Development of conjugated secondary antibodies for wildlife disease surveillance

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Abstract

Disease monitoring in free-ranging wildlife is a challenge and often relies on passive surveillance. Alternatively, proactive surveillance that relies on the detection of specific antibodies could give more reliable and timely insight into disease presence and prevalence in a population, especially if the evidence of disease occurs below detection thresholds for passive surveillance. Primary binding assays, like the indirect ELISA for antibody detection in wildlife are hampered by a lack of species-specific conjugates. In this study, we developed anti-kudu and anti-impala immunoglobulin-specific conjugates in chickens and compared them to the binding of commercially available protein-G and protein-AG conjugates, using an ELISA-based avidity index. The conjugates were evaluated for cross-reaction with sera from other wild herbivores to assess future use in ELISAs.

The developed conjugates had a high relative avidity of > 70% against kudu and impala sera. The commercial conjugates (protein-G and protein-AG) had significantly low relative avidity (< 20%) against these species. Eighteen other wildlife species demonstrated cross-reactivity with a mean relative avidity of > 50% with the impala and kudu conjugates and < 40% with the commercial conjugates.

These results demonstrate that species-specific conjugates are important tools for the development and validation of immunoassays in wildlife, and for the surveillance of zoonotic agents along the livestock-wildlife-human interface.

Keywords: Wildlife species, Adaptive immunity, Avidity, Conjugates, Diagnostics, Enzyme-linked immunosorbent Assay (ELISA), Disease surveillance, Serology

Introduction

With the current increase in emerging and re-emerging disease outbreaks of both veterinary and human importance, there has been an urgent need for evidence-based methods for measuring both infection incidence and prevalence (Lambert et al., 2022). Several techniques and interventions have been employed to mitigate the debilitating effects of disease-causing organisms on livestock and wildlife. However, what determines the choice of intervention to be implemented is the knowledge of the epidemiology governing or influencing these diseases (Artois et al., 2009). Wild animals are known to be hosts and/or reservoirs for pathogens that are of concern for cross-species transmission risk to humans and livestock. Therefore, an understanding of the epidemiology and ecology of pathogens in wildlife will better inform policies and interventions for control. Passive surveillance is currently used in most wildlife settings and is largely dependent on the detection of clinical cases or case mortalities. However, opportunistic collection of mortality data and biases in the detection of carcasses and clinical signs can lead to a distortion of the true incidence, therefore, more active form of surveillance is needed (Garnier et al., 2017). The detection of antibodies against pathogens can provide insights into prior exposure as well as information on the prevalence of a pathogen in an environment and the risk of pathogen spill over (Gardner et al., 1996; Garnier et al., 2017). This approach may be especially useful for diseases with a short infection period like anthrax or those that do not cause mortality like brucellosis.

Several serological techniques have been used to detect exposure to pathogens in African wildlife. These include primary binding assays like the enzyme-linked immunosorbent assay (ELISA) (Kock et al., 1992; Tiziana Lembo et al., 2011; Turnbull et al., 1992a) as well as more historic secondary binding assays like the agar gel immunodiffusion (AGID) test and complement fixation test (CFT)(Blackburn & Swanepoel, 1988). Assays like the indirect ELISA can be highly sensitive and specific for the detection of pathogen specific antibodies in the serum of a host, but they rely on a host-specific enzyme conjugate that limits the cross-species use of the assay. Most commercially available indirect ELISA kits are only validated for use in domestic ruminants. The enzyme-linked detection technique involves a highly specific antigen-antibody (Ag-Ab) interaction and was developed by Engvall and Perlmann (Engvall & Perlmann, 1971). First, an antigen is restricted on a firm surface of a plate, followed by the addition of the sample antibody (if present) which then binds with a secondary antibody that is linked to an enzyme; next, this conjugated enzyme reaction is measured by incubating with a chromogen substrate (ThermoFisher Scientific 2019). Horseradish peroxidase and alkaline phosphatase are the most used enzymes conjugated with secondary antibodies (Payment & Descoteaux, 1978; Rennard et al., 1980; Voller

et al., 1974). These conjugates in a simpler sense, refer to an anti-species immunoglobulin that is linked to an enzyme that facilitates the detection through colour visualization. The ability to use conjugates of high avidity and specificity is therefore very important in measuring immune response through the use of ELISA (Smit, 2017). The interaction and bond that exist between an antibody and an antigen is one that is quite robust. The ability to be reversed and the strength of this bond are often dependent on the nature of the force that exists which could be electrostatic, van der Waals' or hydrogen (van Oss et al., 1987). Some of the binding forces are negatively associated with distance and this makes them highly reliant on how well the molecules bind at the binding site (BioRad, 2021). It is known that the measure of strength (affinity) of hapten-antibody binding (specific binding site) determines how well an antigen binds with an antibody (Hudson et al., 1989). Avidity on the other hand is the total and cross-dependent binding strength of all the binding sites of an antibody to the multivalent antigen (Hudson et al., 1989). It is therefore important to develop secondary antibodies that are of both high affinity and avidity. Species-specific conjugates for wildlife are often not available and the generic conjugates that are used in these assays can vary significantly in binding to wildlife antibodies and results from these unvalidated assays should always be interpreted with caution(Kelly et al., 1993; Kramsky et al., 2003; Pruvot et al., 2013; Stöbel et al., 2002b).

Antibody avidity can be evaluated by means of ELISA in the presence of an immune-complex disruptive or disassociating compound like a chaotropic agent (Dauner et al., 2012; Dimitrov et al., 2011; Hedman & Seppälä, 1988; Hudson et al., 1989; MacDonald et al., 1988; Westerlund et al., 2005). The thiocyanates can impact electrostatic interactions owing to their ionic characteristics making them more widely acceptable (Almanzar et al., 2013; Smit, 2017). There are a few reports about the use of different diluents for the chaotrope, including phosphate buffered saline (PBS) (Dimitrov et al., 2011; Ferreira & Katzin, 1995) and PBS+Tween (Dauner et al., 2012; Smit, 2017).

The paucity of studies around the use of ELISA for surveillance of wildlife diseases is perhaps due to the lack or scarcity of species-specific conjugated secondary antibodies. There are various studies around the use of non-species-specific commercial conjugates such as protein A (protA), protein G (protG) and protein AG (pAG) for wildlife serological studies (Feir et al., 1993; Kelly et al., 1993; Kramsky et al., 2003; Smit, 2017; Stöbel et al., 2002b) (Table S1). Some commercial conjugates are available for domestic species (BioRad, 2021) and some wildlife species, predominantly those from Europe (Rossi et al., 2014). The variation in binding affinity for the commercial conjugates among various hosts show that developing species-specific conjugates could be important to improving wildlife disease surveillance. Furthermore, the different methods

used in these studies and differences in data interpretation further complicate the synthesis of the results. Thus, it is important to develop conjugates that are specific to African wildlife and not entirely rely on commercial multispecies conjugates.

Because wildlife hosts of pathogens of both veterinary and zoonotic importance are quite diverse globally, manufacturing species-specific conjugates for all host species seems impracticable; however, developing these for a few common hosts could improve disease surveillance efforts. In this study, we developed species-specific conjugates for kudu and impala respectively. These two species have been implicated as hosts for diseases like brucellosis (Godfroid, 2017; Simpson et al., 2021), anthrax(Lizanne Basson, Ayesha Hassim, At Dekker, Allison Gilbert, Wolfgang Beyer, Jennifer Rossouw, & Henriette van Heerden, 2018; De-Vos, 1990) and foot and mouth disease (Letshwenyo et al., 2006; Vosloo et al., 2005; Wittmann, 1990). We evaluated the binding avidity of these conjugates to several wildlife species and compared them to commercially available conjugates. We addressed the following questions: (1) do developed novel species-specific conjugates for kudu and impala have better avidity than the commercial conjugates? (2) do these developed conjugates perform better across a range of related wildlife species? The validation of ELISA assays using conjugates specifically developed for pathogen detection in wildlife, rather than commercially available conjugates, is critical for improving wildlife disease surveillance and research.

