Evaluation of assay procedures for prediction of passive transfer status in lambs

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Objective—To compare 4 assay procedures for prediction of passive transfer status in lambs.

Animals—Thirty-one 1-day-old Sardinian lambs.

Procedure—Serum IgG concentration was determined by use of single radial immunodiffusion. The following were determined: serum total protein concentration as measured by refractometry (ie, refractometry serum total protein concentration), serum γ-globulin concentration as determined by serum protein electrophoresis, and serum γ-glutamyltransferase (GGT) activity as measured by spectrophotometry. Accuracy of these assays for estimation of serum IgG concentration in 1-day-old lambs was established by use of linear regression analysis.

Results—Refractometry serum total protein concentration, biuret method serum total protein concentration, and serum γ-globulin concentration were closely and linearly correlated with serum IgG concentration. The natural logarithm (ln) of serum GGT activity was closely and linearly correlated with serum IgG concentration (ln). Refractometry serum total protein concentration, biuret method serum total protein concentration, and γ-globulin concentration accounted for approximately 85%, 91%, and 95% of the variation in serum IgG concentration, respectively. Serum GGT activity (ln) accounted for approximately 92% of the variation in serum IgG concentration (ln).

Conclusions and Clinical Relevance—For prediction of passive transfer status in 1-day-old lambs, serum GGT activity or biuret method serum total protein concentration determination will allow for passive transfer monitoring program development. Immediate refractometry serum total protein concentration determination is beneficial in making timely management and treatment decisions. Serum γ-globulin concentration can be used as a confirmatory test. (Am J Vet Res 2006;67:593–598)

Lambs are born with negligible serum IgG concentrations, so neonatal lambs depend on the passive transfer of maternal IgG in colostrum to provide humoral immunity during the neonatal period. Failure of the neonatal lambs to obtain and absorb colostral IgG, termed FPT, is a secondary immunodeficiency disorder that has been linked to increased risk of illness and death from bacterial septicemia and common neonatal diseases. Several accepted tests to determine passive transfer status in calves are used in lambs. Most thresholds used with these test procedures are established on the basis of data drawn from studies in calves. Passive transfer status in lambs has been commonly assessed by methods such as SRID, serum protein electrophoresis, refractometry, and zinc sulfate turbidity test. However, no currently available assay procedure is entirely satisfactory. The SRID method developed by Mancini et al and others is currently the gold standard for direct measurement of serum IgG concentration in neonates, although it requires a long diffusion time (18 to 24 hours) and is expensive and laborious for analysis of large numbers of samples. Measurement of serum γ-globulin concentration by use of serum protein electrophoresis provides an accurate estimation of the total immunoglobulin concentrations in the serum of neonates, but it requires specialized equipment and a long analysis time. Refractometry is a commonly used tool in veterinary practice and provides an estimation of the serum protein concentration. Because immunoglobulins constitute a large proportion of the protein in neonatal lamb serum and the albumin concentration of lamb serum is relatively constant, refractometry provides an estimation of the serum immunoglobulin concentration. This is a simple, fast, inexpensive test and is also adaptable for field use but is purported to be inaccurate for dehydrated lambs and has not been fully evaluated. The zinc sulfate turbidity test and its thresholds are well established for lambs. Results of studies on lambs indicate that the measurement of serum GGT activity is an additional useful predictor of
passive transfer status in the early neonatal period, as reported for calves.\textsuperscript{10,15} Algorithms predicting serum IgG concentration as a function of serum GGT activity and lamb age are currently available.\textsuperscript{17,18} The usefulness of the GGT method is enhanced because many practitioners and veterinary clinics have the capability for automated serum biochemical analysis. Information regarding the application of the biuret method as a test to determine passive transfer status in lambs is not available. The biuret method is a reference method for direct measurement of serum protein concentration and is based on a colorimetric assay in an automated biochemical system.\textsuperscript{18,19} This makes the potential usefulness of the biuret method comparable to that of serum GGT activity. To our knowledge, comparison of the various test procedures used for assessment of passive transfer status in lambs has not yet been thoroughly investigated.

