regression with Monte Carlo cross validation to determine the optimum number of PLS factors. The predictive performance of the calibration model was evaluated by the test set.

**Results:** Correlation coefficients for the IR-based assay were 0.93 and 0.87, respectively, for the entire data set and test set. Sensitivity in the diagnosis of failure of transfer of passive immunity (FTPI) ([IgG] <1,000 mg/dL) was 71.4% and specificity was 100% for the IR-based method (test set) as gauged relative to the RID reference method assay.

**Conclusions and Clinical Importance:** This study indicated that infrared spectroscopy, in combination with chemometrics, is an effective method for measurement of IgG in alpaca serum.

**Key words:** Camelid; Failure of passive transfer; Radial immunodiffusion.

Pregnant camelids have an epitheliochorial form of placentation that unlike humans, does not allow for the in-utero transfer of immunoglobulins.<sup>1</sup> Consequently, newborn crias are born agammaglobulinemic and rely upon the transfer of maternal antibodies from the dam's colostrum to acquire protective passive immunity for the first few weeks of life.<sup>2,3</sup> In the newborn cria, transfer of immunoglobulins is considered successful when immunoglobulin G (IgG) concentration in the serum is  $\geq 1,000$  mg/dL.<sup>4</sup> Failure to absorb sufficient amounts of immunoglobulins in the immediate postpartum period leads to a condition known as failure of transfer of passive immunity (FTPI).<sup>1</sup> FTPI has a reported prevalence between 9% and 20.5% in camelids and is recognized as an important disorder that might lead to an increased susceptibility to infectious organisms.<sup>4–7</sup>

The most common clinical reason for measuring camelid immunoglobulins is suspicion of FTPI, there-

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### Abbreviations:

AVC	Atlantic Veterinary College
FTIR	Fourier-transform infrared spectroscopy
FTPI	failure of transfer of passive immunity
IgG	immunoglobulin G
IT	immunoturbidimetric assay
MCCV	Monte Carlo cross validation
PLS	partial least squares
RID	radial immunodiffusion

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fore early and accurate diagnosis is required to provide timely medical intervention.<sup>7</sup> Testing methods available for use in camelids include total serum protein and globulin concentration measurements, zinc sulfate turbidity, glutaraldehyde coagulation, sodium sulfate precipitation, immunoturbidimetric assay (IT), and radial immunodiffusion (RID).<sup>3,4,6,8–11</sup> Of these, RID provides quantitative data and is currently considered the reference method test for IgG quantification in various species.<sup>7</sup> Notwithstanding the benefit of providing quantitative data, RID has a number of disadvantages including a substantial turnaround time of 18–24 h for results,<sup>12</sup> a requirement for enhanced technical skill by the user, high cost compared with other testing methods, and a requirement for specific reagents with a limited shelf life.<sup>7,9,12</sup> An accurate, more rapid, and economic IgG assay for quantifying serum IgG is desirable for camelids.

Fourier-transform infrared spectroscopy (FTIR) has emerged as a powerful diagnostic tool for quantitative and qualitative characterization of biological samples in human and veterinary medicine.<sup>13–15</sup> The basis for this technique is that infrared radiation passing through a sample of interest is absorbed by that sample

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*Submitted June 6, 2013; Revised September 24, 2013; Accepted October 23, 2013.*

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*10.1111/jvim.12258*

more or less strongly as a function of wavelength across the infrared spectral region. The resultant infrared absorption spectrum uniquely characterizes that sample, with the absorbance of radiation measured as a function of wavelength ( $\mu\text{m}$ ) or wavenumber ( $\text{cm}^{-1}$ ), translating to absorption bands across the infrared spectrum.<sup>7,14,15</sup> The infrared absorption patterns reflect the molecular composition of the sample.<sup>7</sup> To the extent that molecular composition is characteristically affected by disease, the absorption patterns for biomedical specimens (biofluids and tissues) might be equated to biochemical “fingerprints.” These “fingerprints” then have the potential to correlate directly with the presence or absence of disease or provide the basis to quantify diagnostically pertinent biological fluid analytes.<sup>7</sup> Thus, infrared spectroscopy could serve as a robust, reagent-free method to quantify certain biofluid components of diagnostic interest.<sup>7,13,14</sup> If it were to prove sufficiently accurate, the ease of use and low cost per sample may make FTIR a desirable testing method for determining serum IgG concentrations.