Materials and Methods

Experimental design and samples

Species-specific immunoglobulin conjugates for kudu and impala were developed by vaccinating Highland brown, Specific Pathogen Free (SPF) chickens (Avi-farms, Centurion, South Africa) with immunoglobulin (Ig) from kudu and impala (4 animals per species), respectively. Anti-species immunoglobulin Y (IgY) were purified from egg yolks and conjugated to horseradish peroxidase. Cross-reactivity and avidity of the new conjugates were evaluated and compared to commercially available protein G (protG) and protein AG (pAG) conjugates using different herbivore species by means of an ELISA-based avidity index (AI). Serum samples from a variety of species (10 samples per species Table 1) were collected from South African National Parks (SANParks) biobanks and from samples banked in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa. Species were classified into subfamilies and tribes based on Hassanin and Emmanuel (1999) and Gatesy et al. (1997) to give an indication of phylogenetic relatedness. Goat, sheep, and cattle samples were also included. Animal and research

ethics from the University of Pretoria was obtained (REC063-19, REC041-19) and permits were obtained from the Department of Agriculture, Land Reform and Rural Development.

Table 1: List of species used for avidity and cross-reactivity tests. Species subfamily and tribe are as described by Hassanin and Emmanuel (1999) and Gatesy et al. (1997)

Common Name	Species	Subfamily	Tribe
Greater kudu	*Tragelaphus strepsiceros*	Bovinae	Tragelaphini
Impala	*Aepyceros melampus*	Antilopinae	Aepycerotini
Burchell's zebra	*Equus quagga burchellii*	Equinae	Equini
Black wildebeest	*Connochaetes gnou*	Alcelaphinae	Alcelaphini
African buffalo	*Syncerus caffer*	Bovinae	Bovini
Giraffe	*Giraffa camelopardalis*	Giraffinae	Giraffini
Blue wildebeest	*Connochaetes taurinus*	Alcelaphinae	Alcelaphini
Nyala	*Tragelaphus angasii*	Bovinae	Tragelaphini
Sable antelope	*Hippotragus niger*	Antilopinae	Hippotragini
Waterbuck	*Kobus ellipsiprymnus*	Hippotraginae	Hippotragini
Gemsbok	*Oryx gazella*	Antilopinae	Hippotragini
Springbok	*Antidorcas marsupialis*	Antilopinae	Antilopini
Hartebeest	*Alcelaphus buselaphus*	Antilopinae	Alcelaphini
Roan antelope	*Hippotragus equinus*	Antilopinae	Hippotragini
Common eland	*Taurotragus oryx*	Bovinae	Tragelaphini
Common tsessebe	*Damaliscus lunatus*	Antilopinae	Alcelaphini
Blesbok	*Damaliscus pygargus phillipsi*	Antilopinae	Alcelaphini
Bushbuck	*Tragelaphus scriptus*	Bovinae	Tragelaphini
Bontebok	*Damaliscus pygargus*	Antilopinae	Alcelaphini
Goat	*Capra hircus*	Caprinae	Caprini
Sheep	*Ovis aries*	Caprinae	Caprini
Domestic cattle	*Bos taurus*	Bovinae	Bovini

Precipitation of kudu and impala immunoglobulins

Immunoglobulin was extracted from kudu and impala by ammonium sulphate precipitation using the method described by Staak et al. (2001). Briefly, respective sera were diluted 1:4 with PBS (total

volume 80 ml), while constantly stirring, 40 ml of saturated ammonium sulphate (Merck, Darmstadt, Germany) was slowly added to achieve a 33% saturation and the pH of the suspension was adjusted to 7.8 using a 2N NaOH (Associated Chemical Enterprises, Johannesburg, South Africa). The suspension was stirred continuously for 3 hours on a magnetic stirrer (Bibby Sterilin LTD, Staffordshire, England) and then centrifuged at room temperature for 30 minutes at 1400 × g using Eppendorf centrifuge 5810R (Eppendorf, Hamburg, Germany) and the supernatant was discarded. The pellet was resuspended to a total volume of 80 ml in PBS and further purified by two additional cycles of precipitations, as described above. The final precipitate was dissolved in PBS in a volume half of the initial serum sample. Ammonium sulphate was removed by desalting spin columns (Thermo Scientific, Rockford, USA). IgG heavy and light chains were confirmed by SDS-PAGE gel electrophoresis (Figure 1).

The total protein concentration of the precipitated immunoglobulins (Ig) was determined using the spectrophotometer (Xpose™ Trinean Spectrophotometer, Trinean, Burladingen, Germany). The SDS-PAGE gel electrophoresis was performed as described by Laemmli (1970) with a few modifications. Samples were diluted with the protein solvent buffer to a final concentration of 2 µg/µl. To determine the molecular size of the Ig, the protein was loaded into the wells of the SDS-PAGE at a concentration of 2 µg/µl. Samples were placed in Eppendorf tubes and put into digital dry bath (Labnet Accublock Digital Dry Bath, Labnet International Inc, Woodbridge, USA) for 10 minutes at 100 $^{\circ}$C after which they were spun using the mini centrifuge (Wealtec E-centrifuge, Wealtec corporation, Sparks, USA) for 10 seconds at 1400 × g. Gel reagents were mixed in volumes indicated in Table S2 and the solution was added between the clamped glass slides. The gel was allowed to polymerize for 30 minutes and then the stacking gel (Table S2) was added and incubated for 30 minutes. The gel was run at 100 V for 2 hours after which it was stained with blue stain (GelCodeTM Blue stain, Themo Scientific^{TM}, Massachusetts, USA). After washing steps, the gel was viewed on the transilluminator (Univetec Cambridge transilluminator, Univetec, Cambridge, UK) for the presence of bands. Subsequently, the gel was transferred to the molecular image gel document system (Bio-rad molecular image gel document system, Bio-rad, California, USA) using the Image Lab software for analysis.

Immunisation of chickens and extraction of IgY from eggs

Preparation of vaccines for immunizing chickens and extraction of IgY from egg yolk was adapted with modifications from Staak et al. (2001). Preparations of purified Ig from kudu and impala were made up to 200 µg/ml (w/v) in PBS. One ml of vaccine (100 µg/ml) was prepared by emulsifying

equal volumes (0.5 ml) of protein and Montanide ISA 50 V 2 adjuvant (SEPPIC, Paris, France) and injected into both sides of the breast muscles. Inoculation was performed on Days 0, 23 and 42(Figure S1). During this period development of specific IgY was monitored by testing the yolks in an ELISA (see antibody tires and method in Supplementary methodology 1 and Figure S2).

Egg yolks representing peak levels of anti-kudu or anti-impala IgY were harvested by separating the yolk from the albumin and diluting the yolk to 1:5 in distilled water before freezing at -20 ºC for 72 hours. The suspension was thawed slowly at 4 °C and centrifuged at 2800 x g for 20 minutes and the supernatant was collected. Ammonium sulphate was added in a concentration of 0.27g per ml of the supernatant and stirred for two hours at room temperature. Afterwards, it was centrifuged at 2800 x g for 20 minutes and the supernatant was discarded. The pellet was resuspended in 24 ml of 2 M ammonium sulphate per egg yolk and stirred for 40 minutes at room temperature, this was followed by centrifugation as before. The precipitate was resuspended in 2.5 ml of PBS for each egg yolk and dialysed against PBS at 4 °C for 48 hours. Finally, the concentration of the immunoglobulin solution was measured and stored at -20 °C (Figure S1).

Affinity chromatography using the polystyrene granulate method as described by Staak et al. (2001) was used to further purify the recovered IgY. Briefly, 150mg of impala and kudu IgG were immobilised separately on the granulated polystyrene using 0.05M carbonate buffer (pH 9.6) and free binding sites on the matrix were blocked using the blocking buffer (PBS; 0.005% Tween 20, PBST). Subsequently, the packed columns were equilibrated using PBST and the chicken IgY were run through the columns using very slow rates to allow for optimal binding. Specific IgY were eluted by means of a glycine/hydrochloric acid elution buffer with a pH of 2.5. The affinity purified IgY were used for the final conjugation.

A western blot was used to confirm the specificity of IgY produced against the respective Ig of kudu and impala. The western blot was performed as described by Howell et al. (2002). The western blots were performed before and after affinity purification.

Horseradish peroxidase conjugation to IgY

The periodate method as described by Wilson and Nakane (1978) and adapted by Staak et al. (2001) was used to conjugate the horseradish peroxidase (HRPO) to IgY. The activity of the conjugate was tested using a checkerboard titration between the kudo or impala serum respectively (Supplementary methodology 2 and Figure S2, Figure 1).

