Use of Fourier-Transform Infrared Spectroscopy for the Diagnosis of Failure of Transfer of Passive Immunity and Measurement of Immunoglobulin Concentrations in Horses

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Background: The economic, accurate, and rapid screening of foals for failure of transfer of passive immunity (FPT) is essential to ensure timely intervention.

Hypothesis: Infrared (IR) spectroscopy of foal sera and pattern recognition may be used to diagnose FPT and quantify serum IgG.

Samples: Sera from 194 foals (24–72 hours) with serum immunoglobulin G (IgG) concentrations determined previously by radial immunodiffusion assay (RID) were used.

Methods: IR spectra were recorded for the serum samples, and the data were randomly divided into training and independent test sets, each containing both FPT-positive (IgG <400 mg/dL) and non-FPT samples. A genetic optimal region selection algorithm and linear discriminant analysis were used to partition the training spectra, and the resulting classifier was then validated by comparing the IR-predicted FPT status for each of the test samples to that provided by the RID IgG assay. A quantitative IR-based assay for IgG was developed using partial least squares (PLS) and validated by testing its ability to predict IgG concentrations.

Results: Specificity, sensitivity, and accuracy for the combined data were 92.5, 96.8, and 95.9%, respectively. Corresponding positive (88.1%) and negative (98.0%) predictive values determined a success rate of 95–97% as compared to RID-based IgG concentrations. The IR-based quantitative assay yielded correlation coefficients for IR spectroscopy versus RID-based IgG concentrations of 0.90 and 0.86 for the training and test sets, respectively.

Conclusions and Clinical Importance: The overall performance of the IR-based test was similar to that of the colorimetric assay and was superior and more economic than other available tests.

Key words: Accuracy; Qualitative; Quantitative; Sensitivity; Specificity.

Equine neonates are born agammaglobulinemic and rely upon colostrum intake for immunoglobulins, predominately immunoglobulin G (IgG). When a foal fails to absorb sufficient maternal antibody the condition is called failure of transfer of passive immunity (FPT). The reference range for normal foal IgG concentrations was established using radial immunodiffusion assay (RID), which is considered the gold standard for the quantitative measurement of IgG. The majority of neonatal foals have serum IgG concentrations >1,000 mg/dL after intestinal absorption of colostrum: foals with concentrations <400 mg/dL are classified as having FPT and those with concentrations between 800 and 400 mg/dL as having partial FPT. Neonates with FPT have an increased risk of morbidity and mortality due to septicemia, requiring early diagnosis and intervention.

The prevalence of FPT is reported to be between 3% and 20% of foals. Economical and accurate screening of foals and other animals for FPT is essential to ensure timely medical intervention and to minimize morbidity, mortality, and financial loss. Consequently, many tests have been developed for the diagnosis of FPT and are widely used in the equine, and to a lesser degree, in the bovine and other species. Several commercial test kits are available. Enzyme-linked immunosorbent assays (ELISAs) have been used as screening tests and measure the neonate’s IgG concentration with a species-specific anti-IgG antibody. In contrast, tests such as the glutaraldehyde coagulation test are nonspecific because they detect other serum proteins in addition to immunoglobulins. There are several reports on the accuracy of these screening tests as compared with RID, but most do not provide a quantitative analysis, and many suffer from poor sensitivity, poor specificity, or both. Although the RID assay provides quantitative data, it requires 18–24 hours to obtain results, requires more technical skill, is not amenable to automation, and often is more expensive than the alternatives. Ideally, a test for FPT should be both highly sensitive and highly specific.