The objective of this study was to develop a FTIR-based assay for the measurement of IgG concentrations in alpaca serum and to compare its performance to that of the RID assay.

## Materials and Methods

### *Experimental Animals*

Serum samples collected from privately owned alpacas ( $n = 175$ ) between 2009 and 2011 were used for this study. Eighty-two alpaca samples were from multiple farms located in Ontario and New Brunswick, Canada, whereas the remaining 93 were from properties in the Adelaide Hills region of South Australia. All alpacas were considered healthy based on general physical examination and no observable signs of disease. Age and sex were collected for 129 of the alpacas.

### *Serum Sampling Protocol*

Blood samples were collected via jugular venipuncture in accordance with the University of Prince Edward Island’s Animal Care Committee animal utilization protocol and the Animal Ethics Committee of the University of Adelaide. After collection, blood samples from Canada were centrifuged and the serum collected into a cryovial and frozen at  $-20^{\circ}\text{C}$  until transported on ice by overnight courier to the Atlantic Veterinary College (AVC) where they were stored at  $-80^{\circ}\text{C}$  before use. Blood samples collected in Australia were taken directly to a diagnostic laboratory for hematologic analysis as part of a separate study. These samples were centrifuged and the serum collected and refrigerated at  $4^{\circ}\text{C}$  for 24–48 h until biochemical analysis was performed. The remaining serum was then stored frozen at  $-80^{\circ}\text{C}$  before being couriered on ice to the AVC.

### *RID Assay for IgG Antibodies*

Commercial RID assays (Triple J Farms, Bellingham, WA) were used as the reference method for determining IgG serum concentrations. RID assays were performed according to manufacturer’s instructions. The zones of precipitation diameters were read at 22–24 h by the same individual to the nearest tenth of a

millimeter using a handheld calliper. Samples exceeding the manufacturer’s stated upper testing limit (3,000 mg/dL) were diluted with 0.85% saline and rerun. Each sample and standard was tested in replicates of five (one replicate per RID plate). The average of the 5 replicates of the assay standards was fitted by linear regression, and that equation was used to determine the concentration of IgG for the unknown serum samples. Regression equations for  $R^2 > 0.9$  were accepted for analysis. The average of the 5 replicate results for each sample was used to determine the IgG concentration of that sample.

### *FTIR for IgG Antibodies*

Thawed serum samples were diluted 1:1 with a 4 g/L potassium thiocyanate solution. After dilution, six replicate dry films were made for each serum sample by evenly spreading 12  $\mu\text{L}$  aliquots of diluted sample onto 5 mm wells within a custom-made, adhesive-masked, 96-well silicon microplate.<sup>8</sup> Each loaded microplate was allowed to dry at room temperature before being loaded into a multisampler<sup>a</sup> interfaced with a FTIR spectrometer<sup>b</sup> and controlled with proprietary software.<sup>c</sup> Absorbance spectra in the IR range of 400–4,000  $\text{cm}^{-1}$  were recorded (resolution 4  $\text{cm}^{-1}$ , 512 scans) for each replicate of each sample, using the single beam spectrum of an empty well as the background.

### *Data Processing*

Absorbance spectra were converted into printable format by GRAMS software package,<sup>d</sup> then imported into MATLAB<sup>®e</sup> for further analysis. Calculations were performed using scripts written by the authors.