Avidity index for cross-reactions between different conjugates and wildlife sera

The respective AIs for the binding of anti-kudu IgY and anti-impala IgY conjugates to kudu and impala sera as well as to the sera of the species listed in Table 1 were compared. The binding of all the sera to protG- and pAG conjugates were also compared as described by Smit (2017)

Briefly, a direct ELISA was employed by coating each microtiter plate (Thermo Scientific™ Pierce 96-well Plates-Corner, USA) with 10 sera samples per species at a dilution of 1:2000. Each plate was coated by adding 50 µl of the serum diluted in PBS in rows A-D of columns 1-10 for the 10 individual animals of the same species. Rows E-H of columns 1-10 were similarly filled with 50 µl of the next 10 sera of the second species. Columns 11 and 12 were filled with 50 µl of the control serum (kudu serum for anti-kudu conjugates, impala serum for anti-impala conjugates, cattle serum for pAG (Inoshima et al., 1999; Smit, 2017) and goat serum for protG (ThermoFisher, 2023)) at a concentration of 1:2000. Following incubation at 37 °C for 1 hour on an orbital shaker, the plates were washed twice with PBS supplemented with 0.05% Tween-20 (PBST; Thermo Fisher Scientific, Waltham, MA USA) using a plate washer (Bio-Rad PW40, Mamesla-Coquette, France). Subsequently, all wells were loaded with PBST supplemented with 5% skimmed milk powder as a blocking step for 30 minutes at 37 °C and afterwards, the wells were washed twice. The conjugates were diluted with PBSTM at a final concentration of 1:400 (as determined in Supplementary methodology 2 and Figure S2) for anti-kudu IgY and anti-impala IgY HRPO, 1:10000 for protein A/G and protein G as prescribed by the manufacturer. Afterwards, 50 µl of PBS was added into the wells of rows A, B, E and F, and rows C, D, G and H were loaded with potassium thiocyanate as a chaotropic agent (CT) at a final concentration of 0,25 M. The plates were incubated for 1 hour at 37 °C on the shaker and followed by a wash step. Colour was developed by the addition of the ABTS substrate (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; Thermo Scientific 1-Step ABTS, USA) and incubated in the dark for 30 minutes. The absorbance was read at 405 nm using the plate reader (Biotek Powerwave XS2,Vermont, USA) (Figure 1). The avidity between the conjugate and serum was calculated as the reduction in colour between wells without CT and those with CT and presented as the AI for each serum. AI was calculated as the mean ELISA absorbance values (ODs) from wells treated with the dissociating chaotrope (NH_4SCN) divided by the mean ODs from wells without chaotrope and multiplied by 100.

Statistical analysis

To present differences between the developed species-specific conjugates for kudu and impala and the commercial conjugates, we calculated the mean, standard deviation for kudu and impala. A t-

test was performed to measure the differences in the means of the ODs and AI for both the test samples and the controls. The AI was defined as the ratio of both the OD of the CT-treated wells and the PBS-treated wells; the AI was calculated for each species and conjugate. The AI values for all species were normalised by subtracting them from the AI of their corresponding controls in order to measure how they differed from the respective control. A multivariate generalised linear model coupled with the Tukey's Honestly Significance Difference (HSD) test for multiple mean comparisons was performed to compare the relationships between the AIs of the conjugates for the subfamily and tribes of the different species. The predictor variables included an interaction between conjugates and the subfamily and also between conjugates and tribes while the response variable was proportion (0-1) of the AI. All statistical analyses were done in R Console version 3.2.1 (R Core Team, 2017) with significance assessed when alpha was <0.05.

Results

Ammonium sulphate precipitation of IgG from Kudu and Impala Sera

The SDS-PAGE analysis confirmed the presence of two protein bands with molecular weights of around 50 and 25 KDa (for both kudu and impala) representing the heavy and light chains of IgG. (Figure 1A).

Western Blot

The western blot analysis confirmed the specificity of the IgY against the IgG of impala (Figure 1B) and kudu (Figure 1C). Figures 1B and C (before affinity chromatography) and 2C (after affinity chromatography) show the specificity of the immunoglobulins produced. Only binding to the 50 KDa heavy chain was observed to confirm the specificity of the secondary antibodies.

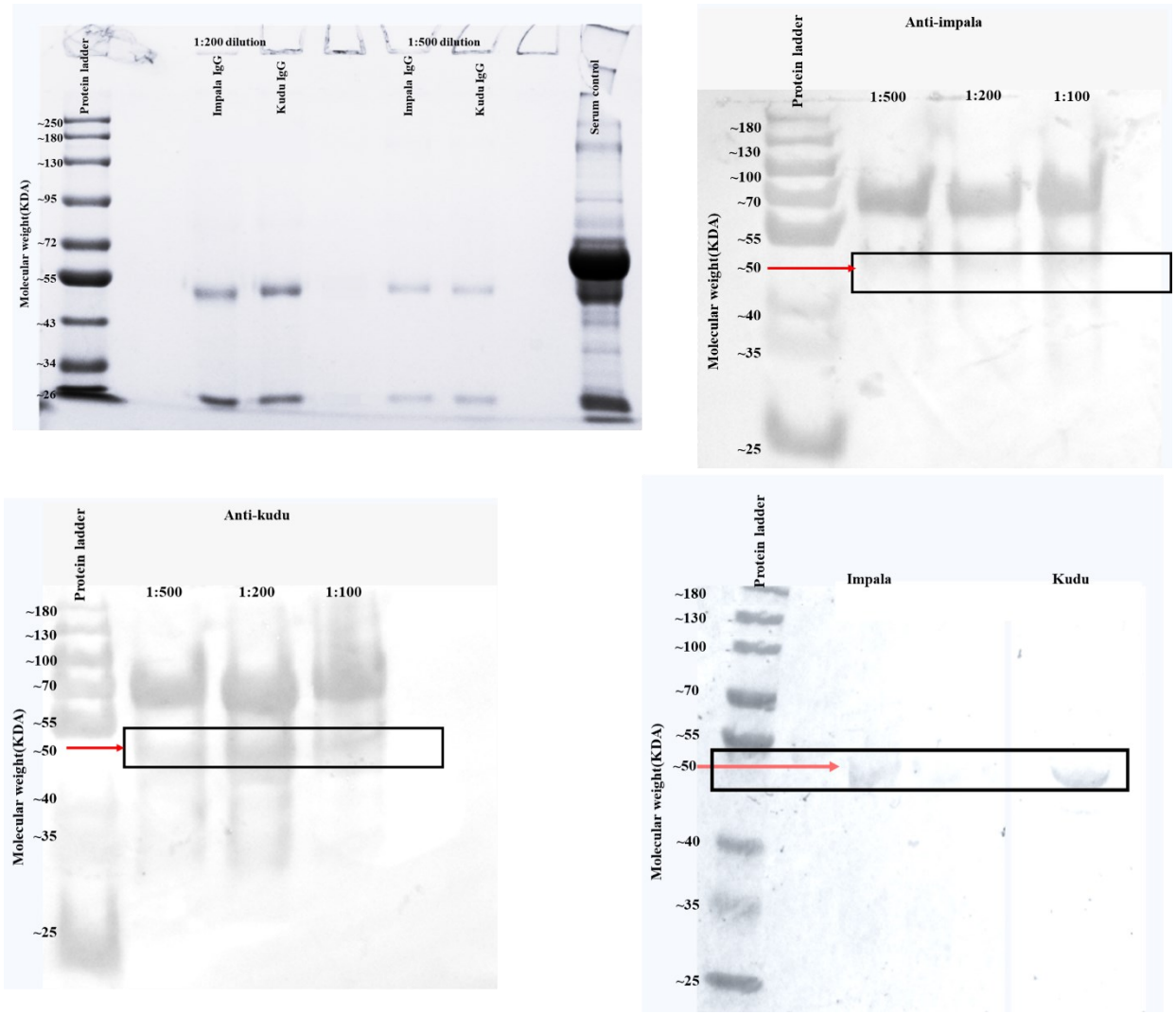


Figure 1: (A) Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) image from the ammonium sulphate precipitated immunoglobulin fractions from kudu and impala sera. The protein bands at 50 and 25 KDa correspond to the heavy and light chains of IgG. Kudu serum was used as the serum control. (B) Western blot image indicating the binding of impala immunoglobulin G (IgG) to the chicken anti-impala IgY directly from the ammonium sulphate precipitated egg yolk without affinity chromatography before conjugation. (C) Western

blot image indicating the binding of kudu IgG to the chicken anti-kudu IgY directly from the ammonium sulphate precipitated egg yolk without affinity chromatography before conjugation. (D) Western blot image showing binding of impala (left) and kudu (right) IgG against the corresponding chicken affinity-purified IgY before conjugation. Red arrows with solid rectangles highlight the molecular weight of interest.