Infrared (IR) spectroscopy is emerging as a powerful quantitative and qualitative technique for diagnostic characterization of biological molecules in fluids and tissues. IR radiation is transmitted through the sample of interest, and the IR spectrometer records the wavelength dependence of radiation absorption by the sample. Simple, small molecules yield simple spectra with well resolved absorption bands that reflect their chemical structure and concentration. In complex samples, the number and size of molecular species increase, causing the number of absorption bands and the extent of band overlap to increase. Although the IR spectra of body fluids or tissues are complex, the information conveyed by these spectra remains conceptually simple. The spectra reflect both the structure of the individual IR active constituents and their relative...
The absorption patterns within the IR spectra of biological samples may be viewed as biochemical fingerprints that correlate directly with the presence or absence of diseases. We hypothesized that IR spectroscopy of foal sera, with pattern recognition approaches applied to these data, could be used to diagnose FPT and to quantify IgG concentrations. The objective of the present study, therefore, was to develop accurate and economical diagnostic and quantitative FPT tests using IR spectroscopy.

Materials and Methods

Samples

Sera used for this project were obtained during a previous study and stored at −80°C. The samples were collected from foals (predominantly Standardbred) of the Maritime Provinces of Canada according to a protocol approved by the Animal Care Committee of the University of Prince Edward Island. Blood was collected from foals (N = 194) 24–72 hours of age, to quantify serum IgG concentrations as part of a routine postpartum foal examination. Blood was allowed to clot, and serum was separated and stored in an −80°C freezer until assayed. An equine RID IgG test was used as the gold standard to determine each foal’s serum IgG concentration. Each serum sample was tested in duplicate, and the average of the results was used to determine IgG concentration. Sera with IgG concentrations >1,600 mg/dL (the RID manufacturer’s stated upper testing limit) were diluted to within the performance range of the assay. ELISA tests as well as a noncommercial glutaraldehyde coagulation test also were performed on these sera, and the results were published elsewhere.

Fourier-Transform IR Spectroscopy

Sera (N = 194) were thawed at 20°C and vortexed. The samples were prepared as described previously with the following modification. Briefly, for each sample, an aliquot was drawn and diluted in 4 g/L potassium thiocyanate solution in the ratio 1 part serum:1 part potassium thiocyanate (KSCN) solution. Duplicate dry films were made for each sample by applying 8-μL aliquots of the diluted serum spread evenly in circular motion into 5-mm wells within a custom-made, adhesive-masked, 96-well silicon microplate. Once the films were thoroughly dried (12 hours at 20°C), the microplate was mounted within a multisampler interfaced with a Fourier-transform infrared spectrometer equipped with a deuterium tryglicine sulfate detector to acquire the IR spectra. Absorbance spectra in the IR range of 400–4,000 cm⁻¹ were recorded. For each acquisition, 512 interferograms were signal-averaged and Fourier-transformed to generate a spectrum with a nominal resolution of 4 cm⁻¹.

Data Preprocessing

Differentiation and smoothing procedures (Savitsky Golay 2nd order derivatives using 2nd degree polynomial functions, with 9-point smoothing) were performed on all spectra to resolve and enhance weak spectral features and to remove baseline variations using spectral manipulation software. The spectra then were normalized using a vector normalization script written in MatLab; the 2nd derivative spectra were scaled by calculating for each spectrum the square root of the sum of square intensities over the wavenumber range of 1,600–1,800 cm⁻¹ and then dividing that spectrum by this factor. With this procedure completed, all spectra had the same integrated intensity (unity) within the specified range. These normalized spectra then provided the basis to develop both quantitative and qualitative diagnostic tests as described in the following section.

Diagnostic and Analytical Test Development

The 194 serum samples were divided into 2 categories: IgG concentrations <400 mg/dL (FPT-positive) and >400 mg/dL (FPT-negative). This resulted in an overweighting of the FPT-negative group, which may create problems in pattern recognition or regression methods in the development phase of IR spectroscopy. To circumvent that possibility, the 55 samples with IgG concentrations >1,600 mg/dL were excluded from the initial data set used to train the pattern recognition (diagnostic) and regression (quantification) methods. Further rationale for this decision was that any method optimized to distinguish FPT-positive from FPT-negative samples in the IgG range of 0–1,600 mg/dL should correctly classify those spectra (samples) with values >1,600 mg/dL as FPT-negative; the latter therefore were considered only during the test phase of the study.

