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Protein G binding to enriched serum immunoglobulin from nondomestic hoofstock species

Joely A. Kramsky, Elizabeth J. B. Manning, Michael T. Collins

Abstract. Quick and cost-effective serologic assays, such as those based on enzyme-linked immunosorbent assay (ELISA) technology, are useful for screening animal populations for infectious diseases. Recombinant protein G is described as an almost universal ELISA conjugate for the detection of antibodies from a wide range of animal species. However, there is limited data documenting the ability of protein G to bind immunoglobulin (Ig) from many captive and free-ranging nondomestic hoofstock (Order Artiodactyla, e.g., elk, antelope, bison). Protein G binding to Ig from 11 species within this taxonomic order (addax, antelope, bison, bontebok, elk, impala, kudu/nyala, muntjac, oryx, sheep, and white-tailed deer) and 2 control species (bovine and chicken) was assessed. A serum Ig enrichment protocol, using high-performance liquid chromatography (HPLC), was optimized in bovids (*Bos taurus*) and then applied to the other study species. Binding assays were performed by adding protein G to microtiter wells coated with titrated dilutions of enriched artiodactyl Ig. Optical densities were measured and binding curves generated. Differences in protein G binding were observed, both within and among species, as well as within taxonomic families. Significant intraspecies binding variation was observed for 7 species tested (antelope, oryx, sheep, muntjac, impala, bontebok, and addax). No statistically significant intraspecies differences in protein G binding were found for Ig from bison, elk, kudu/nyala, white-tailed deer, plus control species (cattle and chicken). Binding of protein G to Ig from impala, muntjac, and elk was statistically different from the positive control (cattle), with muntjac binding curves statistically comparable with the negative control (chicken). For the other 7 species tested, binding curves illustrated the ability of protein G to bind Ig as well as, or better than, the positive control. These findings expand the list of animal species whose Ig is capable of being detected using recombinant protein G, with the caveat that protein G does not bind Ig uniformly in closely related species. It is concluded that recombinant protein G conjugates may serve as useful reagents for serodiagnosis by ELISA in nondomestic hoofstock, although different assay interpretation algorithms and assay protocols may need to be developed on a per species basis for maximum diagnostic effectiveness.

Detection, control, and management of infectious diseases in captive and free-ranging nondomestic ruminant hoofstock species (Order Artiodactyla) often prove challenging. These species, such as okapi, impala, and bontebok, are not often handled, and as a result, samples for diagnostic analysis are infrequently collected. An additional hindrance to disease surveillance is the lack of validated diagnostic assays for these valuable, and in some cases, endangered species.

Reliable, cost-effective, and noninvasive diagnostics are needed to maximize the value of diagnostic samples when they can be collected. In particular, for diseases eliciting a humoral (antibody) immune response, quick, affordable, and effective serologic assays would support disease surveillance in captive and free-ranging animals. Routinely used in agricultural ruminant species, most serological assays are not validated for use in nondomestic hoofstock.

Enzyme-linked immunosorbent assay (ELISA) methodologies are reported to provide higher sensitivity than other serological assays.[32] They may be performed at low cost with high throughput and are readily automated. For many ELISAs, however, the labeled secondary antibody (conjugate) is specific for each species tested. Antisera to many nondomestic artiodactyl ruminants are not uniformly available. The production and evaluation of antisera for use as a conjugate in a separate ELISA for each individual species would prove time consuming and cost-prohibitive. Even if these reagents were available and the assay validated, performing a separate ELISA for each of the numerous species found in the average zoo or wildlife collection would be laborious. To date, this has precluded the use of ELISA technology for antibody detection for the many diverse species of the artiodactyl family.

Protein G, derived from group C and G streptococci, is one of the best-defined immunoglobulin (Ig)-binding bacterial cell wall receptors.[3,5,14] Protein G binds the Fc portion of IgG and IgG subclasses to varied degrees.[7,14,15] For instance, mouse IgG2 is bound strongly by protein G, whereas mouse IgG1 is weakly bound.[5] Recombinant forms of protein G laboratory

From the School of Veterinary Medicine, University of Wisconsin–Madison, Madison, WI 53706.

Table 1. Summary of sera tested, including binding capacity of protein G for each species' Ig (relative to bovine standard binding curve at 4 μg/ml Ig concentration, $P < 0.05$).

Species	Family	Number of animals	Ig–protein G binding
Addax (*Addax nasomaculatus*)	Bovidae	8	equivalent
Antelope* (*Hippotragus* sp.)	Bovidae	7	equivalent
Bison (*Bison bison*)	Bovidae	8	equivalent
Bontebok (*Damaliscus dorcas*)	Bovidae	6	equivalent
Cow (*Bos taurus*)	Bovidae	7	N/A
Impala (*Aepyceros melampus*)	Bovidae	8	lesser
Kudu/nyala (*Tragelaphus strepsiceros/angasi*)	Bovidae	6	equivalent
Oryx† (*Oryx* sp.)	Bovidae	7	lesser
Sheep (*Ovis aries*)	Bovidae	8	equivalent
Elk (*Cervus elaphus*)	Cervidae	8	lesser
Muntjac (*Muntiacus muntjak*)	Cervidae	8	lesser
White-tailed deer (*Odocoileus virginianus*)	Cervidae	8	equivalent
Chicken (*Gallus domesticus*)	Phasianidae	8	lesser