A total of 1,050 (175 samples  $\times$  6 replicates/sample) spectra were smoothed using the Savitzky-Golay method<sup>16</sup> (2nd order polynomial function with 9 points smoothing). This was followed by spectrum region selection chosen as 3,700–2,600  $\text{cm}^{-1}$  and 1,800–1,300  $\text{cm}^{-1}$  (based on previous work for FTIR IgG analysis in horses and further refined to specific areas within those regions that exhibited strong signals).<sup>7</sup> The spectra were processed by the standard normal variate transformation<sup>17</sup> (each absorbance value data point was first shifted by subtracting the mean of all values in that spectrum, and then scaled by ratioing against the SD for all data points in that spectrum). With 6 replicate spectra per serum sample, spectrum outlier detection was carried out by using Dixon’s Q-test<sup>18,19</sup> at each wavenumber with a confidence level set as 95%. Those spectra with over 50% of the absorbance values detected as outliers were excluded from further analysis. Outlier detection was performed for all the 175 serum samples. The spectra for each sample were then averaged and used for succeeding analysis.

### *Calibration Model Development and Assay Validation*

A test set of spectra from serum samples ( $N = 50$ ) was randomly drawn from the pool of 175 samples and set aside for the purpose of later testing the predictive performance of the calibration model. Spectra from the remaining samples ( $N = 125$ ) were again randomly split into a training set ( $N = 75$ ) and a validation set ( $N = 50$ ). Partial least squares (PLS) regression was applied to the training set to develop calibration models with the number of PLS factors ranging from 1 up to 50, evaluating for each model the sum of the squared errors for the validation set samples. This procedure (with new random training/validation splits for each trial, and the same test set throughout) was repeated 5,000 times and the Monte Carlo Cross validation value<sup>20,21</sup> (MCCV) was calculated by the equation

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

where  $N$  denotes the number of repeated procedures ( $N = 5000$ ),  $n_v$  is the number of samples in the validation set ( $n_v = 50$ ), 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 IR spectroscopic data, respectively. The number of PLS factors that gave the lowest MCCV value was chosen as the optimal value. Once the optimal number of PLS factors was determined, the training set and validation were combined and the combined set was used to build a new calibration model. The predictive performance of the new calibration model was then evaluated by the test set that had been set aside in advance. The agreement among the RID and FTIR values for both the test set and combined data set was assessed by scatter plot, the Pearson correlation coefficient, and the concordance correlation coefficient. The differences between the RID and FTIR values for the test set were further characterized by the Bland-Altman plot and the normal probability plot to further evaluate the model assumptions and performance.<sup>22</sup>

### Precision of FTIR Spectroscopic Analyses

With replicate spectra collected in this study, the precision of the FTIR method was evaluated based on the concentrations predicted for the test set samples/spectra. To that end, individual replicate spectra (not the averaged spectra) were used to calculate the IgG concentrations and the relative standard deviations (also called coefficient of variation) were used as the criteria to evaluate precision. For comparison, the relative SD was also calculated for the individual replicate RID assays.

### Diagnostic Sensitivity and Specificity

To assess potential applicability in the clinical diagnosis of FTPI,<sup>7</sup> the diagnostic sensitivity and specificity of FTIR were calculated for the test set and combined data set using IgG <1,000 mg/dL (based on RID) as the cutoff for a positive test for FTPI.

## Results

Of the 129 alpacas for which signalment data were available, 81 were female and 48 were male. Four alpacas were <2 months of age, 5 were 2–3 months of age, 15 were 3–6 months of age, 12 were between 6 months and 1 year of age, and 93 were >1 year of age. Demographic data were unavailable for the remaining 46 alpacas.

The RID IgG concentrations for the 175 serum samples ranged from 394 to 6,327 mg/dL. Twenty-one samples had IgG concentrations below the 1,000 mg/dL cutoff typically used to define FTPI in camelid neonates. Of the remaining 154 samples, 89 had IgG concentrations between 1,000 and 3,000 mg/dL and 65 had IgG concentrations >3,000 mg/dL.