The objective of the study reported here was to derive equations for the estimation of serum IgG concentration in 1-day-old lambs from serum total protein concentration as measured by refractometry (ie, refractometry serum total protein concentration), serum total protein concentration as determined by the biuret method (ie, biuret method serum total protein concentration), serum γ-globulin concentration as determined by serum protein electrophoresis, and serum GGT activity as measured by spectrophotometry in an attempt to compare the ability of these assay procedures for the prediction of passive transfer status. This information is critical in the design of alternative ways to test for FPT in individual lambs and in the development of passive transfer monitoring programs for the flock.

**Materials and Methods**

**Animals**—Passive transfer status was monitored in Sardinian dairy lambs from a commercial farm in central Italy. Only 31 lambs resulting from observed parturitions were included in the study. Lambs were housed on their farm of origin under the standard management protocol. All lambs received colostrum only from their dams within the first 24 hours after parturition. On physical examination, lambs were considered to be healthy. Lamb age, hydration status, and health status were not considered as independent variables in this study.

**Sample collection and processing**—Blood samples were collected by jugular venipuncture from each lamb on day 1 (24 hours) after parturition. Serum was harvested after centrifugation and was stored at 4°C for further analyses. Serum IgG concentration was determined by use of a commercially available SRID assay, according to the specifications of the manufacturer.\textsuperscript{1} Serum total protein concentration was measured by use of a temperature-compensating refractometer\textsuperscript{6} and the biuret method with a commercially available kit\textsuperscript{7} and an automated biochemical analyzer.\textsuperscript{8} Serum γ-globulin concentration was determined by use of a semiautomated agarose gel electrophoresis system\textsuperscript{9} and a commercially available reagent kit for serum protein determination.\textsuperscript{10} Serum GGT activity was measured by use of a commercially available kit\textsuperscript{11} and an automated biochemical analyzer.\textsuperscript{12}

**Statistical analysis**—Mean ± SD values for serum IgG concentration, refractometry serum total protein concentration, biuret method serum total protein concentration, serum γ-globulin concentration, and serum GGT activity were calculated. The accuracy of assay procedures for the estimation of serum IgG concentration in 1-day-old lambs was established by use of standard linear regression analysis.\textsuperscript{13,14} In the first stage, standard scatter diagrams were used to describe the visual display of the data by plotting the serum IgG concentrations on the y-axis against the results for each of the 4 methods on the x-axis. If the linearity assumption between the related variables was not satisfied, an appropriate transformation of the data was performed. Simple linear regression models were generated to predict the mean value of serum IgG concentration in 1-day-old lambs (dependent variable) as a function of refractometry serum total protein concentration, biuret method serum total protein concentration, serum γ-globulin concentration, and serum GGT activity (single independent variables). A separate model was fitted for each assay. The first-order mathematical equation that related the variables in the developed models was expressed by use of the following formula: γ = α + βx, where γ is the predicted measurement of serum IgG concentration in 1-day-old lambs, x is the observed value for a specific assay, α is the estimated y-intercept of the line, and β is the estimated slope of the line. The goodness of fit of the models was established by the coefficient of determination (R²), which was multiplied by 100 and expressed as a percentage to indicate the total variance of serum IgG concentration that was accounted for by variation in the independent variable; the remaining percentage indicated the total variance of serum IgG concentration that was unexplained by the regression (misleading predictions). The 95% CI of the calculated R² values was computed\textsuperscript{15} as suggested in another report.\textsuperscript{15} The derived models were then used to calculate the refractometry serum total protein concentration, biuret method serum total protein concentration, serum γ-globulin concentration, and serum GGT activity that were equivalent to serum IgG concentration strata of 500, 1,000, 1,500, and 2,000 mg/dL for 1-day-old lambs. To determine whether the refractometer was providing an accurate measurement of the total protein in the lamb serum samples, serum total protein concentration results obtained by refractometry and biuret techniques were also compared by linear regression analysis. For all analyses, a value of P < 0.05 was considered significant. Calculations were performed by use of a software package.\textsuperscript{15}