* *H. niger* and *H. equinus* included in species group.
† *O. leucoryx* and *Oryx* spp. included in species group.

reagents have eliminated non–Ig-binding sites (e.g., albumin), thus reducing nonspecific protein binding.[13] Recombinant protein G is widely used as a laboratory reagent for the purification and detection of Ig in numerous well-characterized domestic and laboratory species.[3,5] However, its reactivity with ruminant artiodactyl species has not been well documented beyond cattle, goats, and sheep.[3,20]

Development of a protein G–based ELISA for the detection of antibodies was investigated for use in multiple nondomestic artiodactylid species. Protein G reportedly exhibits broader species specificity with less variation in isotype binding within a species than other nonspecies-specific Ig-binding cell wall proteins such as protein A.[5] Many investigators also report stronger binding affinity between protein G and Ig from several species,[2,3,5,9,10,14] as well as selective reactivity to IgG.[5,12,14,24] Reports on the binding of Fc-receptor bacterial proteins to sera of domestic and wild African mammals,[16] birds, and reptiles[18] found that whole sera from large ungulates (e.g., wildebeest, waterbuck, buffalo, nyala) reacted strongly with the chimeric protein (containing recombinant protein G and protein A) and weakly with protein A alone. These characteristics led to the selection of protein G as the conjugate system for the multispecies assay in this study.

Variation exists in the ability of protein G to bind Ig and Ig subclasses from many domestic species (e.g., rat, mouse), and the extent of Ig structure conservation across species is unknown. Thus, a priori assumptions regarding the uniform capacity for protein G binding to Ig from nondomestic artiodactyl species cannot be made, even within the same taxonomic family. To establish the protein G–binding capacity, it was necessary to acquire Ig from these nondomestic hoofstock species. Because the binding of protein G to Ig from the species included in this study is undocumented and because detailing protein G–binding patterns was the ultimate goal of the study, the initial step of obtaining Ig from these species did not use protein G–based methods. This led to a study design using conventional purification methods to obtain Ig as the first step in defining the use of protein G conjugates in ELISAs for antibody detection in a wide range of artiodactyl species.

Materials and methods

Samples

Eleven diverse species from 2 taxonomic families (Bovidae and Cervidae) within Order Artiodactyla were represented in this study (Table 1). Serum and plasma samples were obtained from 6 to 8 clinically normal adult animals of each species and stored at −70 C until use. Because of limited availability of samples in certain species, some samples were grouped by genus. For example, roan and sable antelope were combined as *Hippotragus* sp., and Arabian and slender-horned oryx were combined as *Oryx* sp. Kudu and nyala were evaluated together because both belong to the subgenus *Tragelaphus*. The total protein and albumin levels of all serum samples used were determined[a]; average values for the sera were within normal ranges for each species (using overall mean values for males and females of all age groups).[4,17,27,31] The positive control was Ig from 7 bovine sera (bovine Ig has previously been proven capable of binding to protein G).[20] The negative control was Ig from 8 chicken sera because Ig of avian species do not bind protein G.[3] Commercially purified bovine IgG[b] (92% purity via fractionation and ion-exchange chromatography) was included for a qualitative binding comparison.

Immunoglobulin enrichment

Frozen serum samples (2–3 ml serum/sample) were treated with 1,1,1-trichlorotrifluoroethane to extract lipids just before use.[1] Immunoglobulin was separated from other serum proteins using a 7.5- × 150-cm BAKERBOND Abx™ (15 μm) mixed-mode ion-exchange column[c] on a Beckman high-performance liquid chromatography (HPLC) system.[d] This HPLC column specifically purifies antibodies by preferentially (and reversibly) binding Ig, allowing a majority of extraneous proteins (including albumin, transferrins, and proteases) to pass through. It is capable of binding all Ig isotypes and their subclasses from any source or species.[21] Preliminary trials determined the optimal HPLC buffers and concentrations for use with bovine sera (data not shown). This protocol was then applied to all other species tested. The mobile phase consisted of 30 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.0), initial buffer, and 20 mM sodium acetate and 1 M sodium chloride (pH 7.0), final elution buffer. Protein was detected by UV absorbance at 280 nm. Delipidated serum samples were diluted up to 10 ml with initial buffer and loaded using a 10-ml sample loop. Samples were held at 2 ml/min and 100% elution buffer during sample loading and void volume elution (typically 11–16 min). This flow rate was maintained until absorbance readings returned to baseline. Immunoglobulin was then removed from the column during an isocratic step of 5% elution buffer at 4 ml/min ("Ig peak"). After absorbances returned to baseline, any protein remaining bound to the ion-exchange column was eluted during an isocratic step of 80% elution buffer ("extraneous protein peak") and the column was reequilibrated. For comparison of methods, a gradient protocol was used for llama (Family Camelidae) serum samples. These samples were held at 2 ml/min and 100% initial buffer during sample loading and elution of void volume, and Ig was removed using a gradient of 0% to 80% elution buffer for 30 min. All collected protein peaks were immediately stored at 4 C and concentrated[e] within 8 hr. Protein concentration of purified peaks was determined using the BCA Protein Assay Kit.[f] The HPLC data were analyzed and chromatograms generated using System Gold chromatography software (Version 8.10).[d]

