Evaluation of the immunocrit method to detect failure of passively acquired immunity in dairy calves

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OBJECTIVE
To evaluate the usefulness of serum immunocrit measurement to detect failure of passively acquired immunity (FPI) in dairy calves.

DESIGN
Diagnostic test evaluation.

ANIMALS
249 female dairy calves (age, 2 to 6 days).

PROCEDURES
A blood sample was collected from each calf, and serum was harvested. Immunocrit was measured in serum samples by use of 55% ammonium sulfate solution and the standard technique. Serum IgG concentration was measured by means of radial immunodiffusion (reference standard), with FPI defined as a result < 1,000 mg/dL. The immunocrit value (cutpoint) that maximized both sensitivity and specificity of the method for detection of FPI was determined by construction of receiver operating characteristic curves, and likelihood ratios for positive and negative test results were calculated.

RESULTS
Immunocrit values were significantly correlated ($\rho = 0.71$) with serum IgG concentration as measured by radial immunodiffusion. An immunocrit cutpoint of 11% was optimal for detection of FPI in the calves. Sensitivity and specificity of the immunocrit method at this cutpoint were 0.88 (95% confidence interval [CI], 0.79 to 0.97) and 0.51 (95% CI, 0.44 to 0.58), respectively. Likelihood ratios for positive and negative test results were 1.80 (95% CI, 1.51 to 2.14) and 0.23 (95% CI, 0.11 to 0.51), respectively.

CONCLUSIONS AND CLINICAL RELEVANCE
The immunocrit method was useful for identifying dairy calves with FPI and was simple and could be quickly performed. Because precipitation of immunoglobulins by ammonium sulfate is not species specific, the immunocrit method should be evaluated for detection of FPI in other veterinary species as well. (J Am Vet Med Assoc 2017;251:702–705)

Ruminants are born with hypogammaglobulinemia as a result of cotyledonary placentaion, which allows minimal transfer of maternal antibodies in utero. Consequently, neonatal ruminants are reliant on ingestion of colostrum for acquisition of important immunoglobulins that will provide protective immunity during the first few weeks after birth. In calves, a serum IgG concentration $\geq$ 1,000 mg/dL is indicative of successful passive transfer of immunity. Failure of passive transfer continues to be an important problem in US heifer dairy calves, with a prevalence of 19.2%. Neonates with FPI are at an increased risk for disease and death, compared with neonates with adequate passive transfer. Accurate and quick measurement of passive immunity status allows for early intervention for calves with FPI through identification of insufficiencies in the herd’s colostrum management program.

The quantitative reference methods for measurement of serum IgG concentrations are ELISA and RID. However, indirect qualitative tests are used more commonly in field settings to assess passive immunity status, and these include measurement of serum total solids concentration by refractometry, sodium sulfite turbidity testing, zinc sulfate turbidity testing, measurement of serum $\gamma$-glutamyltransferase activity or serum globulins concentration, and blood glutaraldehyde coagulation testing. These tests have various sensitivities and specificities, compared with the reference methods, but are commonly used because of their low cost, ease of use, and quick turnaround time for test results.

Despite the availability of the aforementioned tests, researchers continue to evaluate new diagnostic tests for passive transfer status to identify a test that is simple, cost-effective, not species specific, and

ABBREVIATIONS
CI Confidence interval
FPI Failure of passively acquired immunity
RID Radial immunodiffusion
ROC Receiver operating characteristic
highly accurate for prediction of serum IgG concentration. Additional, recently developed tests evaluated for use in dairy calves include cow-side commercial tests, Fourier-transform infrared spectroscopy, and measurement of serum total solids concentration via digital refractometer.\textsuperscript{6,8,9}

The immunocrit method is a novel, simple approach to predicting serum IgG concentration and death before weaning in piglets.\textsuperscript{10} This method involves precipitation of immunoglobulins in serum samples with ammonium sulfate, followed by measurement of the ratio of the precipitate to the sample volume within a microcapillary tube. Piglet serum immunoglobulin concentrations are correlated ($r = 0.86$) with densities of immunoglobulin heavy-chain protein bands on gel electrophoresis.\textsuperscript{10} The immunocrit method needs to be evaluated in more species prior to widespread use.