Diagnostic FPT Test. A pattern recognition technique was used to identify spectroscopic features conveying the diagnostic information of interest. In particular, a genetic optimal region selection (ORS) algorithm was used to seek specific spectral ranges within which the spectra differed systematically for FPT (IgG <400 mg/dL) versus non-FPT (IgG >400 mg/dL) foals. The ORS trials using the training set only as input and using linear discriminant analysis as the basis to partition the spectra into FPT and non-FPT groups. That procedure resulted in a diagnostic classifier that took preprocessed IR spectra as input and provided FPT status as output for both the training and test sets. This classifier was further validated by implementing it to predict FPT status for each of the 55 test samples with IgG concentrations >1,600 mg/dL, and comparing these to the RID diagnoses. The sensitivity (Se) and specificity (Sp) for the IR-based test were calculated using the RID IgG concentrations as the gold standard. Positive (PPV) and negative (NPV) predictive values were calculated at the average reported FPT prevalence (P) in the literature and compared to the same values for other equine FPT tests either directly or as calculated from previously published Se and Sp data.

IR-Based Quantitative IgG Assay. A quantitative assay for IgG was developed using a multivariate technique, partial least squares (PLS). All PLS trials used preprocessed spectra that were split into training and test sets identical to those employed for the diagnostic classification model. Of the 2 separate test sets, one encompassed 47 samples (14 FPT and 33 negative) that spanned the diagnostic classification model. Of the 2 separate test sets, one encompassed 47 samples (14 FPT and 33 negative) that spanned the diagnostic classification model. Of the 2 separate test sets, one encompassed 47 samples (14 FPT and 33 negative) that spanned the diagnostic classification model.
IgG concentrations in the training set only. The PLS quantification algorithm then was validated by its ability to predict IgG concentrations on the basis of spectra in the independent test sets.

Results

The RID IgG concentrations for the 194 samples ranged from <200 mg/dL to >4,700 mg/dL (Figure 1). Forty samples had IgG concentrations below the 400 mg/dL cut-off diagnostic of FPT, whereas 154 were FPT-negative; 55 of these had values >1,600 mg/dL. The prevalence of FPT for the entire data set was 20.6%.

To illustrate the nature of the measurements, a representative IR spectrum is plotted in Figure 2. The most intense features arise from proteins: bands centered at 1,650 cm\(^{-1}\) (amide I) and 1,545 cm\(^{-1}\) (amide II) correspond to stretching and bending vibrations localized on the amide C=O and N-H groups, respectively; the broad band at \(~3,300\) cm\(^{-1}\) also corresponds to the N-H group, but is a stretching vibration called the amide A mode. Highlighted in Figure 2 are 3 spectral regions identified by the ORS algorithm as optimal to distinguish the serum spectra from FPT versus non-FPT foals. Based on the spectroscopic information in these discriminatory subregions, the IR spectra provided a success rate of 95% for the FPT status. This accuracy was maintained for the training spectra set (used to identify the optimal subregions) and the independent test set. When the 55 samples with IgG concentrations >1,600 mg/dL were included in the test set, the test set classification accuracy was 97%. Classification tables, specificity, sensitivity, accuracy, and predictive values are summarized in Table 1.

The variations in PPV and NPV for the IR-based test across the reported range of prevalence are illustrated in Figure 3. Se, Sp, accuracy, PPV, and NPV are compared to their counterparts for other FPT diagnostic tests (from previously published reports) in Table 2. The PPV and NPV in Table 2 are based on an FPT prevalence of 15%, which is the average prevalence from previous reports. The accuracy of the PLS quantification algorithm is summarized by the scatterplots in Figure 4, which compare the IgG concentrations as provided by RID to their counterparts as determined using the IR spectroscopy–based method. These scatterplots indicate comparable accuracy for the training and test sets, with correlation coefficients for RID versus IR spectroscopy–based IgG concentrations of 0.90 and 0.86 for training and test sets, respectively. The optimal PLS calibration model included 9 PLS factors using 2 spectral ranges, 700–1,710 cm\(^{-1}\) and 2,750–3,500 cm\(^{-1}\), that encompassed all of the major absorptions.