Immunoglobulin recovery confirmation

After the HPLC Ig enrichment steps, 2 additional methods were used to substantiate the presence and quantity of Ig. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Mini-protean II Electrophoresis Cell System.[g] Gels were stained with Coomassie Brilliant Blue R and digitized by the Kodak Electrophoresis Documentation and Analysis System 290.[h] 1D Image Analysis Software[h] was used for densitometry analysis. In additional, proteins were separated by molecular weight using a 300- × 7.8-mm Bio-Sil 250 size-exclusion column[g] on a Beckman HPLC system. The mobile phase consisted of 0.10 M sodium phosphate (HPLC grade), 0.15 M sodium chloride, and 0.01 M sodium azide, pH 6.8. Samples were loaded at 1 ml/min using a 20-μl loop. Gel filtration standards[g] measuring from 1.35 to 670 kD (containing vitamin B12, equine myoglobin, ovalbumin, bovine IgG, and thyroglobulin) were included for comparison with each group of purified protein samples. Protein was detected by UV absorbance at 280 nm. Retention times were calculated using System Gold chromatography software (Version 8.10).[d] All Ig peaks and a minimum of 2 extraneous protein peaks from each species were analyzed using the 2 confirmatory methods. Untreated serum samples from 6 species were included in the SDS-PAGE analysis to measure Ig concentration of samples not treated by enrichment protocols.

Immunoglobulin recovery calculation

Immunoglobulin recovery in each species was determined by summing the net intensity value of protein bands representing Ig light chains (20–25 kD) and heavy chains (50–55 kD)[28] from SDS-PAGE gels. This value was then expressed as a percentage of the net intensity of all proteins in the Ig peak. Because the molecular mass of chicken IgG heavy chain is greater than that of mammalian IgG heavy chain (61–68 kD vs. 50–55 kD, respectively),[26] the larger mass proteins were used for Ig calculations in this species.

Binding assays

Plate coating. Purified Ig from each species was bound to 96-well polystyrene microtiter plates.[i] After standardizing all Ig samples based on Ig recovery calculations, samples were diluted in 50 mM sodium carbonate (pH 9.6) to concentrations of 2, 2.5, 3, 3.5, 4, 4.5, and 5 μg/ml. Individual concentrations were obtained separately by dilution from stock (1 mg/ml in phosphate-buffered saline [PBS]). Each well was coated with 50 μl of diluted sample with duplicates assayed on separate plates. Coated plates were incubated overnight at room temperature in a humid chamber. Wells were washed[j] 3 times with wash 1 (0.15 M sodium phosphate, 0.2 M sodium chloride, pH 7.2) and blocked with 0.5% casein in PBS. The wells were filled completely with blocking agent and incubated in a humid chamber at room temperature for 2 hr. Wash 2 was prepared by adding 0.05% Tween 20 to wash 1 and used to wash each plate 3 times after blocking.

Protein G. A commercial protein G conjugate, labeled with horseradish peroxidase (HRP), was obtained.[k] A conjugate dilution of 1:10,000 was chosen

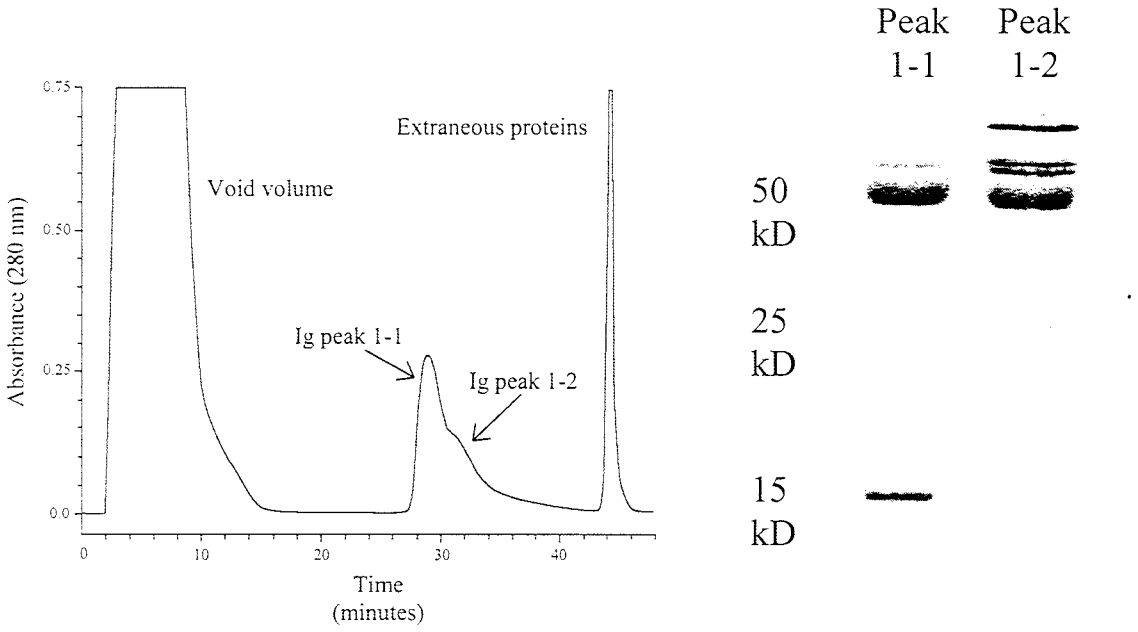

Figure 1. The HPLC chromatogram (left) and SDS-PAGE (right) results from white-tailed deer serum. The HPLC void volume contained albumin, transferrins, proteases, α-globulin, and β-globulin. The Ig peaks (peak 1-1 and peak 1-2) follow a 5% elution buffer step that was administered at 22 minutes. Extraneous proteins were removed with 80% elution buffer. The SDS-PAGE analysis of the Ig peaks shows strong 15-kD proteins in peak 1-1 only (29% of the total protein in the lane). Immunoglobulin heavy chains are 50–55 kD and light chains are 20–25 kD. Peak 1-2 contained more proteins with molecular weights above 50 kD than did peak 1-1.

based on preliminary experiments (data not shown). This single concentration of protein G was added to wells coated with the 7 different concentrations of purified Ig (100 µl protein G/well). Wells were incubated with the protein G conjugate for 1 hr at 37 C. Wash 2 was used (3 times per well) to remove unbound conjugate.