Benefits of the immunocrit method include its potential non–species specificity, quick and simple nature, and ability to be performed anywhere. To the authors’ knowledge, the validity of this method for detection of FPI in dairy calves has not been evaluated. The objective of the study reported here was to evaluate the diagnostic usefulness of the immunocrit method for detection of FPI in dairy calves.

Materials and Methods

Animals

Female calves that were 2 to 6 days of age at 10 dairy farms in 5 counties were eligible for inclusion in the study. Calves were of the Holstein or Jersey breeds. Personnel at all 10 farms fed colostrum only to the newborn calves (ie, no calves were fed colostrum replacers).

The required sample size for the study was estimated on the basis of methods to detect disease (ie, FPI) in a population,\textsuperscript{11} a national mean prevalence of FPI of 19.2%,\textsuperscript{3} an $\alpha$ value of 0.05, an assumed FPI detection probability of 80% by the immunocrit method, and a total available population of approximately one thousand 2- to 6-year-old calves at all 10 farms. The minimum required sample size by this calculation was 161 calves. Two hundred forty-nine calves were included.

Between June and September 2014, a jugular venous blood sample was collected from each calf via an 18-gauge needle into a tube containing no anticoagulant. Samples were centrifuged at 2,880 $X$ g for 5 minutes, and serum was harvested and stored at $-20^\circ$C until assays were performed. The study protocol was approved by the University of California-Davis Institutional Animal Care and Use Committee. Owner consent was obtained for all included calves.

Immunocrit test

Although a 40% ammonium concentration is reportedly effective in precipitating immunoglobulins in piglet serum samples,\textsuperscript{10} the effective ammonium concentration that effectively precipitates immunoglobulins in dairy calf serum samples was unknown and needed to be determined before calf samples could be tested in this study. Therefore, 40 randomly chosen serum samples were first evaluated at ammonium sulfate concentrations of 40%, 45%, 50%, and 55%; solutions for these initial tests were prepared by mixing 40, 45, 50, and 55 g, respectively, of crystalline ammonium sulfate\textsuperscript{6} with 100 mL of deionized water. Serum samples were allowed to thaw at room temperature ($20^\circ$ to $24^\circ$C) for up to 30 minutes and gently mixed by use of a vortex device. A 100-$\mu$L aliquot of each serum sample and 100 $\mu$L of prepared ammonium sulfate solution were mixed in microtubes\textsuperscript{6} containing no anticoagulant. Tubes were then vortexed, and the contents were transferred into two 75-mm nonheparinized capillary tubes.\textsuperscript{6} Negative control samples, consisting of 100 $\mu$L of serum and 100 $\mu$L of deionized water, were also prepared for testing alongside the test samples. Test and control samples were then centrifuged at 12,700 $X$ g for 5 minutes by use of a microhematocrit centrifuge.\textsuperscript{8}

Capillary tubes were removed from the centrifuge, and immunocrit values were read with a microhematocrit reader card from the top of the capillary tube sealant to the intersection between the precipitated IgG and the remaining serum or deionized water sample. The optimum concentration that precipitated immunoglobulin at each of the 4 ammonium sulfate concentrations was determined by creation of ROC curves at an arbitrarily chosen immunocrit cutoffpoint of 6%. The ROC analysis revealed that the 55% concentration yielded maximal sensitivity and specificity when classifying serum samples as having an adequate ($\geq$1,000 mg/dL) or inadequate (< 1,000 mg/dL) IgG concentration. Serum IgG concentration was then measured by use of 55% ammonium sulfate solution in the remaining 209 samples.