Binding activities of anti-kudu IgY, anti-impala IgY and commercial conjugates on kudu and impala sera

Kudu and impala sera bound significantly better with their respective conjugates compared to the commercial conjugates (p<0.0001). There was also a significant drop in optical densities for the commercial conjugates in the presence of the chaotrope (p<0.0001) but not the developed conjugates (p>0.05; Figure 2). For the anti-kudu IgY conjugate on kudu serum, the mean AI was 72.36 ± 1.13 SD compared to 15.23 ± 1.1 SD for pAG and 23.61 ± 0.99 SD for protG. For anti-impala IgY conjugate on impala serum the mean AI was 72.09 ± 0.89 SD, compared to 21.47 ± 0.66 SD for pAG and 23.52 ± 0.56 SD for protG..

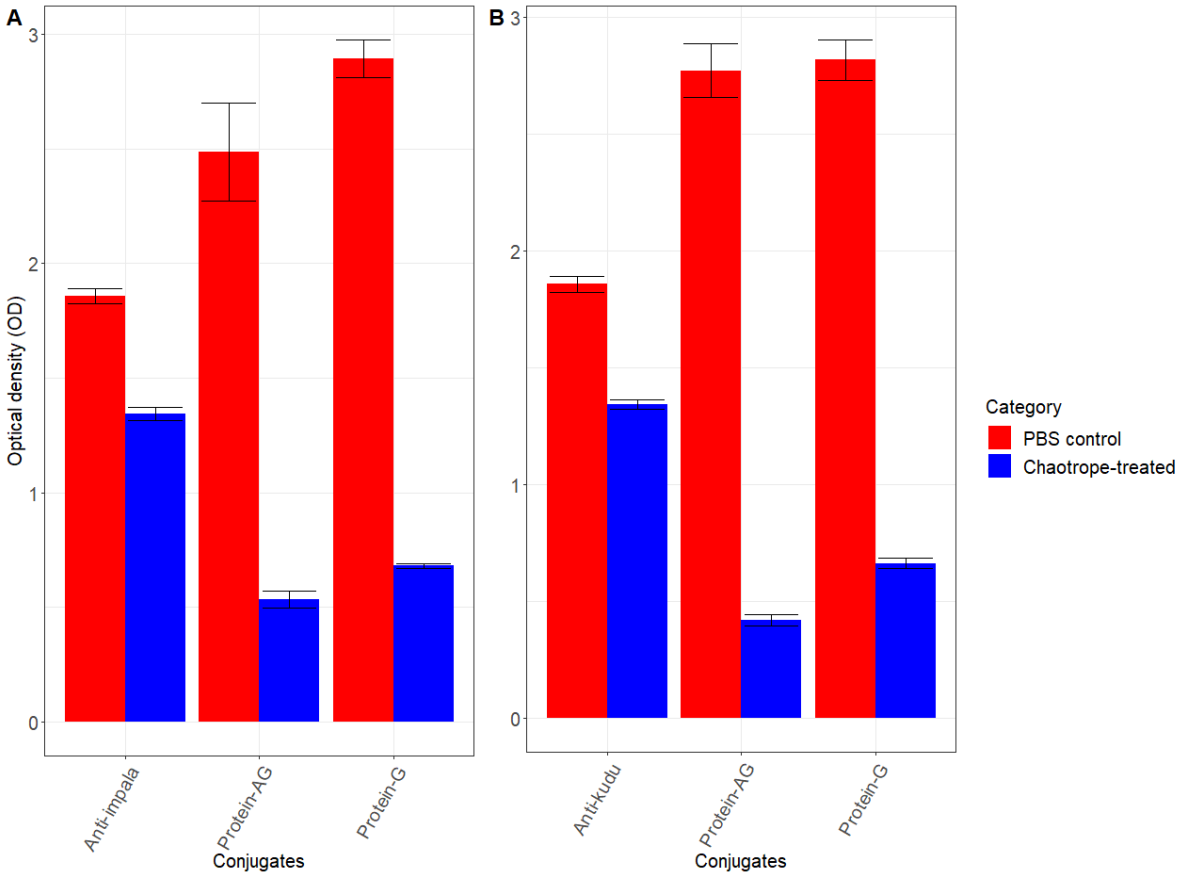


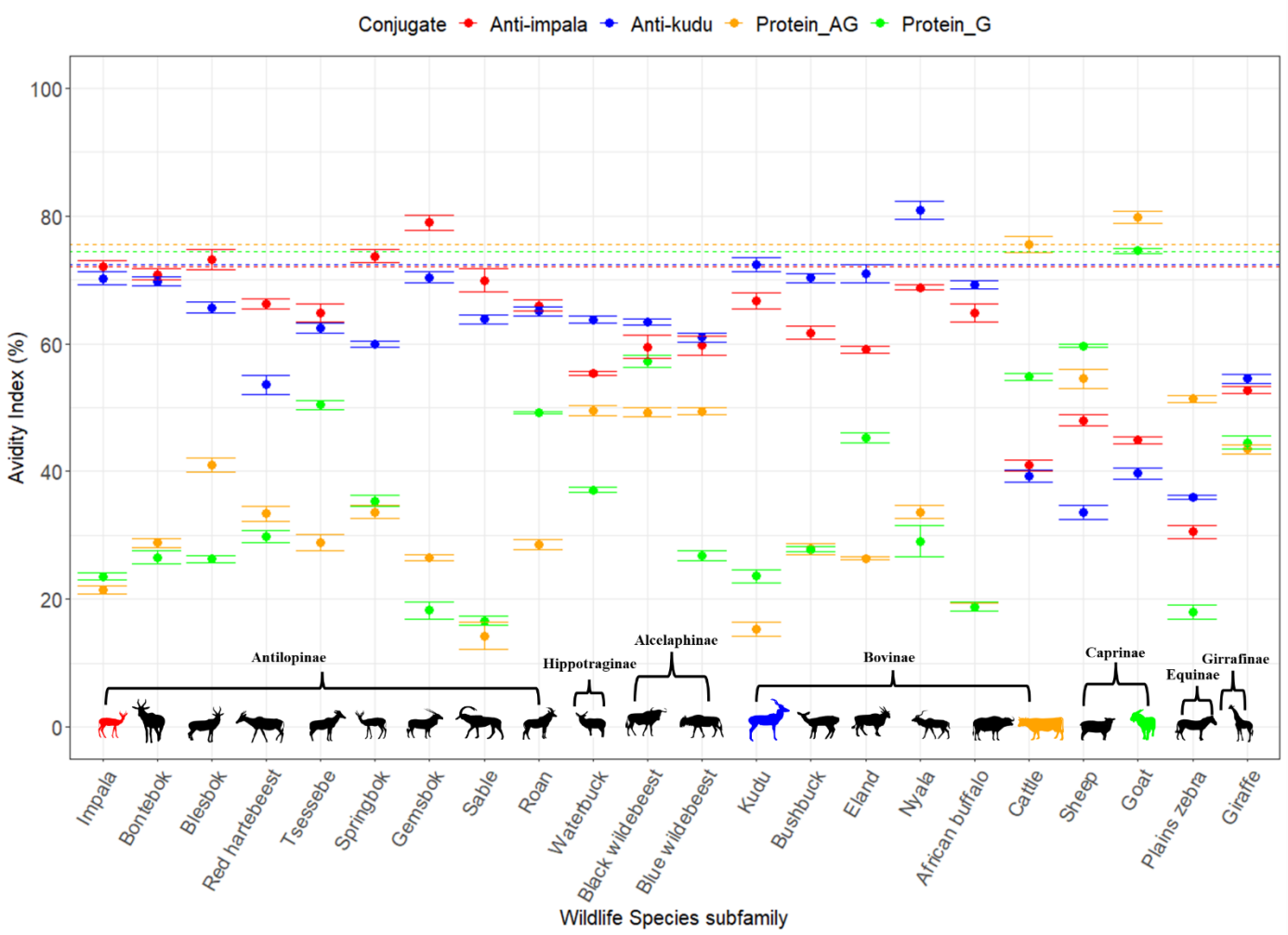
Figure 2: Bar charts with error bars (standard deviation) showing the differences in mean optical densities (OD) for the developed and commercial conjugates, A) impala sera against anti-impala IgY, protein AG and protein G conjugates, and B) kudu sera against anti-kudu IgY, protein AG and protein G conjugates. Red bars represent wells without the chaotrope and the blue bars represent wells that received dissociating chaotrope. For each species, 10 replicates were used for the experiments.