Substrate and stop solution. Color reaction was developed with the addition of 100 µl/well HRP substrate tetramethyl benzidine[k] and plates incubated at room temperature. The reaction was stopped after 15 min with the addition of 100 µl/well hydrogen peroxide.[k] Optical density (OD) was measured at 630 nm.[l]

Data analysis. All individual animal samples were assayed in duplicate. To control for plate-to-plate variation, duplicates were run on separate microtiter plates. To control for day-to-day variation, individual samples within a species were run on different days. A bovine sample was included with all binding assays as a qualitative control for assay conditions. Binding curves were generated by graphing OD versus bound Ig concentration for individual animals within a species. Differences in binding curve slopes (with data from all 7 Ig concentrations) were statistically evaluated to assess intraspecies variation (general linear models). For more detailed analysis of interspecies variation (mixed effects model), comparisons were made at 3 of the 7 individual antigen concentrations (3, 4, and 5 µg/ml). Tukey statistical analysis was used to compare binding at multiple Ig concentrations. Interspecies binding differences were assessed at a P value of 0.05. SAS Statistical Software[m] was used for all analyses.

Results

Immunoglobulin was the predominant component of peaks collected using the 5% elution buffer. Interspecies variation in recovery of Ig was observed with averages ranging from 35% (kudu/nyala) to 95% (elk) of the total purified proteins. Intraspecies variation was also noted. The standard deviation of Ig recovery within species varied from 2.3% (elk) to 14.5% (kudu/nyala). Elk had both the highest level of Ig recovery and the lowest standard deviation. Kudu/nyala had the lowest level of Ig recovery with the highest standard deviation. Elk, sheep, bison, and chicken sera had higher Ig recovery rates than did bovine sera. Immunoglobulin recovery differed in species belonging to the same family (Cervidae: elk, 95%; white-tailed deer, 68%; muntjac, 52%).

The protocol yielded split Ig peaks ("peak 1-1" and "peak 1-2") in 7 of 8 white-tailed deer, 5 of 6 kudu/nyala, 6 of 8 chickens, and 3 of 8 muntjac. With the exception of kudu/nyala, the highest Ig concentration was found in peak 1-1. Also, a number of individual animals had 15 kD proteins in their Ig peaks. Most notably, in all white-tailed deer with split Ig peaks,

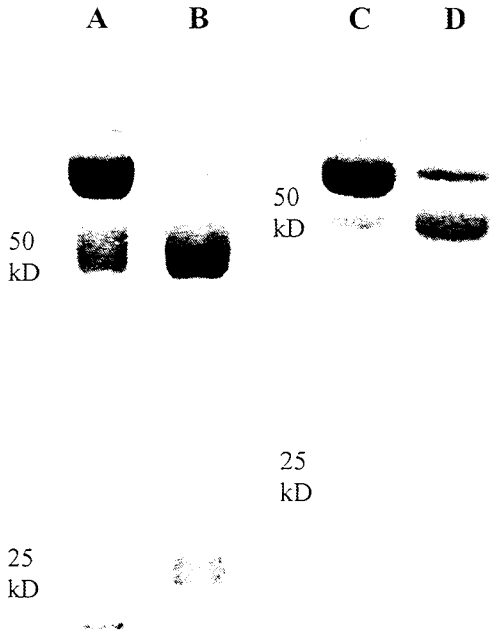

Figure 2. The SDS-PAGE comparison of original sera and enriched Ig samples of representative animals. Immunoglobulin heavy chains are 50–55 kD and light chains are 20–25 kD. Albumin, the predominant protein found in serum, is 66 kD. A majority of serum protein was removed during the enrichment process, whereas Ig heavy and light chain relative percentages increased. **A**, untreated bovine positive control serum contained 29% Ig. Albumin comprised 42% of the total serum protein. **B**, after HPLC Ig enrichment and concentration, the resultant sample contained 83% Ig. Sixty-six–kilodalton proteins were 9% of the total protein measured in the enriched sample. **C**, impala serum contained 21% Ig, with albumin making up 50% of total serum protein. **D**, The HPLC-enriched impala serum contained 69% Ig and 21% albumin.

prominent 15 kD proteins were found in peak 1-1 but not in peak 1-2 (Fig. 1).

Unprocessed bovine serum samples contained an average of 21% Ig; after enrichment and concentration the amount was on average 52% greater (Fig. 2).

Size-exclusion chromatography (SEC) results supported the SDS-PAGE Ig recovery calculations. The HPLC-enriched peaks shown by SDS-PAGE to have large amounts of 25- and 50-kD proteins had peaks matching standard bovine IgG retention times on SEC. Many Ig peaks also had small amounts of high molecular weight protein (>300 kD). The SEC of extraneous protein peaks showed numerous proteins of various sizes, especially of high molecular weight. As seen on SDS-PAGE gels in those species with split Ig peaks, with the exception of kudu/nyala, peak 1-1 contained larger amounts of protein with retention times matching the bovine IgG standard. Representative SEC chromatograms with matching SDS-PAGE gels are shown in Fig. 3.

