Quantitative Indirect ELISA-based Method for the Measurement of Serum IgG in Springbok Calves.

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ABSTRACT

Passive transfer of immunity through maternal colostrum is an important factor in the survival of ruminant neonates, as they essentially lack the ability to receive immunity in utero. Inadequate ingestion of colostrum by neonates can result in increased levels of morbidity and mortality. Several methods are used to determine serum immunoglobulin levels, both before and after administration of colostrum or colostrum replacement. Radial immunodiffusion analysis (RID) is generally considered the reference method for determining serum or colostral immunoglobulins concentrations in ruminants. Unfortunately, RID is labor-intensive, time-consuming, and takes 48 to 72 hours to provide serum immunoglobulin test results. Currently, methods to determine colostral or serum immunoglobulin concentrations in non-domestic ruminants are not available. In this study we describe a method for measuring sera immunoglobulin levels using an indirect enzyme-linked immunosorbent assay (ELISA) that can be completed within one working day. The described method is relatively more accurate, precise, and expeditious and less labor intensive than an RID assay. While this protocol was developed for use with springbok sera, it should be easily adaptable for other non-domestic ruminants species for which commercial antibodies are not readily available.

INTRODUCTION

The term "passive immunity" refers to the transfer of immunoglobulins from the dam to neonate, either in utero or via colostrum, that serve to provide immunological protection until the neonate's immune system develops sufficiently to provide its own response to environmental pathogens. In

ruminants, passive immunity is through the ingestion of colostrum. Due to their synepitheliochorial placenta, minimal quantities of immunoglobulin are transferred in utero.[1] Additionally, there are many environmental and behavioral factors that can lead to insufficient passive transfer of colostral immunoglobulins to calves.[2] These include the volume of colostrum ingested, method of administration, age of calf at feeding, breed of the dam and the concentration of immunoglobulins in the colostrum or replacer.[2,3] In order to reduce morbidity and mortality, it is often necessary to provide calves with a colostrum supplement or replacer. These colostrum replacers or supplements can be derived from natural colostrum collected from related dams, colostrum collected from a related species, or blood or blood products.[4,5]

The evaluation of efficacy of passive transfer is through determination of serum immunoglobulins after ingestion of colostrum. Several qualitative and quantitative methods have been used to evaluate the efficacy of passive transfer of colostral immunoglobulins in calves. The methods have been reviewed and include total serum protein concentration as determined by refractometry, sodium sulfite test, radial immunodiffusion (RID), zinc turbidity assay, whole blood glutaraldehyde coagulation, γ-glutamyl transferase,[2] and an enzyme-linked immunosorbent assay (ELISA).[6] Of the various methods mentioned, only the RID and ELISA are considered reliably quantitative.

Radial immunodiffusion analysis has been considered the reference method for determination of serum or colostral immunoglobulin concentrations.[4,7,8] However, the prolonged incubation time (48 – 72 hours) results in a delay in the observation of the test results and also subjects the test to the possibility of microbial contamination if sodium azide is not included in the medium. An ELISA protocol for determining IgG levels in sera and colostrum in bovines that relies on commercially available reagents has been reported.[6]

Studies assessing passive transfer status in wild ruminants using quantitative ELISA methods are currently unavailable. A reliable, accurate method to evaluate passive transfer of colostral immunoglobulins status in non-domestic ruminants must be established where commercial tests are not available. Thus, the objective of this study was to develop such an assay for springbok. The test would be anticipated to be adaptable to other non-domestic ruminant species.