**Results**—For the 31 lambs, serum IgG concentration ranged from 125 to 5,240 mg/dL (mean ± SD, 2,218 ± 1,785 mg/dL), refractometry serum total protein concentration ranged from 4.0 to 8.2 g/dL (5.9 ± 1.4 g/dL), biuret method serum total protein concentration ranged from 3.9 to 8.1 g/dL (5.8 ± 1.4 g/dL), serum γ-globulin concentration ranged from 0.1 to 3.8 g/dL (1.5 ± 1.2 g/dL), and serum GGT activity ranged from 38 to 16,026 U/L (3,290 ± 4,066 U/L).

Refractometry serum total protein concentration, biuret method serum total protein concentration, and serum γ-globulin concentration were linearly and significantly (P < 0.001) associated with the estimated serum IgG concentration in 1-day-old lambs (Figure 1). The model predicting serum IgG concentration in 1-day-old lambs as a function of refractometry serum total protein concentration (P < 0.001; R² = 0.848) had the following form: serum IgG (mg/dL) = −5,713 + refractometry total protein (g/dL) × 1,350. The 95% CI of the calculated R² was 0.706 to 0.925. The model predicting serum IgG concentration in 1-day-old lambs as a function of biuret method serum total protein concentration (P < 0.001; R² = 0.909) had the following form: serum IgG (mg/dL) = −5,417 + refractometry total protein (g/dL) × 1,150. The model predicting serum IgG concentration in 1-day-old lambs as a function of biuret method serum total protein concentration (P < 0.001; R² = 0.909) had the following form: serum IgG (mg/dL) = −5,417 + refractometry total protein (g/dL) × 1,150.
The 95% CI of the calculated $R^2$ was 0.818 to 0.956. The model predicting serum IgG concentration in 1-day-old lambs as a function of serum $\gamma$-globulin concentration ($R^2 = 0.946; P < 0.001$) had the following form: serum IgG (mg/dL) = $-83 + \gamma$-globulin (g/dL) $\times 1,528$. The 95% CI of the calculated $R^2$ was 0.890 to 0.974.

A positive curvilinear relationship was detected between serum IgG concentration and serum GGT activity. This relationship was made linear by taking the ln (to base $e$) of each variable. Models that did not use the ln transformations were screened and rejected because these models had lower $R^2$ values. The ln[GGT] was linearly and significantly ($P < 0.001$) associated with the estimated ln[IgG] in 1-day-old lambs (Figure 1). The model predicting the ln[IgG] as a function of the ln[GGT] ($P < 0.001; R^2 = 0.920$) had the following form: ln[IgG] (mg/dL) = 2.346 + ln[GGT] (U/L) $\times 0.688$. The 95% CI of the calculated $R^2$ was 0.839 to 0.961.

The refractometry serum total protein concentration, biuret method serum total protein concentration, serum $\gamma$-globulin concentration, and serum GGT activity equivalent to serum IgG concentration strata of 500, 1,000, 1,500, and 2,000 mg/dL for 1-day-old lambs were calculated (Table 1). Results obtained by refractometry were closely and significantly ($P < 0.001; R^2 = 0.949$) correlated to the biuret method results, and the linear relationship was described by the equation $y = -0.273 + 1.023x$, where $y$ is the biuret method serum total protein concentration and $x$ is the refractometry serum total protein concentration.