As expected, protein G bound to bovine Ig in a linear fashion with increasing Ig concentrations and did not bind to negative control chicken Ig at any concentration (OD values near background levels) (Fig. 4A). The binding curves for enriched bovine Ig samples mirrored those of commercially purified bovine IgG (data not shown). An Ig concentration of 4 μg/ml was considered representative of overall protein G binding and was thus chosen as the basis for statistical comparisons of binding curves made between species (Table 1).

Binding curves for 8 species (addax, antelope, bison, bontebok, kudu/nyala, oryx, sheep, and white-tail deer) demonstrated that their Igs bound to protein G as well as or better than bovine Ig (Fig. 4C). The OD values for all concentrations in bontebok and kudu/nyala exceeded those of the bovine control (Fig. 4B). (Statistically, however, the kudu/nyala Ig–binding curve was not different from that of bovine Ig, and bontebok Ig binding differed statistically from that of the bovine control at 3 μg/ml only.) Ig-binding curves of addax, antelope, bison, white-tailed deer, kudu/nyala, and sheep were not statistically different from those of bovine Ig at all 3 concentrations evaluated.

Binding curves for 3 species demonstrated that their Igs bound less well than the bovine Ig control. Mean OD readings for impala, elk, and muntjac Ig were lower than those for bovine Ig control at every concentration (data not shown), and the OD values for the Igs of these 3 species were statistically different from those of bovine Ig at all concentrations analyzed (i.e., 3, 4, and 5 μg/ml). Muntjac Ig–binding curves had the lowest slope of all the evaluated species (Fig. 4B), and muntjac OD values did not significantly differ from those of the negative control at any statistically evaluated Ig concentration.

Binding of Ig to protein G varied among the 3 Cervidae species tested (elk, white-tailed deer, and muntjac) (Fig. 4D). Minor differences were found within species at various Ig concentrations but were not considered biologically significant overall. No significant variation in Ig binding to protein G among individual animals within a species was observed in bison, elk, kudu/nyala, and white-tailed deer, as well as the bovine and chicken controls (data not shown).

The alternative HPLC gradient method used for llama sera samples did not appear to affect Ig enrichment and binding to protein G. Llama Ig–binding curves were not statistically different from those of bovine Ig, indicating that llama Ig binds protein G as well as bovine Ig (data not shown).

Discussion

Characterization of protein G binding to Ig of these artiodactyl species implies that this conjugate may be used in a multispecies ELISA for any disease that elicits serum antibody, regardless of the causative organ-