Discussion

We demonstrated that the IR spectra of serum can be used for the diagnosis of FPT. This may be accomplished either by means of diagnostic classification of the
Table 1. Classification table for training, test, and combined data sets comparing radial immunodiffusion assay to infrared spectroscopy–based diagnosis of failure of transfer of passive immunity.

<table>
<thead>
<tr>
<th>Classification of Failure</th>
<th>Calibration Data</th>
<th>Validation Data</th>
<th>Combined Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radial Immunodiffusion</td>
<td>Iris-based Diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG &lt;400 mg/dL</td>
<td>184 spectra; 92 samples</td>
<td>94 spectra; 47 samples</td>
<td>204 spectra; 102 samples</td>
</tr>
<tr>
<td>IgG &gt;400 mg/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>66</td>
<td>204</td>
</tr>
<tr>
<td>Sp (%)</td>
<td>92.3</td>
<td>92.9</td>
<td>92.5</td>
</tr>
<tr>
<td>Se (%)</td>
<td>96.2</td>
<td>97.0</td>
<td>96.8</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>95.1</td>
<td>96.6</td>
<td>95.9</td>
</tr>
<tr>
<td>PPV (%)</td>
<td>86.3</td>
<td>89.5</td>
<td>88.1</td>
</tr>
<tr>
<td>NPV (%)</td>
<td>98.0</td>
<td>98.1</td>
<td>98.0</td>
</tr>
</tbody>
</table>

- Sp, specificity; Se, sensitivity; PPV, positive predictive value; NPV, negative predictive value.
- PPV is the ratio of true positives over the sum of true and false positives and NPV is the ratio of true negatives over the sum of true and false negatives. PPV and NPV calculations are based on study failure of transfer of passive immunity prevalence of 20.6%.
- Training data including 184 spectra (92 samples) with IgG concentrations in the range 0–1,600 mg/dL.
- Test data including 94 spectra (47 samples) with IgG concentrations in the range 0–1,600 mg/dL.
- Test data as above, but also including an additional 110 spectra (55 samples) with IgG concentrations >1,600 mg/dL.

Table 2. Comparison of recently evaluated tests for failure of transfer of passive immunity. (IgG <400 mg/mL) with prevalence = 15%.

<table>
<thead>
<tr>
<th>Diagnostic Test</th>
<th>Se (%)</th>
<th>Sp (%)</th>
<th>Accuracy (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Source of Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infrared</td>
<td>92.5</td>
<td>96.8</td>
<td>95.9</td>
<td>83.4</td>
<td>98.7</td>
<td>In-house</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>95.0</td>
<td>80.0</td>
<td>72.8†</td>
<td>95.7†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>88.9†</td>
<td>79.4†</td>
<td>43.2†</td>
<td>97.6†</td>
<td></td>
<td>EquiZ equine FPT test kit, VMRD Inc, Pullman, WA</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>89.0</td>
<td>91.0</td>
<td>63.6†</td>
<td>97.9†</td>
<td></td>
<td>In-house</td>
</tr>
<tr>
<td>Enzyme-linked</td>
<td>94.4</td>
<td>83.4</td>
<td>49.4†</td>
<td>98.7†</td>
<td></td>
<td>In-house–refractometry</td>
</tr>
<tr>
<td>Immunosorbent</td>
<td>94.4</td>
<td>72.4†</td>
<td>37.2†</td>
<td>98.6†</td>
<td></td>
<td>In-house–visual method</td>
</tr>
<tr>
<td>visual method</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Turbimetric</td>
<td>63.3</td>
<td>92.3†</td>
<td>58.1†</td>
<td>93.4†</td>
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<td>In-house</td>
</tr>
<tr>
<td>Immunoassay</td>
<td>88.9†</td>
<td>78.1†</td>
<td>41.7†</td>
<td>97.5†</td>
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<td>Midland 4, Plasma Foal IgG Quick test kits, Midland Bioproducts Corp, Boone, IA</td>
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<td>Lateral flow</td>
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<tr>
<td>Assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme-linked</td>
<td>76.0†</td>
<td>95.0</td>
<td>58.2†</td>
<td>93.4†</td>
<td></td>
<td>SNAP foal IgG, Idexx laboratories, Westbrook, ME</td>
</tr>
<tr>
<td>Immunosorbent</td>
<td>88.9†</td>
<td>95.8†</td>
<td>78.9†</td>
<td>95.8†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorimetric</td>
<td>90.0†</td>
<td>79.1†</td>
<td>43.1†</td>
<td>97.8†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunoassay</td>
<td>100.4</td>
<td>96.4†</td>
<td>81.5†</td>
<td>100.4</td>
<td></td>
<td>DVM Stat, VDx Inc, Belgium, WI</td>
</tr>
</tbody>
</table>