The overall patterns for blood/serum/plasma infrared spectra are characterized by strong absorptions in two regions (3,600–2,750  $\text{cm}^{-1}$  and 1,800–400  $\text{cm}^{-1}$ ), the former corresponding to stretching vibrations of the X-H moieties (X=C, N, O), and the latter corresponding to bending vibrations and stretching modes involving heavier atoms (eg the C=O stretch cited

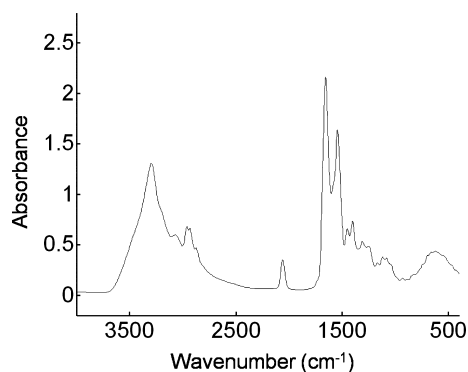
above; Fig 1).<sup>7</sup> Within each spectrum, the most intense features result from proteins (the absorptions centering around 3,300  $\text{cm}^{-1}$  are attributed to N-H stretching and those around 1,650  $\text{cm}^{-1}$  correlate with C=O stretching of backbone protein amide linkages).<sup>7</sup>

As a first step toward development of the IR-based analytical method, 10 spectra were identified as outliers (using the method described above) and removed from further analysis. The PLS regression trials yielded an optimal model (based on the lowest MCCV) with 15 PLS factors. A scatter plot (Fig 2) demonstrates the level of agreement between IgG concentrations obtained via RID and FTIR. The Pearson correlation coefficients for the combined set (training and validation sets) and test set were 0.93 and 0.87, respectively. The concordance correlation coefficients were 0.93 and 0.86, respectively.<sup>23,24</sup> Both correlation coefficients indicated that the FTIR-predicted IgG concentrations had good to excellent correlation with RID determined IgG concentrations.

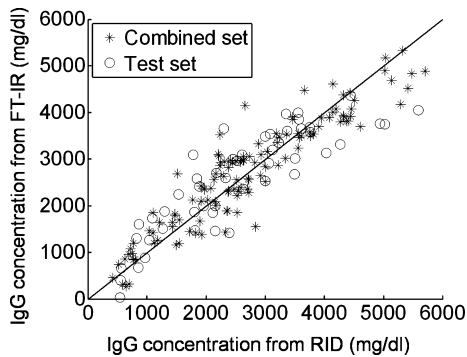
The mean value of the differences (FTIR – RID) is –20.4 mg/dL, which is small compared to the IgG concentration values, indicating no significant systematic difference (bias) between the 2 methods (Fig 3). However, the 95% confidence intervals on the Bland-Altman plot indicated a high variability ( $\text{SD} \cong 600$  mg/dL) between FTIR and RID methods. A normal probability plot for the differences (Fig 4) demonstrates the majority of data points are distributed closely around the reference line. No obvious non-normality was observed in the error distribution, indicating the model assumption was not violated and further validating the developed model.

### Precision of the FTIR Spectroscopic Analyses

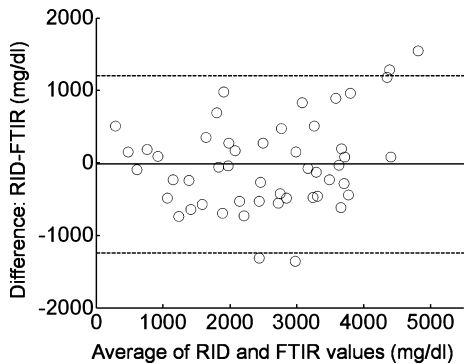
The relative SDs for the RID did not show an obvious correlation with the IgG concentration but the relative SDs for the FTIR method decreased with an increase in the IgG concentration (Fig 5). The relative SDs for the RID and FTIR methods overlapped when



**Fig 1.** Representative infrared spectrum of alpaca serum. The strongest features arise from proteins with relatively less abundant serum components contributing relatively weak fingerprints. The absorption at 2,062  $\text{cm}^{-1}$  originates with the  $\text{KSCN}^-$  internal standard.