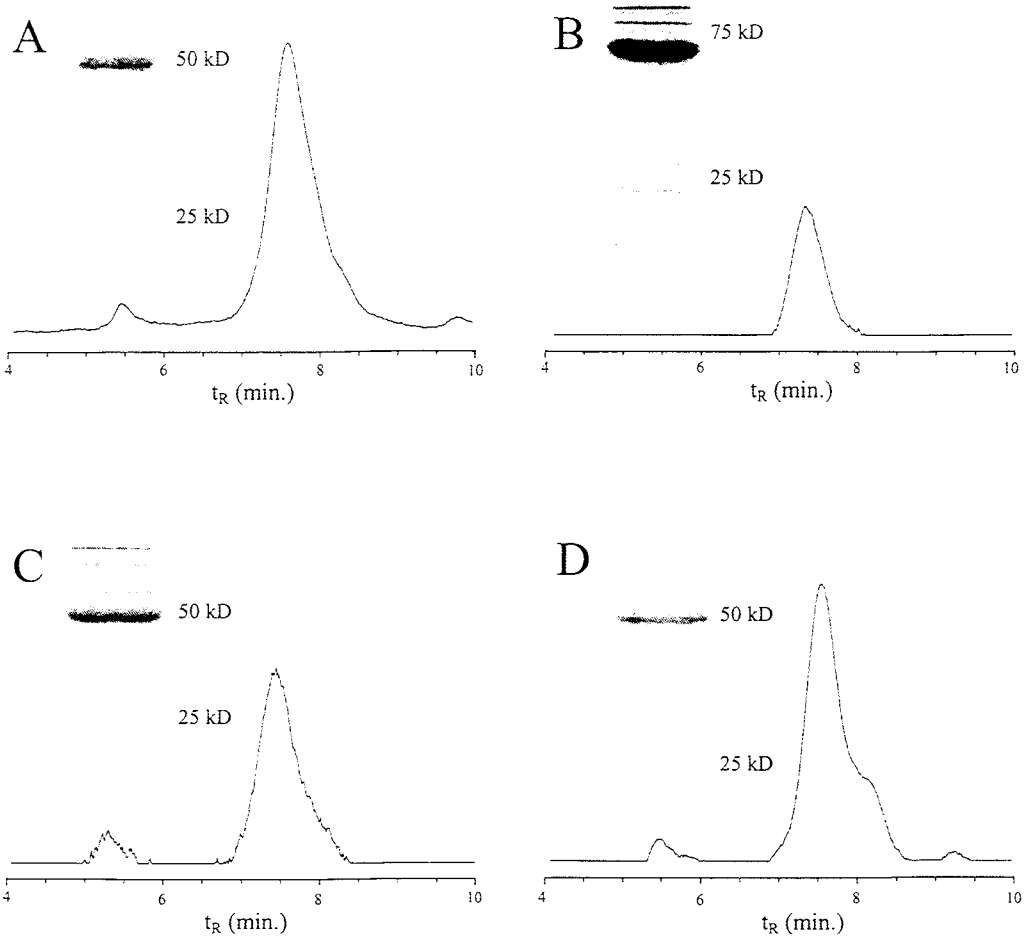

Figure 3. Size-exclusion chromatograms and matching SDS-PAGE gels from the Ig peaks recovered by HPLC from serum of representative species. Percent Ig recovery was calculated from SDS-PAGE results (see "Materials and methods"). **A**, enriched Ig from bovine serum positive control. Predominant SEC peak t_R = 7.58 minutes (bovine IgG standard t_R = 7.59 minutes). Immunoglobulin recovery = 90%. **B**, enriched Ig from chicken serum negative control. Predominant SEC peak t_R = 7.32 minutes (bovine IgG standard t_R = 7.68 minutes). Immunoglobulin recovery = 76%. **C**, enriched oryx (family Bovidae) Ig. Main SEC peak t_R = 7.46 minutes (bovine IgG standard t_R = 7.73 minutes). Immunoglobulin recovery = 81%. **D**, enriched elk (family Cervidae) Ig. Main SEC peak t_R = 7.53 minutes (bovine IgG standard t_R = 7.59 minutes). Immunoglobulin recovery = 91%.

ism. Testing the binding of protein G to Ig from 11 species within Order Artiodactyla confirms its potential application as a conjugate in a wide variety of species. However, just as differences in Ig yield using a single standard enrichment protocol were seen, variation in protein G binding was also observed. Although protein G does bind Ig from a variety of species, this study has confirmed that Ig for all species may not be bound uniformly, or at all, by this conjugate.

In keeping with other studies,[9,16] variability among animals within a species was observed. Because differences were also noted in the capacity of protein G to bind Ig from different species of the Cervidae family, it may be that protein G–Ig-binding characteristics do not follow phylogenic lines. In addition, there appeared to be no correlation between phylogeny and Ig-enrichment results. The grouping of kudu and nyala illustrates possible immunologic variation between species within a subgenus. This may account for the observed higher standard deviation of Ig recovery. These findings further emphasize the importance of optimization in each species investigated and warn against assuming that Ig properties within taxonomic families are similar. Species within the same family may have distinct differences in many facets of their immune systems (for example, rats and mice).[6]

The enrichment protocols described in this study provided an effective method for obtaining Ig from the species in question for the purposes of this study. The Ig concentrations obtained were sufficient to assess the capacity of protein G to serve as a serum ELISA antibody conjugate. Although these findings support the use of protein G–based diagnostics for various nondo-