MATERIALS AND METHODS

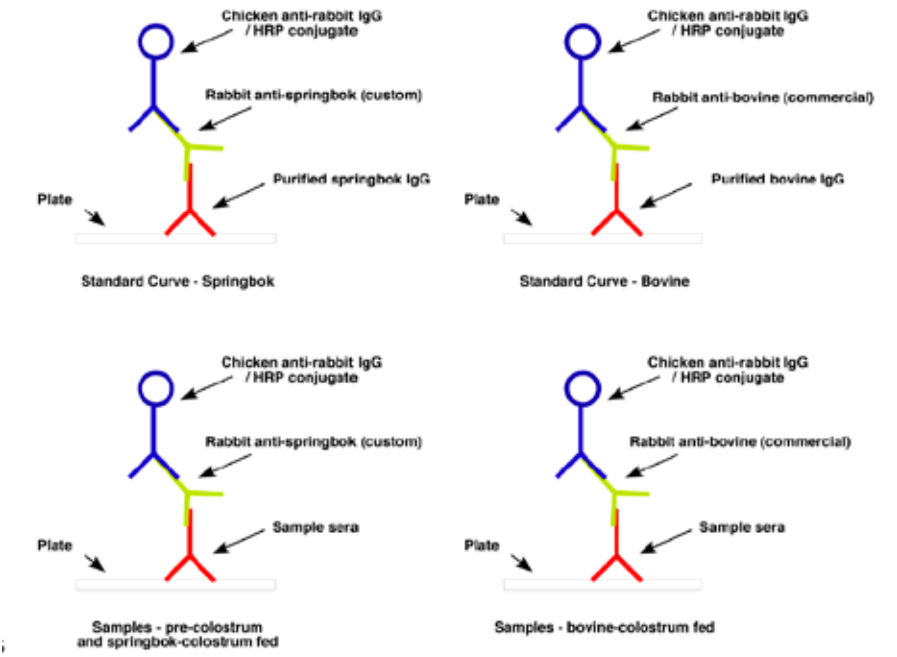
Production of Standard Springbok and Anti-springbok Immunoglobulin G (IgG)

Serum from an adult male springbok (Antodorcas marsupialis) was collected by jugular venipuncture. The springbok was housed at the San Diego ZooSafari Park (SDZSP) (Escondido, CA, USA). The procedures used in this study were reviewed and approved by the SDZSP's Institutional Animal Care and Use Committee. The serum was provided to a commercial provider (Antibodies Incorporated, Davis, CA, USA) to isolate total springbok IgG, using a proprietary method. The relative purity of the antibody was determined using SDS-PAGE gel analysis (Antibodies, Inc., Davis, CA, USA). A portion of the purified IgG was provided for use as a standard in the ELISA, while the remainder was used as inoculum for two rabbits, following accepted protocols for development of polyclonal antibodies.[9]

Collection of Serum Samples from Springbok Calves

The developed ELISA was used to establish serum springbok specific IgG levels in 36 samples from springbok calves held at the SDZSP. Blood was collected via jugular vein from springbok calves born through November 2009 to June 2010. Three milliliters of blood was placed in a red top tube (Becton Dickson, Franklin Lakes, NJ, USA) and allowed to clot for 60 minutes prior to centrifugation. The serum was then collected and stored at -20° C until subsequent analysis of IgG concentration (Thomp-

Figure 1: *Graphic representation of the indirect ELISA protocols used in determining serum IgG in springbok calves*



son, et al, submitted for publication). The samples analyzed consisted of springbok calves in three different categories. The first 19 samples were taken prior to any colostrum consumption (< 24 hours of age). Another seven samples were from calves that were allowed to consume maternal colostrum and were 48-72 hrs of age. The final 10 samples were from calves that were fed >4.68 g of IgG during their first 24-36 hours of life, from a commercially available lacteal-derived, United States Department of Agriculture-regulated bovine-based colostrum replacer (Land 'O'Lakes, St. Paul, MN, USA) and were 48-72 hrs of age.

ELISA Assay Development and Determination of Serum IgG in Springbok Calves

The protocol described herein is a variation of the common "sandwich ELISA" (Harlow and Lane, 1988). However, in this case, the antigen was bound to the assay plate before the introduction of the "primary antibody" (Figure 1). In this case, the antigen was an antibody, and the "primary antibodies" were specific to the immunoglobulin of interest. All controls (known concentration of purified springbok IgG) and samples (springbok sera) were assayed in triplicate. Initial dilutions of all controls and serum samples were done using positive-displacement pipetters. This compensated for the relative viscosity of the sample. While this was not a major concern in evaluation of sera, positive displacement pipettes had the advantage of minimizing volume errors when handling viscous samples such as colostrum.