Binding activities of anti-kudu IgY, anti-impala IgY and commercial conjugates on kudu and impala sera

When comparing how each host species reacted to the conjugates, we found that kudu serum had mean AIs of 72.36 ± 1.07 with anti-kudu IgY, 66.67 ± 1.17 with anti-impala IgY. There was a significant difference between the anti-kudu IgY, anti-impala IgY, pAG and protG conjugates for kudu sera (p<0.0001; Figure 4). Similarly, impala serum had AIs of 72.08 ± 0.88 with anti-impala IgY, 70.20 ± 0.99 with anti-kudu IgY, 21.47 ± 0.62 with pAG and 23.52 ± 0.53 with protG conjugates, respectively (Figure 3). There was also a significant difference among all the conjugates for impala sera (p<0.05).

Our developed IgY conjugates out-performed the commercial conjugates for all wildlife species except for zebra specifically with an AI of less than 50 (anti-kudu IgY=30.54 ± 1.04; anti-impala IgY= 35.97 ± 0.37). The average AI for anti-impala IgY across all the species was 61.73 ± 11.25 (Table S3), for anti-kudu IgY was 63.25 ± 11.51 (Table S4), pAG was 37.71 ± 17.25 (Table S5) and protG was 36.08 ± 15.78 (Table S6) . All wildlife sera tested with the protG conjugate had an AI of less than 50, except for black wildebeest (57.24 ± 0.88) and tsessebe (50.38 ± 0.64) (Figure 3). Also, all the wildlife sera that were tested for pAG conjugate, demonstrated an AI of less than 50%, except for plains zebra (51.35 ± 0.48). The individual AIs for the wildlife sera are captured in Figure 3.

There were significant differences (p<0.05) between each species and its respective controls, except for impala and blesbok (p=0.088; Table S3). All the animals had avidity index below the respective controls, except for gemsbok and nyala which were higher than kudu (anti-kudu), goat which was higher than cattle (pAG); and springbok which was higher than impala (Figure 3). Details of all the normalised AIs, are shown in Table S3. Values above the zero threshold indicate higher avidity than the respective control while negative values indicate lower avidity compared to the control. Comparing the differences in avidity of the developed conjugates to the different wildlife species, there was a significant interaction between the developed conjugates (anti-impala IgY and anti-kudu Ig conjugates) and the subfamily of the wildlife species (p<0.0001; Figure 4A and B). Antilopinae and Caprinae subfamilies did significantly better with anti-impala, while the Bovinae, Alcelaphinae and Hippotraginae subfamilies did better with anti-kudu (p<0.0001; Figure 4A and B).Tribes and subfamilies more closely related to kudu performed better with anti-kudu conjugate than anti-impala. And wildlife species more closely related to impala performed better with anti-impala. There was a wider variation in tribes than in subfamilies as in Figure 4A. Animals that share the same tribe such as the domestic cattle and the African buffalo demonstrated significant variation (p<0.0001) in their avidity to both the commercial and developed conjugate. Domestic cattle performed significantly better with pAG and protG than the African buffalo while the African buffalo demonstrated significantly better avidity than domestic cattle (p<0.0001).



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58 **Figure 3:** (A)Scatter plot with error bars (standard deviation) showing the avidity index for each of the conjugates (red anti-impala, blue antikudu,
59 yellow protein AG and green protein G) determined for different wildlife species. The avidity between the conjugate and different sera was
60 calculated as the reduction in colour between wells without a chaotropic agent (CT) and those with CT and presented as the AI for each serum. .
61 The silhouettes in colour connect species and conjugate colours to denote the species used as control for each conjugate: impala for anti-impala
62 IgY, kudu for anti-kudu IgY, cattle for protein AG and goat for protein G. The horizontal dotted lines indicate the avidity index of the respective

63 controls, and the colours correspond to the conjugates. Species were grouped into subfamilies as described by Hassanin and Emmanuel (1999),
64 however, ordering of the species was not done by phylogenetic relationships.

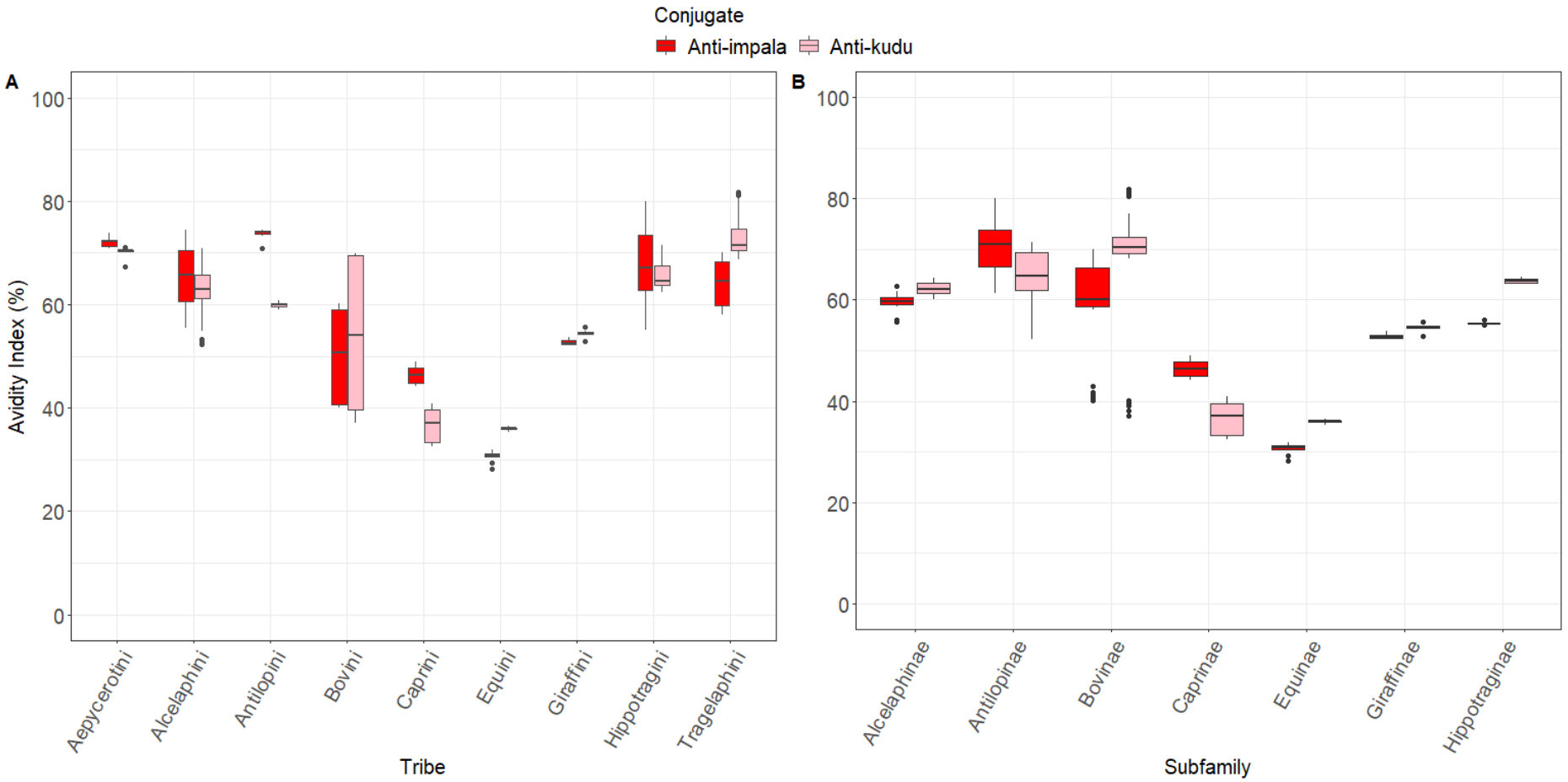

Figure 4: Box plots showing the avidity index for the wildlife species grouped by A) the tribe they belong to and B) their subfamilies. These species were classified based on the work described by Hassanin and Emmanuel (1999) and Gatesy et al. (1997). Red indicates anti-impala IgY and pink is anti-kudu IgY conjugate.

DISCUSSION

We developed conjugates for kudu (IgY anti-kudu) and impala (IgY anti-impala), two important hosts in disease transmission in wildlife in South Africa. We show that the conjugates are specific to their respective species and have better avidity than the commercially available protG and pAG conjugates. This is the first study to develop species-specific conjugates for antibody detection in kudu and impala with quantitative evidence of cross-reactions with antibodies of other species of African wildlife, providing the tools for the development and validation of primary binding assays like the indirect ELISA. These assays can improve sero-surveillance for infectious diseases in wildlife.