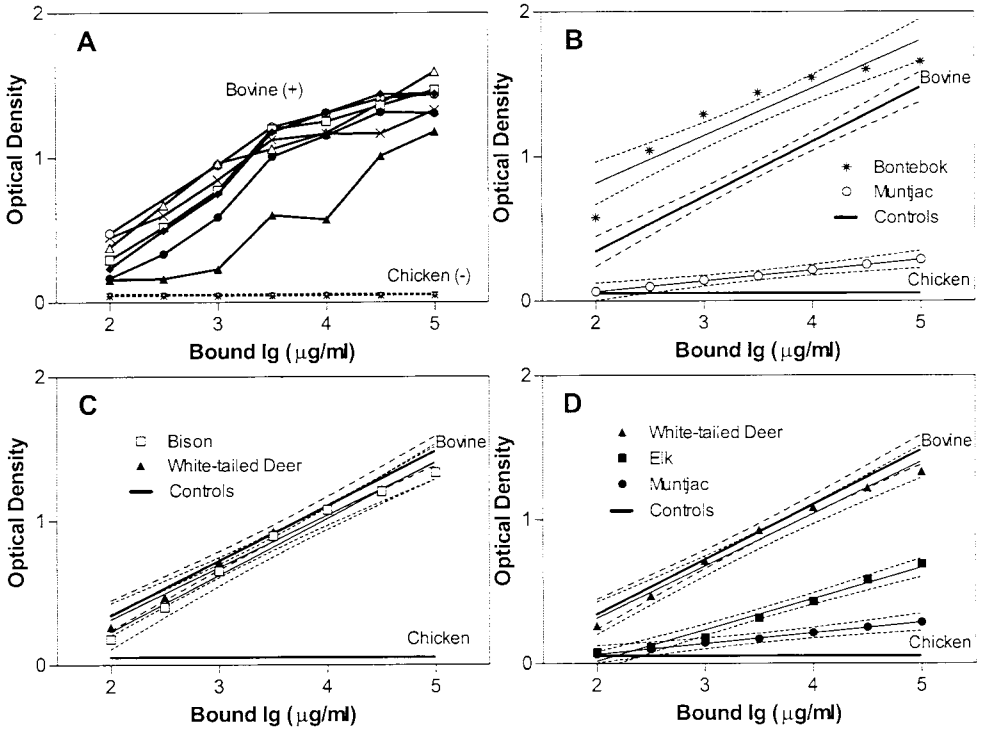

Figure 4. Binding curves illustrate binding of protein G to Ig from various species. A uniform concentration of protein G was added to microtiter wells coated with enriched artiodactyl Ig (2–5 μg/ml). Regressed lines represent 6–8 individual animals. **A**, positive and negative control species, bovine (n = 7) and chicken (n = 8). **B, C**, binding curves from representative species illustrating similarity to and difference from control species. Bontebok binding curve (r^2 = 0.625) had a higher intercept than the positive bovine control, muntjac (r^2 = 0.269) had the lowest slope of all species (**B**). **C**, two of 7 species that were statistically similar to the bovine control (bison r^2 = 0.712, white-tailed deer r^2 = 0.711). **D**, binding variation among 3 species from family Cervidae (muntjac, r^2 = 0.269; elk, r^2 = 0.729; white-tailed deer, r^2 = 0.711). Bovine r^2 = 0.792, chicken r^2 = 0.007. Confidence levels of 95% are illustrated.

mestic species, the interspecies variation noted confirms the need to establish assay interpretation criteria that may in fact need to be developed on a per species basis.

Because ion-exchange chromatography separates proteins on the basis of their net charge,[11] elution time and separation are ultimately dependent on the isoelectric point (pI) of the Ig. The pI naturally varies among proteins, and by species, and will therefore affect Ig recovery. The relationship between buffer pH and Ig pI is critical to maximizing separation of proteins. Binding affinity to BAKERBOND Abx™ has been shown to vary among species; thus slightly different chromatographic conditions may be needed for optimal Ig recovery from each species.[22] Few efforts to purify, quantify, or characterize Ig in artiodactyl species have previously been reported. The presence and relative amounts of Ig isotypes, subclass profiles, Ig structures, and pIs of most artiodactylids also have not been extensively characterized. It is likely that greater Ig yield and purity could have been achieved if enrichment protocols had been optimized for each study species. The goal of this study, however, was not to maximize Ig recovery in each artiodactyl species but to compare the effectiveness of a standardized protocol across multiple species and obtain sufficient Ig to assess protein G binding. The enrichment process described in this study, however, does provide the groundwork for future research to determine the optimal buffers, concentrations, and pH needed for maximal Ig recovery. The 15 kD bands found in various samples may represent the J-chain of IgM or possibly IgA.[29] Similarly, proteins of high molecular weight (>300 kD) observed on SEC may represent intact IgM (900 kD)[8] or IgA (up to 600 kD).[30] It should be noted, however, that the methodologies used for verification of Ig presence in purified peaks (SDS-PAGE and SEC) have inherent limitations. The molecular weight of predominant proteins alone may not provide positive identification of Ig. Definitive identification would require assays using species-specific antisera, which are unavailable for the majority of these artiodactyl species.

A number of factors may limit binding of protein G to Ig. They include the lack of, or variation in, the Fc region of Ig necessary for protein G binding, insufficient levels of total Ig or IgG in the original serum sample (e.g., an immunocompromised animal), or the presence of binding inhibitors. Use of enriched Ig from

serum with normal protein and globulin levels eliminated most of these factors in the present study.

Binding assay results may have been affected by the dependence of binding on buffer pH. Optimal binding of Ig to polystyrene and protein G may occur at different pHs in different species.[2] Because only 1 pH prevailed for the coating of the microtiter plates and protein G–binding assays, binding conditions may not have been uniformly optimized for each species.

Similarities exist between protein G binding to Ig of many of the species tested and the bovine positive control. This information may provide a starting point for ELISA development in these species. However, for those species with protein G–binding patterns that are statistically different from the bovine control, additional adjustment in ELISA protocols (e.g., serum or conjugate dilutions and interpretive cutoff values) may be necessary. Variability in protein G binding to Ig among species also may affect interpretation of the final result in a multispecies assay. Finally, because protein G selectively binds IgG versus other isotypes, an immune response predominated by another Ig isotype would not be detected in an assay using this protein G conjugate system.

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Sources and manufacturers

a. VITROS® 250 Chemistry System, Ortho-Clinical Diagnostics, Raritan, NJ.
b. Sigma–Aldrich Chemical Co., St. Louis, MO.
c. J. T. Baker Inc., Phillipsburg, NJ.
d. Beckman Instruments, Inc., Fullerton, CA.
e. Centriprep®-50, Amicon Inc., Beverly, MA.
f. Pierce Chemical Company, Rockford, IL.
g. Bio-Rad Laboratories, Hercules, CA.
h. Eastman Kodak Company, Rochester, NY.
i. MaxiSorp, Nalge Nunc International, Naperville, IL.
j. Mini-wash, Skatron Instruments, Inc., Sterling, VA.
k. IDEXX Laboratories Inc., Westbrook, ME.
l. Bio-Tek EL312, Bio-Tek Instruments, Inc. Winooski, VT.
m. SAS Inc., Cary, NC.