Controls and samples ("antigen" - neonatal springbok serum IgG in this study) were diluted to the appropriate level in "coating buffer" (50 mM sodium carbonate, pH 9.0), and 100 µl of appropriate dilutions were bound in triplicate to Fisher Immulon IV microtiter plates (Fisher Scientific, Pittsburgh, PA, USA) for either 1 hour at room temperature with gentle agitation using an agitator (Orbitron rotator I, Boekel Scientific, Feasterville, PA, USA). Each immunoassay plate included a standard curve to

minimize plate-to-plate variation.

Following the initial binding step, plates were washed 5 times with TBS (25 mM Tris pH pH 7.4, 150 mM Nacl, 2 mM KCl) using a BioTek plate washer (BioTek Instruments, Winooski, VT, USA) following a program designed for this application (protocol available upon request). Non-specific binding sites were blocked using 300 µl of blocking buffer (25 mM Tris pH pH 7.4, 150 mM Nacl, 2 mM KCl, 1.35% fish skin gelatin) for 1 hour at room temperature with gentle agitation. This was followed by five washes, as described above.

An initial dilution at 1:750 of primary antibody, a polyclonal rabbit anti-springbok IgG (Antibodies Inc., Davis, CA, USA) or rabbit anti-bovine IgG (Bethyl Laboratories, Montgomery, TX, USA) was diluted in BSA diluent (Cygnus Technologies, Southport, NC, USA) and 100 µl of the diluted rabbit anti-bovine IgG or rabbit anti-springbok IgG was added to the plates. Subsequent adjustments in dilution were sometimes required in order to receive an appropriate level of signal. The plates were then incubated at room temperature for 1 hour with gentle agitation and washed as above. Chicken anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Bethyl Laboratories, Montgomery, TX, USA) was diluted 1:1,500 in horseradish peroxidase diluent (Cygnus Technologies, Southport, NC, USA) and was used as secondary antibody, and 100 µl of this was added to each well and incubated with gentle agitation for 1 hour at room temperature. The plates were then washed five times as described above.

Colorometric determination was performed by addition of 100 µl of TMB (3, 3', 5, 5' tetramethyl benzidine), (Moss, Inc., Pasadena, MD, USA). The plates were allowed to develop for 30 minutes at room temperature in the dark. The reaction was then quenched by addition of 100 µl of 0.5 M sulfuric acid. The plates were then read using a Spectromax 250 microplate spectrophotometer at 450nm (Molecular Devices, Sunnyvale, CA, USA).

The data derived were subjected to a 4-parameter nonlinear regression analysis.[10] Immunoglobulin G concentration determination of serum from calves allowed to nurse their dams were determined against a springbok standard curve. Serum IgG concentration in calves fed the commercial bovine colostrum replaced were determined against a bovine standard curve. The coefficient of determination (R2) for the standard curves was uniformly > 0.990 indicating acceptable sample IgG concentration prediction by the standard curves.

RESULTS AND DISCUSSION

The objective of this study was to describe an adaptable method for determining IgG in non-domestic ruminants where commercial antisera are not available. Although this method was developed for IgG, it can be adapted for IgM and IgA, which are also present in colostrum. Determination of IgG was considered in this study because it is the predominant immunoglobulin (85-90 %) in ruminant colostrum.[11] While the method required specialized equipment and several steps, it provided more reliable and repeatable results than comparable methods. Additionally, the technique had a faster turn-around of test results compared to RID. Previous studies reported a 94 % agreement between RID and ELISA in determining serum IgG concentration in dairy calves.[12] We recommend that the ELISA described in this study be used as a reference method to evaluate sensitivity and specificity of methods that are used in the field in non-domestic ruminant neonates.

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