Chicken anti-kudu and chicken anti-impala conjugates developed in this study confirm the importance of using IgY in developing secondary antibodies against mammalian sera IgY from eggs is that it is cheap to produce in large volumes and ethically preferable as no blood collection from animals is required (Amro et al., 2018).This study showed that the developed anti-kudu IgY and anti-impala IgY conjugates had higher AIs (>70%) as compared to commercial pAG and protG conjugates with AIs less than 30%. This confirms stronger binding of the secondary IgY antibodies which is an important parameter in the development of primary binding assays like the indirect ELISA (Dimitrov et al., 2011). The weak binding observed for protG and pAG conjugates to impala sera in this study was also observed in other studies(Feir et al., 1993; Kramsky et al., 2003; Smit, 2017; Stöbel et al., 2002b), and is also in agreement with the findings of Smit (2017). However, it contradicts other findings that reported strong reactivity with either protG or pAG (Feir et al., 1993; Kelly et al., 1993; Kramsky et al., 2003; Stöbel et al., 2002b).This could be due to the differences in the methods used. In this study, we measured the binding strength of the antibodies in the presence of a dissociative agent while other studies only compared binding of conjugates under normal physiological conditions. Similar to this study Smit (2017) also recorded high OD values for protA and pAG but showed that the avidity was weak and binding could easily be disrupted under stringent binding conditions, like in the presence of the chaotropic agent.

Sera from the different species reacted differently with the two developed IgY conjugates and the two commercial conjugates. Wildlife species had stronger binding to the IgY conjugates than to the commercial conjugates, except for the plains zebra. Although the wildlife species demonstrated good avidity with both anti-kudu IgY and anti-impala IgY conjugates, there appeared to be a phylogenetic preference between the two IgY conjugates. The antelopes more closely related to kudu had better avidity to the anti-kudu IgY conjugate and the ones more closely related to impala had better avidity to the anti-impala IgY conjugate (Figure 4A and B). Species-

specific conjugates can also bind with good avidity to closely related species (Smit, 2017). This means that the more distantly related they are to the species for which the conjugate was developed, the less avidity. For example, sable, roan, tsessebe, blesbok and bontebok had better avidity with anti-impala IgY as they all belong to the *Antilopinae* family as described by Hassanin and Emmanuel (1999). Similarly, the members of the Tragelaphini tribe such as nyala, eland, and bushbuck (Hassanin & Emmanuel, 1999) had better avidity with the anti-kudu IgY conjugate (Figure 3 and Figure and B). A weaker avidity was seen in more distant related species like cattle, goat, plains zebra and giraffe (Figure 3 and Figures 4A and B).

There are reports in the literature where assays developed for livestock were used for antibody detection in wildlife. These include studies were assays that have been developed for horses were used for zebra(Abdelgawad et al., 2015), domestic dogs for African wild dog(Kat et al., 1995), domestic cats for lions (Gumbo et al., 2022) and domestic cattle for African buffalo (Sarangi et al., 2022). However, in this study we report a significant variation between domestic cattle and African buffalo within the bovini tribe. African buffalo reacted strongly with anti-kudu and anti-impala conjugates with an avidity of greater than 60% but had a poor avidity of less than 20% with pAG and protG conjugates. Whereas domestic cattle on other hand had a stronger avidity with pAG and protG conjugates but demonstrated poor avidity index with anti-kudu and anti-impala conjugates (≤40%). These results emphasise the need to develop and validate serological assays that are specific for wildlife species and caution against interspecies use of assays without proper validation even if they belong to the same tribes.

The conjugates developed here are important tools for the development of validated assays for the surveillance of emerging and re-emerging diseases of veterinary and human importance. And the concept of a diagnostic test being fit and validated for specific host species is one that is critical and promoted by the World Organization for Animal Health (OIE)(Gardner et al., 2019). Wildlife diseases are often understudied, and little is known about the accuracy of the diagnostic techniques employed(Jia et al., 2020). One pertinent question that has remained is about the accuracy of the of diagnostic tests validated in domestic stocks when used in wildlife species. Majority of the wild animals tested in this study are important host to various pathogens responsible for a range of diseases in the wild. And these animals demonstrated strong avidity with either ant-impala or anti-kudu, this is therefore an indication that developing multi-species polyclonal conjugate consisting of a cocktail of immunoglobulins could further improve active surveillance and facilitate the validation of immunoassays in these species.

The pAG conjugate tested in this study demonstrated an avidity index of less than 40% with most wildlife species, with the exception of the plains zebra, black and blue wildebeest and waterbuck. These results corroborate the findings of Smit (2017) who reported similar AIs in these species. The poor reactivity seen in the majority of the wildlife species could be attributed to a genetic predisposition that could make pAG bind weakly with the IgG of the wildlife species. Except for black wildebeest and tsessebe, all the wildlife species had an AI of less than 50% with the protG conjugate used in this study. Factors that could influence the binding of conjugates in primary binding assays could include variation in antibody structure between species, a limited amount of IgG in the original serum, as seen in immunocompromised individuals or the presence of inhibitors(Kelly et al., 1993; Kramsky et al., 2003). Also, pAG and protG could selectively bind to the subclasses or isotypes of IgG as seen in mice, where IgG2 is bound more strongly to protG, while IgG1 binds very weakly(Björck & Kronvall, 1984). Therefore, when an immune response is predominantly of a different subclass, these subclasses may not be detected in an immunoassay that is utilizing these conjugates. The variation in the avidity of conjugates to the immunoglobulins of different species emphasises the importance of proper species-specific validation of diagnostic assays.

The level of avidity of the conjugates impacted the outcome of the antiPA ELISA significantly. The antiPA ELISA ODs for developed conjugates were about 50% less than that of the commercial conjugates. This agrees with the assertion that the conjugate will only optimally bind specifically to the species for which it was developed for or for most closely related species (Feir et al., 1993; Smit, 2017). The samples tested with protG showed higher ODs than those tested with the developed conjugates. As this study is a continuation of a previous study where we looked at antiPA antibodies in wildlife species in the Kruger and Etosha National Parks (Ochai et al., 2022), we noticed that only the animals (kudu and impala in KNP) that were positive for both ELISA and Toxin Neutralisation Assay (TNA) still remained positive following the drop in the OD values.

CONCLUSION

Results of this study demonstrate the need to develop conjugates for immunoassays that are specific to African wildlife, as they are important hosts to many pathogens of human, animal, and zoonotic importance in KNP and parks like it. Kudu and impala sera demonstrated better avidity to their corresponding conjugates than to the commercial conjugates. The wildlife species tested in this study showed stronger avidity to the developed conjugates than to the commercial conjugates. This could also be achieved through a multi-species polyclonal conjugate consisting

of a cocktail of immunoglobulins from various wildlife species. Such evidence-based methods could allow for more accurate validation of diagnostic assays for the detection of incidence and prevalence of wildlife and zoonotic diseases.

SUGGESTIONS FOR FUTURE RESEARCH

Future studies to examine the development of polyclonal cocktail conjugated secondary antibodies for other African wildlife could establish immunodiagnostic assays that would be more specific to identify pathogens of veterinary and human diseases. Secondly, owing to the varying reports of avidity and binding ability of commercial conjugates, we suggest studies that evaluate these conjugates on a wider selection of wildlife species beyond what is covered in this study. Finally, we advocate more studies focused on how the use of different conjugates affects the outcome of disease surveillance and screening.

Data Availability Statement

At the time of publication, data were not publicly available from the authors. The raw data supporting the conclusions of this article will be made available by the corresponding author, without undue reservation.

Ethics Statement

This study was reviewed and approved by University of Pretoria Research Ethics Committee, Animal Ethics Committee (REC 049-21), Department of Agriculture, Forestry and Fisheries (DAFF) in South Africa (Ref 12/11/1/1/6 (2382SR)) in South Africa, South African National Parks (SANParks), South Africa (Ref: BMTA 006/22).

Author Contributions

SO, and HH conceived the ideas of the study. SO, HH, and JC designed the study. SO collected the data. SO, JC and HH designed the methodology. SO analysed the data. SO, and HH wrote the first draft of the manuscript. All authors contributed significantly to manuscript revision, read, and gave approval for publication.