### Table 1

<table>
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<th>Serum IgG concentration (mg/dL)</th>
<th>Refractometry</th>
<th>Biuret</th>
<th>Serum $\gamma$-globulin concentration (g/dL)</th>
<th>Serum GGT activity (U/L)</th>
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### Discussion

In our study, no attempt was made to determine the test performance by use of standard epidemiologic methods because of the lack of a serum IgG concentration that is universally accepted by the veterinary community as an optimal threshold value, below which FPT occurs in lambs. The choice of an arbitrary threshold value was ill-advised. Information regarding the risk of illness or death associated with varying strata of serum IgG concentrations, as determined by SRID, is sparse for lambs. Results of observational studies indicate that serum concentrations of IgG $< 800$ and $< 1,500$ mg/dL and serum concentrations of IgG$_3$ $< 600$ and $< 1,000$ mg/dL in 24- to 36-hour-old lambs are linked to an increased risk of death. In studies that used the zinc sulfate turbidity test to evaluate passive transfer status in lambs, zinc sulfate turbidity readings $< 10^{11}$ and $< 12$ units in 1- to 2-day-old lambs were associated with an
increased risk of illness and death. A model predicting zinc sulfate turbidity units as a function of serum protein concentration by refractometry was generated for 1-day-old lambs. From application of that formula, serum protein concentrations of 5.2 and 5.4 g/dL are estimated to be equivalent to zinc sulfate turbidity readings of 10 and 12 units, respectively. Application of our model in the prediction of serum IgG concentration in 1-day-old lambs as a function of refractometry results indicates that serum protein concentrations of 5.2 and 5.4 g/dL are approximately equivalent to serum IgG concentrations of 1,332 and 1,521 mg/dL, respectively. In an observational study in 1-day-old lambs, the mortality rate was higher in lambs with serum γ-globulin concentrations ≤ 0.5 g/dL, compared with lambs with a higher serum γ-globulin concentration. Application of our model in the prediction of serum IgG concentration in 1-day-old lambs as a function of serum γ-globulin concentration indicates that a serum γ-globulin concentration of 0.5 g/dL is equivalent to a serum IgG concentration of 681 mg/dL. On the basis of these findings and consistent with results of observational studies, the serum IgG concentration that provides the optimal threshold value, below which FPT occurs in 1- to 2-day-old lambs, should be between 600 and 1,600 mg/dL. Optimal selection of each stratum of passive transfer will be governed by the health and management system of the flock. Lower passive transfer strata may be adequate under conditions of maximal sanitation, and higher strata may be required for conditions of poor sanitation and high infection pressure.

Test results for passive transfer status have 2 major applications. From a flock management standpoint, the first application is to evaluate the success of passive transfer in lambs of a given health and management system. Alternatively, tests for passive transfer may be used in the diagnosis and treatment of individual lambs where FPT is suspected. The ability to provide accurate test results is only the first in a series of steps that might lead to improved passive transfer monitoring programs for flocks. The ability to obtain immediate test results on-farm is imperative in making timely management and therapeutic decisions. Additionally, suitability and economic considerations are also important because they may limit the application of certain assay procedures in veterinary practice.

Results of our study indicate that each of the examined test procedures is valuable for evaluating the status of passive transfer of immunoglobulins in neonatal lambs. Measurement of serum γ-globulin concentration by serum protein electrophoresis is the most accurate tool for estimation of passive transfer status in lambs. In our study, the model that was developed to predict serum IgG concentration as a function of serum γ-globulin concentration was extremely accurate; approximately 93% of the variation in serum IgG concentration in 1-day-old lambs was explained by an association with serum γ-globulin concentration. Limits, within which the true accuracy of this method for IgG estimation was contained, ranged from 89% to 97%. Even the lower limit of this CI was indicative of a fairly strong linear association between the 2 variables. Although this method has been used to assess passive transfer status in neonates, the requirement of a specialized laboratory and the long time before results are available to the veterinarian, as well as the associated cost, could reduce the on-farm application of electrophoresis. However, the excellent predictive ability of this method, as observed in our study, indicates that it may be valuable as a confirmatory test for the diagnosis of FPT in suspect individual lambs.