- Sp, specificity; Se, sensitivity; PPV, positive predictive value; NPV, negative predictive value; FTP, failure of transfer of passive immunity.
- Superscript letters and numbers refer to references and footnotes.
- † Calculated by authors based on published specificity and sensitivity data, allowing for a prevalence of FPT = 15%.
Hemolysis may result in inaccurate IgG concentrations. Because no reagents are required, ELISAs are limited by the need for confirmatory testing. Through use of IR assays, the need for confirmatory testing may be eliminated. An important advantage of having the quantitative capability of the IR test is that borderline cases may be identified as such. To illustrate, the scatterplot is divided into quadrants categorizing results as true positive, true negative, false positive, and false negative (Figure 4). The majority of cases were designated correctly by the IR-based assay. Where the IR and RID designs disagree, the RID assay did not provide quantitative information.

![Fig 4](image)

**Fig 4.** Scatterplot comparing serum IgG concentrations as determined by the infrared-based method to radial immunodiffusion (RID) assay. Open circles denote the 184 training spectra (92 samples), whereas the triangles represent the set of 94 independent test spectra (47 samples). The line of identity and guidelines at the 400 mg/dL level are included for reference. Note that for samples with IgG concentrations <200 mg/dL, the RID assay did not provide quantitative information.

processed spectra or by use of a quantification algorithm (in the present case, PLS) to quantify IgG concentrations directly from the spectra. Both procedures were validated by their ability to accurately characterize large, independent sets of test samples.

The diagnostic accuracy of the IR-based test meets or surpasses currently available alternatives (Table 2), and the methodology carries several practical advantages. For example, hemoglobin (eg, from hemolysis) and other chemical interferents may compromise the accuracy of some testing modalities. In the present approach, such interferents are accounted for by ensuring that the spectral data set used to develop the diagnostic algorithms encompasses the full range of possible interferents, preventing the possibility of chemical interference. Because no reagents are required, the per-test cost is very low and repeat testing is inexpensive. No standards need be prepared before testing, and results are available within minutes. Samples were allowed to dry for 12 hours in the present study so that a large number of samples could be run at once. In practice, the authors have dried samples within a few minutes. IR spectroscopy may be performed immediately after the sample is dry. As an added benefit unique to this testing modality, the same spectrum in principle can simultaneously provide results for serum total protein, albumin, cholesterol, glucose, triglyceride, and urea concentrations at no additional cost. Finally, the IR test, in common with RID, zinc sulfate turbidity, and colorimetric assays, also can quantify IgG concentrations.

An important advantage of having the quantitative capability of the IR test is that borderline cases may be identified as such. To illustrate, the scatterplot is divided into quadrants categorizing results as true positive, true negative, false positive, and false negative (Figure 4). The majority of cases were designated correctly by the IR-based assay. Where the IR and RID designs disagree, the RID assay did not provide quantitative information.