**Fig 2.** A scatter plot comparing the immunoglobulin G (IgG) concentrations obtained from radial immunodiffusion (RID) and Fourier-transform infrared spectroscopy (FTIR) methods. The asterisks denote samples used in building the calibration model and the circles indicate the samples in the test set. 15 partial least squares (PLS) factors were retained. If RID and FTIR give comparable results, the data points should distribute closely around the reference line of 45°.

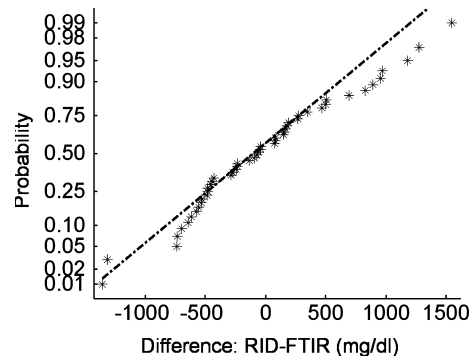


**Fig 3.** A Bland-Altman plot of the differences in the immunoglobulin G (IgG) concentrations in the test set as obtained by radial immunodiffusion (RID) and Fourier-transform infrared spectroscopy (FTIR) methods. The solid horizontal line represents the mean difference between RID and FTIR assays (−20.4 mg/dL) and the dashed lines represent the 95% confidence interval. If there is no systematic bias between RID and FTIR method, 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 distribute around the mean of the difference randomly. Narrow dispersion of the data points means low measurement uncertainties.

the IgG concentration was high, but when the IgG concentration was low, the FTIR method had larger relative SDs.

### Diagnostic Sensitivity and Specificity

For the test set ( $n = 50$ ), 7 samples had IgG concentrations below the 1,000 mg/dL diagnostic cutoff value generating a FTPI prevalence of 14% in the test set. Sensitivity for this data set was 71.4% (95% confidence interval [95% CI] = 30.3–94.9) and specificity was 100% (95% CI = 89.8–100). Using the entire data



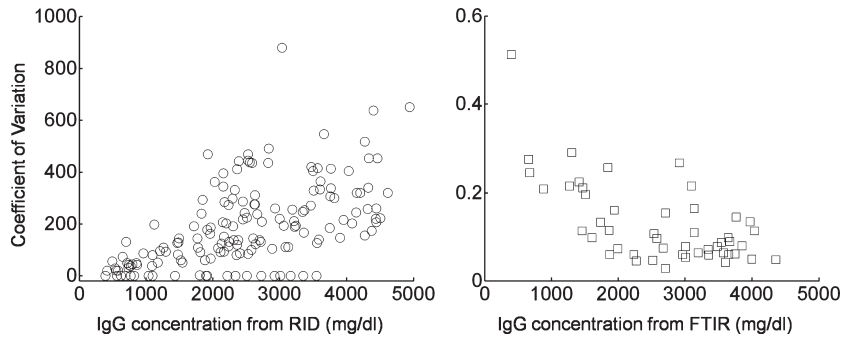
**Fig 4.** Normal probability plot for the differences of the immunoglobulin G (IgG) concentrations in test set obtained from radial immunodiffusion (RID) and Fourier-transform infrared spectroscopy (FTIR) methods. If the measurement errors follow a normal distribution, the data points should be largely located in the reference line.

set ( $n = 175$ ; 21 samples with IgG concentrations below 1,000 mg/dL), sensitivity was 81% (95% CI = 57.4–93.7) and specificity was 100% (95% CI = 97–100). Within the entire data set, there were no false positives and 4 false negatives identified (Table 1).