Results of our study indicate that measurement of serum GGT activity may be a useful method for prediction of passive transfer status in lambs in the early neonatal period, similar to results obtained from other studies in lambs and calves. This enzyme is concentrated in colostrum of ruminants as well as in ewe colostrums and is readily absorbed across the non-selective intestinal barrier of ruminant neonates within the first 24 to 48 hours after parturition. Consequently, passive transfer of immunoglobulins in lambs is significantly associated with that of the serum GGT activity. Age-adjusted serum GGT activities have been established to predict passive transfer status in neonatal lambs. In our study, approximately 92% of the variation in serum IgG concentration in 1-day-old lambs was explained by an association with serum GGT activity and the CI around this estimation was between 84% to 96%. The test was performed similarly to the biuret method and was more accurate than refractometry for the prediction of passive transfer status in neonatal lambs. Measurement of serum GGT activity is an attractive assay procedure because it is inexpensive, rapid, and readily available to many practitioners and should not be affected by dehydration. Thus, measurement of serum GGT activity could be useful for prediction of serum IgG concentration in sick neonatal lambs. However, the most valuable application of serum GGT activity is in the development of passive transfer monitoring programs. The documentation that GGT activity does not deteriorate with storage in serum from humans, horses, and neonatal calves suggests that samples may be obtained, stored, and batch-processed at a later time.

To our knowledge, no study has been performed to critically estimate the relationships among refractometry serum total protein concentration, biuret method serum total protein concentration, and serum IgG concentration, as determined by SRID in neonatal lambs. An estimated relationship has been presented only between refractometry and the zinc sulfate turbidity test results in lamb serum. Results of our study indicate that a strong association exists between refractometry and the biuret method results in neonatal lamb serum, indicating that refractometry provides an accurate estimation of the actual serum protein concentration in lambs. The fit of the models that used either refractometry serum total protein concentration or biuret method serum total protein concentration to predict serum IgG concentration in 1-day-old lambs was adequate. However, regression analysis indicated that the biuret method provides a superior prediction than refractometry for passive transfer status in lambs. Approximately 91% of the variation in serum IgG concentration was explained by an association with the biuret method serum total protein concentration, and...
the CI around this estimation was between 82% to 96%. Conversely, refractometry serum total protein concentration accounted for approximately 85% of the variation in serum IgG concentration, and the CI around this estimation was wider (ie, between 71% to 92%). As a result, a larger proportion of the variation in serum IgG concentration in 1-day-old lambs was unexplained by the refractometry serum total protein concentration and was caused by something other than the actual measurement. We have a few theories for this observation. First, the use of a colorimetric method and a spectrophotometer, such as that used in the biuret method, is more accurate because a greater control exists in assay measurements. Second, although the presence of lipemia or severe hemoglobinemia or bilirubinemia in the sample may interfere with either refractometry or spectrophotometry measurements of serum total protein concentration, high concentrations of other serum nonprotein solids, such as glucose, urea, sodium, or chloride, may also falsely increase the refractometry serum total protein concentration. 22,23,25 Finally, the use of refractometry in moribund or dehydrated calves32,33,38 and lambs5,19 has raised some concerns because the clinical disease, and hence dehydration, can increase the serum total protein concentration and create the potential for misclassification of calves and lambs with FPT. However, this effect has not been fully evaluated in clinically ill neonatal ruminants.33,38 In our study, the effect of hydration status on the accuracy of refractometry as a measure of passive transfer status was not evaluated. Because the dehydration status can artificially increase either refractometry or spectrophotometry measurements of serum total protein concentration,22,23 the discrepancy in accuracy between these 2 methods in our study should be ascribed to sample-related causes of error in serum total protein concentration measured by refractometry, as well as to the greater control in measurement of biuret method serum total protein concentration, rather than hydration status. However, these potential influences are hypothesized but have not been documented. Thus, the effects of high concentrations of serum nonprotein solids in vivo (ie, in clinically ill lambs) and the hydration status on the accuracy of refractometry measurements to predict passive transfer status in neonatal lambs remain open to debate.