References

1. Agnese ST, Spierto FW, Hannon WH: 1983. Evaluation of four reagents for delipidation of serum. Clin Biochem 16:98–100.
2. Akerstrom B, Bjorck L: 1986. A physicochemical study of protein G, a molecule with unique immunoglobulin G-binding properties. J Biol Chem 261:10240–10247.
3. Akerstrom B, Brodin T, Reis K, Bjorck L: 1985. Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. J Immunol 135:2589–2592.
4. Baumgartner W, Pernthaner A: 1994. Influence of age, season, and pregnancy upon blood parameters in Austrian Karakul sheep. Small Ruminant Res 13:147–151.
5. Bjorck L, Kronvall G: 1984. Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. J Immunol 133:969–974.
6. Butler JE: 1986. Biochemistry and biology of ruminant immunoglobulins. *In:* Progress in veterinary microbiology and immunology, ed. Pandey R, pp. 1–53. Karger, New York, NY.
7. Christensen P, Johansson BG, Kronvall G: 1976. Interaction of streptococci with the Fc fragment of IgG. Acta Pathol Microbiol Scand [C] 84:73–76.
8. Dorrington KJ, Mihaesco C: 1970. Subunit structure of human γM-globulins. Immunochemistry 7:651–660.
9. Eliasson M, Andersson R, Olsson A, et al.: 1989. Differential IgG-binding characteristics of staphylococcal protein A, streptococcal protein G, and a chimeric protein AG. J Immunol 142: 575–581.
10. Eliasson M, Olsson A, Palmcrantz E, et al.: 1988. Chimeric IgG-binding receptors engineered from staphylococcal protein A and streptococcal protein G. J Biol Chem 263:4323–4327.
11. Fahey JL, Terry EW: 1978. Ion exchange chromatography and gel filtration. *In:* Immunochemistry, ed. Weir DM, pp. 8.1–8.16. JB Lippincott Company, Philadelphia, PA.
12. Fahnestock SR: 1987. Cloned streptococcal protein G genes. Trends Biotechnol 5:79–83.
13. Goward CR, Murphy P, Atkinson T, Barstow DA: 1990. Expression and purification of a truncated recombinant streptococcal protein G. Biochem J 267:171–177.
14. Guss B, Eliasson M, Olsson A, et al.: 1986. Structure of the IgG-binding regions of streptococcal protein G. EMBO J 5: 1567–1575.
15. Kato K, Lian LY, Barsukov IL, et al.: 1995. Model for the complex between protein G and an antibody Fc fragment in solution. Structure 3:79–85.
16. Kelly PJ, Tagwira M, Matthewman L, et al.: 1993. Reactions of sera from laboratory, domestic and wild animals in Africa with protein A and a recombinant chimeric protein AG. Comp Immunol Microbiol Infect Dis 16:299–305.
17. Kie JG, White M, Drawe DL: 1983. Condition parameters of white-tailed deer in Texas. J Wildl Manag 47:583–594.
18. Kronvall G, Seal US, Finstad J, Williams RC Jr: 1970. Phylogenetic insight into evolution of mammalian Fc fragment of gamma G globulin using staphylococcal protein A. J Immunol 104:140–147.
19. Mestecky J, Zikan J, Butler W: 1971. Immunoglobulin M and secretory IgA: evidence for a common polypeptide chain different from light chains. Science 171:1163–1165.
20. Myhre EB, Kronvall G: 1981. Specific binding of bovine, ovine, caprine and equine IgG subclasses to defined types of immunoglobulin receptors in gram-positive cocci. Comp Immunol Microbiol Infect Dis 4:317–328.
21. Nau DR: 1987. ABx: a novel chromatographic matrix for the purification of antibodies. *In:* Commercial production of monoclonal antibodies: a guide for scaling-up antibody production, ed. Seaver S, pp. 247–275. Marcel Dekker, New York, NY.
22. Nau DR: 1989. Optimization and scale-up of linear and step gradient elution techniques for preparative antibody purification on a mixed-mode matrix. BioChrom 4:266–278.
23. Page M, Thorpe R: 1985. IgG purification. *In:* Immunochemical

protocols. ed. Pound JD. pp. 95–111. Humana Press Totowa. NJ.
24. Peng Z. Becker AB. Simons FE: 1994. Binding properties of protein A and protein G for human IgE. Int Arch Allergy Immunol 104:204–206.
25. Phillips TM: 1992. Ion-exchange chromatography. *In:* Analytical techniques in immunochemistry. ed. Phillips TM. pp. 22–39. Marcel Dekker. New York. NY.
26. Ratcliffe MJH: 1996. Chicken immunoglobulin isotypes and allotypes. *In:* Weir's handbook of experimental immunology. ed. Weir DM. pp. 24.1–24.7. Blackwell Science. Cambridge. MA.
27. Ritchie BW. Harrison GJ. Harrison LR: 1997. Biochemistries. *In*: Avian medicine: principles and applications. ed. Ritchie BW. Harrison GJ. Harrison LR. p. 236. Wingers Publishing. Lake Worth. FL.
28. Rose NR. de Macario EC. Folds JD. et al.: 1997. Electrophoretic and immunochemical characterization of immunoglobulins. *In*: Manual of clinical laboratory immunology. ed. Rose NR. de Macario EC. Folds JD. et al.. p. 112. ASM Press. Washington. DC.
29. Schrohenloher RE. Mestecky J. Stanton TH: 1973. Molecular weight of a human J chain. Biochim Biophys Acta 295:576–581.
30. Schubert D: 1970. Carbohydrate attachment to immunoglobulin subunits. J Mol Biol 51:287–301.
31. Teare A: 1996. Electrophoretic and immunochemical characterization of immunoglobulins. *In*: ISIS physiological data reference values. ed. Teare S. pp. 1–374. American Association of Zoo Veterinarians and International Species Information System. Media. PA.
32. Walters NJ: 1996. Basic immunology and serology. *In*: Basic medical laboratory techniques. ed. Walters NJ. Estridge BH. Reynolds AP. p. 419. Delmar Publishers. Albany. NY.