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Supplementary information

Table S 1: Summary of results obtained from publications that reported the use of commercially available Protein A, Protein G, and Protein AG in some African wildlife species and domestic stock. The table contains references of publications, methods used in parenthesis, and different interpretations of results. NA here stands Not Applicable

		Kelly et al. (1993) (Direct ELISA)		**Stöbel et al. (2002b) (Indirect ELISA)**		**Feir et al. (1993) (Indirect ELISA)**		**Kramsky et al. (2003)* (Direct ELISA)**	**Smit (2017) (Direct Avidity ELISA)**
Species	**Common name**	**Protein A**	**Protein AG**	**Protein A**	**Protein G**	**Protein A**	**Protein G**	**Protein G**	**Protein AG**
Loxodonta africana	African elephant	Weak	Weak	Medium	Low	NA	NA		Weak
Giraffa camelopardalis	Giraffe	Weak	Weak	Low	High	NA	NA		Weak
Aepyceros melampus	Impala	Weak	Strong	None	Low	None	None	Lesser to control	No reaction
Connochaetes taurinus	Blue wildebeest	Weak	Strong	NA	NA	NA	NA		Moderate
Syncerus caffer	African buffalo	Weak	Strong	None	Low	NA	NA		Strong
Tragelaphus angasii	Nyala	Weak	Strong	None	Low	NA	NA		Weak
Taurotragus oryx	Common eland	Weak	Strong	NA	NA	NA	NA		Weak
Tragelaphus scriptus	Bushbuck	Weak	Strong	NA	NA	NA	NA		Weak
Hippotragus niger	Sable antelope	Weak	Strong	None	High	None	Reacted	Equivalent to control	Moderate
Kobus ellipsiprymnus	Waterbuck	Weak	Strong	None	High	NA	NA		Moderate
Equus quagga burchellii	Burchell's zebra	NA	NA	NA	NA	NA	NA		Strong
Alcelaphus buselaphus	Red hartebeest	NA	NA	NA	NA	NA	NA		Weak
Connochaetes gnou	Black wildebeest	NA	NA	None	low	NA	NA		Strong

Damaliscus lunatus	Common tsessebe	NA	NA	NA	NA	NA	NA		Weak
Damaliscus pygargus phillipsi	Blesbok	NA	NA	None	Medium	NA	NA	Equivalent to control	
Antidorcas marsupialis	Springbok	NA	NA	High	High	None	Reacted		Strong
Hippotragus equinus	Roan antelope	NA	NA	None	Low	NA	NA	Equivalent to control	Moderate
Oryx gazella	Gemsbok	NA	NA	None	Low	NA	NA		Moderate
Tragelaphus strepsiceros	Greater kudu	Weak	Strong	Medium	Medium	None	Reacted	Equivalent to control	No reaction
Bos taurus	Domestic cattle	Weak	Strong	NA	NA	NA	NA	Control	
Capra hircus	Goat	Weak	Strong	NA	NA	NA	NA		
Ovis aries	Sheep	Weak	Strong	NA	NA	NA	NA	Lesser to control	
Damaliscus pygargus	Bontebok							Equivalent to control	

Table S 2: SDS-PAGE gel (8%) reagents and volume of separating and stacking gel (Laemmli, 1970)

Reagents	**Separating gel (ml)**	**Stacking gel (ml)**
Distilled water	7	2.1
30% Acrylamide	4	0.5
1.5 M Tris (pH 8.8)	3.8	0.380
10% SDS	0.150	0.030
10%APS	0.150	0.030
TEMED	0.009	0.003

Supplementary Methodology 1: Briefly, the microtiter plates (Thermo Scientific™ Pierce 96-well Plates-Corner, USA) were coated overnight with 25 µg/ml of the extracted IgG from the respective species (impala and kudu) as described by Staak et al. (2001). Plates were washed twice with Phosphate Buffered Saline (PBS) supplemented with 0.05% Tween-20 (Thermo Fisher Scientific, Waltham, MA USA) (PBST) using a plate washer (Biorad PW40, Mamesla-Coquette, France). The plates were blocked blocked with PBST supplemented with 5% skimmed milk powder (PBSTM) and then incubated for 1 hour at room temperature. This was followed by washing the plates twice. A 100 µL of the the egg yolk from each chicken was added into the plate with a starting dilution of 1:20 in PBSTM starting from the first column of each plate. This was followed by 30 minutes incubation on a rotatory incubator (Environmental Shaker-Incubator ES-20, Biosan Ltd, Germany). Afterwards, the plates were washed five times and a 100 µL of a 1:10000 dilution of goat anti-chicken horseradish peroxidase conjugate (Invitrogen goat anti-chicken, USA) was added to each well and incubated for 30 minutes on the rotary incubator. This was followed by a wash step and subsequently, the substrate 2,2'-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) (Thermo Scientific 1-Step ABTS, USA) was added and incubated in the dark for 45 minutes. The absorbance was read at 405 nm using the plate reader(Biotek Powerwave XS2 reader, Vermont, USA).

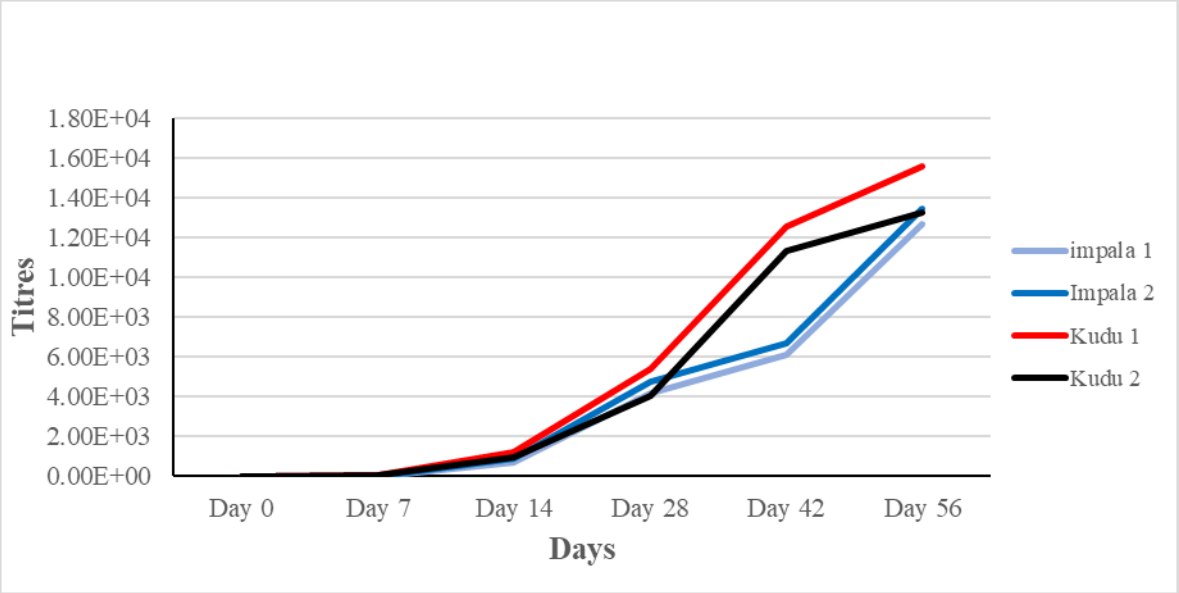

Figure S 1**:** Line chart showing the increase in titres over days of antibodies against impala and kudu Immunoglobulin G (IgG). The first vaccination was given on "Day 0", the second dose was given on "Day 23" and the last dose was given on "Day 42"

Supplementary Methodology 2: A pooled sera for each species (impala and kudu) were coated to microtiter plates with a starting dilution of 1:1000 from column 1 to 11 in coating buffer (bicarbonate buffer) left overnight at 4oC to incubate. This was followed by a blocking step with the blocking buffer (200 µL) containing PBST and 5% skimmed milk powder (PBSTM) and then incubated at room temperature for 1 hour. The developed conjugates were tested against each species with a starting dilution of 1:200 row A to row G. The blanked wells are row H and column 11. The plates were incubated at room temperature for 30 minutes. Subsequently, the plates were washed and after which the ABTS substrate (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; Thermo Scientific 1-Step ABTS, USA) was added and allowed in the dark for 45 minutes. The absorbance was read at 405 nm using the plate reader (Biotek Powerwave XS2 reader, Vermont, USA).