### Discussion

This study showed that infrared spectroscopy, in combination with a quantification algorithm developed using PLS regression, is a promising technique for the measurement of IgG concentrations in camelid serum. This method was validated by its ability to accurately quantify IgG concentrations in an independent test set of samples.

Within this study, the specificity was excellent but the sensitivity of the FTIR method was slightly lower than desired for diagnosis of FTPI in neonates. However, when compared with reported data for other methods available to quantify camelid serum IgG concentrations, these results are equivalent to or better than most.<sup>3,6</sup> One study looked at using a commercially available sodium sulfate turbidity test for the diagnosis of FTPI in crias. The authors found that when compared with RID and used according to manufacturer's instructions, the sensitivity for this test was only 36% and specificity was 100%.<sup>6</sup> However, they also noted that by lowering the test's endpoint, the performance of the test could be significantly improved.<sup>6</sup> The same study also found that serum total protein concentration, at an endpoint of 5.0 g/dL, yielded a sensitivity of 71% and specificity of 80% and that serum globulin concentration, at an endpoint of 2.5 g/dL, produced a sensitivity of 64% and specificity of 100% when compared with RID.<sup>6</sup> The authors concluded that measurement of serum albumin, total solids or gamma-glutamyltransferase activity was not predictive of FTPI in crias.<sup>6</sup> Other researchers have found that total serum protein measurement, at an endpoint of 5.15 g/dL, had a



**Fig 5.** Coefficient of variance plots for the radial immunodiffusion (RID) and Fourier-transform infrared spectroscopy (FTIR) methods. Lower coefficient of variance means high precision of the test methods; this comparison shows that the precision of the RID and FTIR methods are roughly comparable.

**Table 1.** FTIR IgG false negative samples compared to IgG RID results identified within the entire data set ( $n = 175$ ).

Sample	Data Set	FTIR IgG Value (mg/dL)	RID IgG Value (mg/dL)
A	Calibration	1,075	719
B	Calibration	1,192	773
C	Test	1,602	861
D	Test	1,307	822

FTIR, Fourier-transform infrared spectroscopy; IgG, immunoglobulin G; RID, radial immunodiffusion.

sensitivity of 87.5% and specificity of 87.9% when compared with RID.<sup>3</sup> Serum globulin concentration, at an endpoint of 1.6 g/dL, had a test sensitivity of 96.1% and specificity of 91.2% when compared with RID.<sup>3</sup> This study also compared zinc sulfate turbidity and 10% glutaraldehyde coagulation tests with RID and found sensitivities of 100% and 78.4%, respectively, and specificities of 54.3% and 91.2% for these testing methods.<sup>3</sup>

A recent study of the diagnostic agreement between the currently available camelid RID kit and 2 new commercially available IT found a lack of agreement between the 3 methods with the significant differences between assay results mainly attributed to their use of different standards.<sup>11</sup> Of the 3 assays, RID was found to be the most imprecise leading the authors to question if the RID assay should continue as the “gold standard” for camelid serum IgG quantification or whether a more automated and precise method, such as IT, should be adopted.<sup>11</sup> These considerations raise an important question relevant to the present study: Might the accuracy of the FTIR-based assay be improved if an IT assay was used as the reference standard rather than the RID assay? This issue is potentially worth exploring in future studies. However, it must be emphasized that imprecision does not equate with inaccuracy and that the above referenced study did not determine the accuracy of the RID and IT assays – only their precision and agreement.