It should be mentioned that the models created in our study to make predictions are applicable only in the specified range of independent variables used in the development of regression analyses and only for lambs whose important characteristics, including the criteria for sample selection, are within the range of original data.11 Consequently, we are able to recommend their use to predict passive transfer status in clinically normal 1-day-old lambs. Selection of a sample collection time of 24 hours after parturition allows easy assessment of the amount of passive transfer and potentially allows for treatment before the onset of infections.11,12 Admittedly, our calculated R² values for all of the equations are higher than those published for lambs8–9 and other ruminant species.31,32,35,38 This observation warrants further comments. The relationship between serum GGT activity and serum IgG concentration is curvilinear for calves32 and lambs35, the ln transformation of each variable, as seen in our study, allows the relationship between serum GGT activity and serum IgG concentration to be linear. In studies on calves,31,32,35 lambs,27–29 and crias,42 regression models that were developed to predict serum IgG concentration as a function of serum GGT activity included dependent and independent variables that were transformed in a different manner by use of the ln or were untransformed. In addition, associations between serum IgG concentration and serum total protein concentration or serum GGT activity reportedly are age-sensitive for calves31,35 and lambs27,28 and appear to decline as calves and lambs age. In some studies in calves31,35 and lambs27–29 regression models that were developed to predict serum IgG concentration as a function of age and either serum GGT activity or serum protein concentration yielded larger R² values when the study population was restricted to the early neonatal period. In our study, the lambs were sampled on day 1 (24 hours) after parturition and the lamb age was not considered as an independent variable to include in the regression analysis. This eliminates the potential variation in serum sample measurements (ie, IgG concentration, total protein concentration, and GGT activity) resulting from the normal decay of passively acquired immunity, endogenous production, physiologic response, normal feeding, and many other factors. Clearly, the usefulness of models that permit the prediction of passive transfer status in neonates of varying ages is valuable because practitioners rarely have the opportunity to restrict the examined population to neonates in the first few days after parturition. However, it should be mentioned that such models could provide a lower estimation accuracy.

Overall, our study provides useful information for the practicing veterinarian in validating alternative ways of testing for FPT in individual lambs and in the development of passive transfer monitoring programs. Optimal application of each of these tests will be governed by their accuracy, cost, and suitability to veterinary practice. Usefulness of the laboratory methods can be compromised by the requirement of specialized equipment and the extended time required for results to become available to the veterinarian. Thus, the most suitable application of serum protein electrophoresis should be as a confirmatory test. Measurements of serum GGT activity and biuret method serum total protein concentration are more widely used test procedures because a number of private veterinary clinics have an automated serum biochemical analyzer and test results are rapidly available; this may be valuable in the development of passive transfer monitoring programs in lambs. The use of refractometry to determine serum protein concentration is the most expedient procedure when attempting to determine appropriate treatment of lambs with suspected FPT. Compared with other tests, it does not require expensive instrumentation, provides an immediate result, and is also adaptable to field use. These advantages should be of benefit in making timely management and therapeutic decisions. However, it should be mentioned that refractometry yields a larger proportion of misleading pre-
dictions of serum IgG concentrations, compared with all of other methods described. We were not able to evaluate the ability of the tests to predict passive transfer status in clinically ill lambs, as the lambs in our study were apparently healthy. Thus, the relevance of these 4 tests procedures in clinically ill lambs remains to be evaluated.

a. Sheep IgG VET-RID kit, Bethyl Laboratories Inc, Montgomery, Tex.
b. Leica VET 360, Leica Microsystems Inc, Buffalo, NY.
c. Roche reagent for total protein, Roche Diagnostic Systems, Branchburg, NJ.
d. BM/Hitachi 917 analyzer, Boehringer Mannheim Corp, Indianapolis, Ind.
e. HYDRA SYS agarose gel electrophoresis system, Sebia Inc, Norcross, Ga.
g. Roche reagent for GGT, Roche Diagnostic Systems, Branchburg, NJ.
h. GraphPad Prism, version 4.01 for Windows, GraphPad Software Inc, San Diego, Calif.

References