RID test

The IgG concentration was measured in all 249 serum samples (40 samples analyzed by all 4 ammonium sulfate concentrations and 209 samples analyzed only by the 55% ammonium sulfate concentration) by use of a commercial RID kit\textsuperscript{7} (detection range, 196 to 2,748 mg/dL), in accordance with the manufacturer’s recommendations. Briefly, RID plates (stored in a refrigerator at $4^\circ$C) containing specific anti-bovine IgG, agarose gel, 0.1M phosphate buffer (pH, 7.0), 0.1% sodium azide as a bacteriostatic agent, and 1 $\mu$g of amphotericin B/mL as an antifungal agent were warmed at room temperature for 30 minutes. A 5-$\mu$L aliquot of the provided reference serum at each of 3 concentrations (196, 1,402, and 2,748 mg/dL) was transferred via pipette into individual wells on each RID plate used. A 5-$\mu$L aliquot of each serum sample was also transferred via pipette into individual wells, and then plates were incubated at room temperature for 24 hours. Diameters of the observed zones of precipitation were measured by use of a digital RID plate reader.\textsuperscript{7}
Serum IgG concentrations were determined by comparing the diameter of the zones of precipitation with a standard curve generated by use of the reference serum data. The regression equation generated in this manner ($R^2 = 0.97$ to 0.99) accurately predicted the inoculum IgG concentration. Minimum detectable serum IgG concentration by the RID was 196 mg/dL. For study purposes, calves with values $< 196$ mg of IgG/dL of serum were assumed to have a concentration of 195 mg/dL. Serum samples containing IgG concentrations $> 2,748$ mg/dL were diluted 1:2 in PBS solution and reincubated into the RID plates.

**Statistical analysis**

The RID assay was considered the reference standard for comparison with the immunocrit method of serum IgG measurement. A serum IgG concentration $\geq 1,000$ mg/dL was considered indicative of adequate transfer of passive immunity. Initially, immunocrit ranges from 6% to 18% were considered as potential cutpoints for indicating FPI, but immunocrit cutpoints of 10% through 15% were considered in the final analysis. At each cutpoint, sensitivity and specificity of the immunocrit method for detection of FPI in dairy calves were calculated. Sensitivity was defined as the probability of a test result indicating an inadequate ($< 1,000$ mg/dL) serum IgG concentration as determined by RID (ie, FPI). Specificity was defined as the probability of a test result indicating an adequate ($\geq 1,000$ mg/dL) serum IgG concentration as determined by RID. Sensitivity and specificity at each immunocrit cutpoint (10% through 15%) were determined, followed by ROC curve construction, which allowed determination of the optimum cutpoint that maximized both sensitivity and specificity. The Spearman correlation coefficient ($\rho$) was calculated to determine the correlation between RID results (serum IgG concentration) and immunocrit values.

As a result of differences in prevalence of serum samples with inadequate serum IgG concentrations in calves from the various farms used, likelihood ratios (prevalence independent) for a positive or negative test result were determined instead of predictive values (prevalence dependent) for a positive or negative test result. These values and their 95% CIs were calculated by use of standard methods as described elsewhere. \textsuperscript{12} Likelihood ratios were interpreted on the basis of their magnitude. A likelihood ratio $> 1$ was considered to indicate that the tested calf had FPI, and a ratio close to 0 was considered to indicate the tested calf had no FPI. \textsuperscript{13-15} A likelihood ratio of 1 indicated no effect on the odds of FPI. Statistical software\textsuperscript{9} was used for all data analyses, with values of $P < 0.05$ considered significant.

**Results**

Mean $\pm$ SD IgG concentration in 249 serum samples as determined by RID was 1,741 $\pm$ 793 mg/dL (range, 257 to 4,751 mg/dL). The proportion of samples with positive RID results for FPI (serum IgG concentrations $< 1,000$ mg/dL) was 19.7% (49/249).

In immunocrit assays, all negative control samples had no precipitation measurable on the microhematocrit card reader. The area under the ROC curve was 1.00 ($P < 0.001$; 95% CI, 0.92 to 1.00). An immunocrit cutpoint of 11% was chosen for detection of FPI because it provided optimal sensitivity (0.88; 95% CI, 0.79 to 0.97) and specificity (0.51; 95% CI, 0.44 to 0.58). Sensitivity of the immunocrit method at cutpoints of 10%, 12%, 13%, 14%, and 15% was 0.82, 0.88, 0.92, 0.96, and 0.98, respectively, and specificity was 0.66, 0.36, 0.35, 0.14, and 0.08, respectively. The correlation between the immunocrit values and serum IgG concentration as measured by RID was 0.71 ($P < 0.001$). The likelihood ratio for a positive test result was 1.80 (95% CI, 1.51 to 2.14), and the likelihood ratio for a negative test result was 0.23 (95% CI, 0.11 to 0.51).