The PPV and NPV values vary with the population prevalence of FPT, which must therefore be considered when using this test. The population studied here was predominantly Standardbred foals in Atlantic Canada, and the prevalence of FPT was similar to that previously reported for Standardbred foals. In populations in which the prevalence of FPT is low, foals with FPT may require verification by the quantitative IR-based IgG test or by using another confirmatory test. The overall performance (accuracy) of the IR-based test was similar to that of the colorimetric assay and was superior to other tests currently available. The sensitivity was superior to that of ELISAs and turbidometric assays, similar to that of glutaraldehyde coagulation and zinc sulfate assays, and lower that that of the colorimetric assay. Specificity was similar to the colorimetric assay, but superior as compared to published results for all other assays. The PPV is high, as is the case for most of the other testing modalities. The most obvious distinguishing feature of the IR-based approach is the high PPV, which was superior to that reported for other tests. A high PPV is difficult to achieve in the diagnosis of FPT and is of particular importance in reducing the unnecessary and expensive treatment of otherwise healthy foals.

Currently available tests have both strengths and weaknesses. Although accurate enough to be considered the gold standard in FPT testing, recent work has revealed that RID assays may vary, depending on the standards used to establish the concentration curve, and typical turnaround times are 18–24 hours. As a consequence, it has been proposed that universal standards for RID assays be used. Although glutaraldehyde coagulation is fast and inexpensive, it has been reported to have generally poor or variable specificity, especially for IgG concentrations <800 mg/dL, thus prompting other workers to recommend confirmatory testing to verify a positive test result. Hemolysis may result in false positive results for the zinc sulfate turbidity and glutaraldehyde coagulation assays. Although ELISA-based tests offer the convenience of on-site testing, they have poor PPV as compared to the IR test and involve the use of heat- and contamination-sensitive reagents.

The ELISA tests also are among the more expensive options, which decreases their appeal for screening large
numbers of animals. An economic comparison of different tests for FPT has recently been published. The cost per test varied from $2.00 to $13.65, with the cost of some tests varying depending upon the number of samples tested at any one time (eg, RID). The IR-based test is reagent free (no kit is required), standards are not required, and the estimated costs of each test is <$1 in disposables, regardless of the number of samples evaluated.

Although the diagnostic method reported here focused on the 400 mg/dL IgG concentration as the FPT cut-off point to provide a ready comparison with other published methods, the methodology could be readily adapted to further distinguish between FPT and partial FPT (IgG >400 mg/dL but <800 mg/dL). Introduced in the mid-1980s, the term “partial FPT” does not indicate the need for IgG supportive therapy, but results within this range are considered clinically relevant in sick foals. Although the relevance in clinically normal foals remains controversial, many clinicians regard foals with concentrations <800 mg/dL as candidates for IgG supplementation.

This proof-of-concept study has successfully established IR spectroscopy as a candidate methodology for FPT diagnosis. Additional study with close scrutiny of discrepant diagnoses would clarify the performance characteristics of the technique. One concern is samples that are (according to the gold standard of RID) close to the FPT cut-off point. These samples are close enough to 400 mg/dL to suggest that imprecision inherent to the test under scrutiny (in this case the IR-based test) or the gold standard against which it is compared (the RID test), may cause confusion. In conclusion, IR spectroscopy, in conjunction with classification or quantification algorithms, is an accurate means to diagnose FPT in foals.

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### Footnotes

3. RID, VMRD Inc. Pullman, WA
4. CITE test and SNAP IgG foil test, Idexx Inc, Portland, ME
5. KSCN, SigmaUltra, Sigma-Aldrich Inc, St.Louis, MO
6. HTS-XT autosampler, Bruker Optics, Milton, ON, Canada
7. Vector 22, Bruker Optics, Milton, ON, Canada
8. GRAMS/AI 7.02, Thermo Galactic, Salem, NH
9. MATLAB 6.5, The Math Works Inc, Natick, MA
10. RJD, VMRD Inc. Pullman, WA

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### References