Overestimation of IgG concentrations (false negatives) is a problem sometimes encountered with testing methods such as the zinc sulfate turbidity or the glutaraldehyde coagulation test.<sup>25</sup> With these tests, chemical interference from other proteins, such as albumin or hemoglobin, may occur resulting in inaccurate results.<sup>7</sup> These erroneous test results can have clinical consequences for the neonate in question, including an increased risk of pneumonia, septicemia, enteritis, omphalitis, and death, if no medical treatment is provided because of a false negative test result.<sup>4</sup> With FTIR, the possibility for chemical interference can be reduced by ensuring that potential interferants are accounted for within the spectral data set used to develop the diagnostic algorithms.<sup>7</sup> Within the test set of this study, 4 false negatives were identified. Upon gross examination of the serum samples, no hemolysis or lipemia was noted indicating interference from these components was not a major factor with these samples.

For the 4 false negatives within our data set, the RID-derived IgG concentrations were >700 mg/dL indicating only partial FTPI in these cases. Studies have shown that foals with serum IgG concentrations <400 mg/dL (complete FTPI) are at a significantly greater risk of developing septicaemia and other debilitating diseases; however, no similar association has been shown in foals with partial FTPI (serum IgG concentrations between 400 and 800 mg/dL).<sup>26–28</sup> While comparable studies have not been performed in camelid species, it is reasonable to assume that those crias with very low or complete FTPI may have an increased risk of morbidity and mortality when compared to crias with only partial FTPI. Accordingly, while the false negatives in this FTIR study are an issue, the partial FTPI concentrations were less likely to result in serious clinical consequences. We also note that discrepancies between concentrations determined by IR spectroscopy and RID may arise from errors in the RID method; it is possible that better reference measurements would resolve the apparent discrepancies and reduce the apparent false negative rate accordingly.

A drawback of this study is the relatively small number of serum samples with low IgG concentrations

in the data set. This limitation led to only 21 of the 175 samples, a 12% prevalence, having a serum IgG concentration below the 1,000 mg/dL cutoff with only 7 of those 21 randomly assigned into the test set. As a result, the small number of samples with IgG concentrations <1,000 mg/dL may affect the algorithm's accuracy in predicting IgG values at lower concentrations as algorithm development was weighted toward higher IgG concentrations. In future studies, it would be desirable to obtain another data set with a higher proportion of samples with IgG concentrations <1,000 mg/dL, which would allow further modification in the algorithm and possibly better performance at lower IgG concentrations.

A variety of previous studies have demonstrated the potential for FTIR in a clinical laboratory setting.<sup>29,30</sup> The unique absorption pattern displayed for each unique analyte allows for the possibility to distinguish among different sample components and separately quantify them, thus providing additional useful clinical data.<sup>29,30</sup> For example, at least 6 serum constituents of importance in both veterinary and human medicine have been proven suitable for FTIR-based analysis in prior studies. These include total protein, albumin, glucose, triglycerides, urea, and cholesterol.<sup>29,30</sup> Consequently, it might be possible to acquire extra clinical pathological data from each spectrum without further time requirements or additional costs.<sup>7</sup> In addition to diagnosing FPT or measuring serum constituents, FTIR may also prove useful in both clinical and laboratory settings to quantify IgG in adult camelid plasma donors.

In conclusion, FTIR is a promising technique to rapidly quantify IgG concentrations in alpaca serum. Further studies with a larger data set of low IgG serum concentrations are warranted to further refine the algorithm for screening crias with FTPI.

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### Footnotes

<sup>a</sup> HTS-XT autosampler, Bruker Optics®, Milton, ON, Canada

<sup>b</sup> Tensor 37, Bruker Optics®

<sup>c</sup> OPUS® ver. 6.5, Bruker Optics®

<sup>d</sup> GRAMS software ver. 7.02, Thermo Fisher Scientific Inc, Waltham, MA

<sup>e</sup> MATLAB MathWorks R2011b, Natick, MA

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### Acknowledgments

The authors acknowledge Cynthia Mitchell for her technical assistance and Professor Michael Reichel for providing some of the samples used in this study. This study was supported by the Atlantic Canada Opportunities Agency and the Atlantic Veterinary College Research Fund.

*Conflicts of Interest:* The authors disclose no conflict of interest.

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