**Discussion**

The immunocrit method has many benefits that make it a good option for on-site testing by food animal producers or practicing veterinarians. The test requires minimal equipment and training, similar to the demands for Hct measurement, and related supplies are inexpensive and easily acquired for testing of large numbers of animals. Indeed, ammonium sulfate is a commercially available compound, which practicing veterinarians or producers can acquire.

The 11% cutpoint of the immunocrit method yielded a sensitivity of 0.88 for detection of FPI, which would allow a minimal number of calves with FPI to be misclassified as having adequate passive transfer of immunity (ie, a false-negative result). This would also result in a high proportion of neonatal calves with FPI being correctly identified and would allow appropriate intervention, such as changes to the herd’s colostral management program tailored to individual affected calves. The lower specificity of 0.51 would result in an overestimation of the proportion of calves with FPI and therefore an increased number of unnecessary interventions, potentially leading to unnecessary expenditures. If this test had a low sensitivity, then the proportion of calves with adequate passive transfer would be underestimated, resulting in calves with FPI failing to receive appropriate treatment and possible failure to detect deficiencies in the herd’s colostral management program.

A likelihood ratio for a positive test result of 1.80 meant that calves with FPI ($< 1,000$ mg of IgG/dL of serum) were 1.80 as likely to have an immunocrit of $< 11\%$ as were calves without FPI. A likelihood ratio for a negative test result of 0.23 meant that calves without FPI were 0.23 times as likely to have an immunocrit $\geq 11\%$ as were calves with FPI. These findings suggested that the immunocrit method would be a suitable screening test for FPI in dairy calves. Identification of FPI would allow dairy producers to evaluate their colostrum management practices and target areas in need of improvement.
We also found the immunocrit method could be improved for calves by using a 55% ammonium sulfate solution rather than the suggested 40% solution used for piglets. Use of the 55% solution resulted in more effective immunoglobulin precipitation than with the other concentrations (40%, 45%, and 50%), thereby improving the immunocrit test results. We would therefore suggest that if the immunocrit method is to be used for other veterinary species, the effective ammonium concentration for those species should first be determined.

Other cow-side testing options have less promising results. For example, measurement of serum total solids concentration by optical refractometry with a cutpoint of 5.5 g/dL has a sensitivity of 0.800 and specificity 0.807, and digital refractometry has a sensitivity of 0.855 and specificity of 0.825 at an 8.3% cutpoint. The immunoassay test has the advantage of being easy for producers to perform, but it is qualitative and the individual cost of each test is fairly substantial. Additionally, the immunoassay test does not appear to offer any advantage with respect to sensitivity (0.73 to 0.82) or specificity (0.65 to 0.71). On the other hand, the immunocrit method had a superior sensitivity of 0.88 but inferior specificity of 0.51 at a cutpoint of 11% for dairy calves in the present study. The main advantages of the immunocrit method over other tests include the relatively low cost, small volume of sample required, and quick turnaround time for results (6 to 7 minutes) and the ability of ammonium sulfate to specifically precipitate immunoglobulins rather than all serum proteins. It should be noted that practicing veterinarians are more likely to have access to and use a microhematocrit machine than are dairy producers, so veterinarians could be expected to use the immunocrit method more often than dairy producers.

Because IgG is the predominant immunoglobulin in colostrum (90%), only IgG was measured in serum samples to assess passive immunity status in calves and sensitivity and specificity of the immunocrit method in the present study. Serum IgM and IgA concentrations were not measured. Consequently, for calves or other species that receive immunoglobulins through sources other than colostrum, such as serum- or plasma-based colostrum replacers, measurement of serum IgM and IgA concentrations would be recommended instead.

The prevalence of FPI (19.7%) in dairy calves of the study reported here was consistent with the national average of 19.2%. Ammonium sulfate is specific for precipitation of immunoglobulins, and this precipitation is not species specific; therefore, the immunocrit method may be useful for detecting FPI in other veterinary species as well. Because predictive values of diagnostic tests are prevalence dependent, likelihood ratios should be calculated when assessing the diagnostic usefulness of this method in other species.

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Footnotes

References