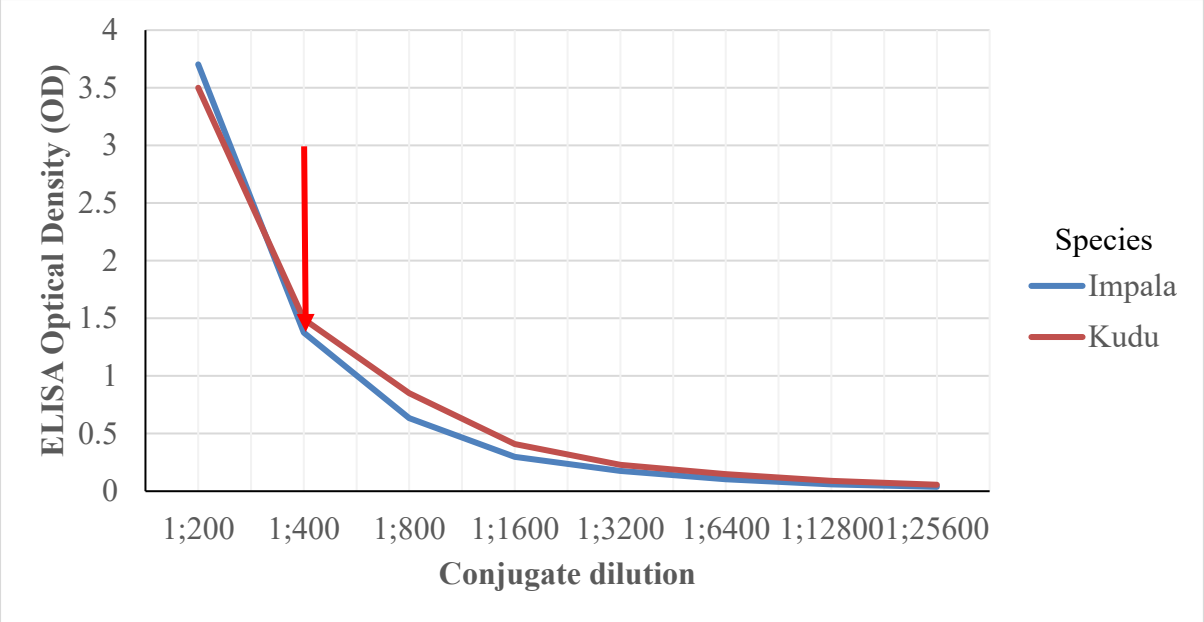

Figure S 2: Line graph showing the concentration (Optical Density) of chicken anti-kudu and chicken anti-impala conjugates at different dilutions. The blue line represents anti-impala conjugate while the orange line represents anti-kudu conjugate. The red arrow depicts the optimal dilution (1:400) for the conjugates.

Table S 3: Anti-impala summary statistics showing mean OD of PBS and potassium thiocyanate treated wells, mean OD of PBS and chaotrope treated wells for the goat control, percentage reduction in OD, and the mean avidity index and standard deviation (SD) and the p-value of the independent T-test comparing avidity index of each species and the impala control.

Species	Mean OD ± SD PBS	Mean OD ± SD chaotrope	Mean OD impala Control	Mean OD control chaotrope	Percentage reduction	Mean Avidity index ± SD	P- value
African buffalo	1.64 ± 0.03	1.06 ± 0.02	1.67	1.03	35.18	64.82 ± 1.37	4.58E-10
Black wildebeest	1.37 ± 0.05	0.81 ± 0.05	1.62	1.34	40.50	59.5 ± 1.86	9.77E-11
Blesbok	1.46 ± 0.04	1.07 ± 0.02	1.54	1.15	26.82	73.18 ± 1.62	0.088907
Blue wildebeest	1.8 ± 0.04	1.07 ± 0.04	1.86	1.35	40.30	59.7 ± 1.55	3.10E-12
Bontebok	1.16 ± 0.02	0.82 ± 0.01	1.86	1.35	29.12	70.88 ± 0.87	0.007503
Bushbuck	1.16 ± 0.02	0.78 ± 0.02	1.68	1.18	32.93	67.07 ± 0.39	1.48E-09
Cattle	0.82 ± 0.02	0.34 ± 0.01	1.84	1.35	59.05	40.95 ± 0.9	5.17E-23
Eland	1.26 ± 0.03	0.74 ± 0.02	1.72	1.21	40.94	59.06 ± 0.51	1.07E-15
Gemsbok	1.24 ± 0.02	0.87 ± 0.02	1.86	1.35	29.66	70.34 ± 0.86	0.000329

Giraffe	1.41 ± 0.05	0.74 ± 0.02	1.62	1.34	47.29	52.71 ± 0.52	3.85E-18
Goat	0.94 ± 0.03	0.42 ± 0.01	1.84	1.35	55.11	44.89 ± 0.5	8.84E-20
Impala	1.96 ± 0.03	1.41 ± 0.02	1.96	1.36	27.91	72.09 ± 0.89	NA
Kudu	1.52 ± 0.03	1.01 ± 0.02	1.86	1.35	33.33	66.67 ± 1.23	5.30E-09
Nyala	1.64 ± 0.03	1.13 ± 0.01	1.84	1.35	31.19	68.81 ± 0.4	1.71E-07
Plains zebra	0.36 ± 0.03	0.11 ± 0.01	1.86	1.35	69.45	30.55 ± 1.04	5.32E-24
Red hartebeest	1.42 ± 0.04	0.76 ± 0.02	1.54	1.15	46.44	53.56 ± 1.46	3.41E-15
Roan	1.47 ± 0.02	0.96 ± 0.02	1.72	1.21	34.92	65.08 ± 0.72	7.79E-13
Sable	1.08 ± 0.02	0.76 ± 0.02	1.84	1.35	30.14	69.86 ± 1.81	0.004318
Sheep	0.41 ± 0.05	0.24 ± 0.03	1.68	1.18	41.47	58.53 ± 2.14	4.74E-10
Springbok	1.55 ± 0.02	1.14 ± 0.02	1.85	1.36	26.31	73.69 ± 1.04	0.002179
Tsessebe	1.64 ± 0.03	1.06 ± 0.02	1.67	1.03	35.18	64.82 ± 1.37	4.58E-10

Waterbuck	1.84 ± 0.04	1.02 ± 0.03	1.67	1.03	44.68	55.32 ± 0.29	2.16E-14

Table S 4: Anti-kudu summary statistics showing mean OD of PBS and potassium thiocyanate treated wells, mean OD of PBS and chaotrope treated wells for the goat control, percentage reduction in OD, and the mean avidity index and standard deviation (SD) and the p-value of the independent T-test comparing avidity index of each species and the kudu control. Abbreviation such as NA means Not Applicable.

Species	**Mean OD ± SD PBS**	**Mean OD ± SD chaotrope**	**Mean OD kudu Control**	**Mean OD control chaotrope**	**Percentage reduction**	**Mean Avidity index ± SD**	**P- value**
African buffalo	1.72 ± 0.06	1.19 ± 0.04	1.83	1.33	30.73	69.27 ± 0.64	2.91E-06
Black wildebeest	1.75 ± 0.03	1.11 ± 0.01	2.47	1.80	36.59	63.41 ± 0.49	2.80E-11
Blesbok	1.76 ± 0.06	1.15 ± 0.03	1.76	1.31	34.37	65.63 ± 0.86	5.77E-11
Blue wildebeest	0.85 ± 0.02	0.52 ± 0.01	1.43	1.09	39.03	60.97 ± 0.71	6.58E-14
Bontebok	0.74 ± 0.02	0.52 ± 0.02	1.46	1.20	30.23	69.77 ± 0.71	2.07E-05
Bushbuck	0.87 ± 0.04	0.61 ± 0.02	1.65	1.21	29.74	70.26 ± 0.72	0.000188
Cattle	0.66 ± 0.03	0.26 ± 0.01	1.65	1.18	60.72	39.28 ± 0.99	4.67E-22
Eland	2.15 ± 0.06	1.53 ± 0.03	2.18	1.59	29.07	70.93 ± 1.38	0.022406
Gemsbok	1.02 ± 0.05	0.81 ± 0.04	1.86	1.34	20.78	78.92 ± 1.18	3.03E-10
Giraffe	1.34 ± 0.04	0.73 ± 0.02	2.47	1.80	45.49	54.51 ± 0.69	1.47E-16
Goat	0.51 ± 0.06	0.2 ± 0.02	1.64	1.18	60.28	39.72 ± 0.87	1.39E-21

Impala	1.61 ± 0.04	1.13 ± 0.02	1.78	1.30	29.80	70.2 ± 1.05	0.000364
Kudu	1.86 ± 0.03	1.34 ± 0.02	1.86	1.35	27.64	72.36 ± 1.13	NA
Nyala	1.85 ± 0.04	1.5 ± 0.03	1.86	1.34	18.68	80.82 ± 1.42	3.76E-11
Plains zebra	0.56 ± 0.02	0.2 ± 0.01	1.43	1.09	64.03	35.97 ± 0.38	1.81E-17
Red hartebeest	1.51 ± 0.05	1 ± 0.03	1.76	1.31	33.77	66.23 ± 0.83	1.63E-10
Roan	2.14 ± 0.06	1.41 ± 0.03	2.18	1.59	34.03	65.97 ± 0.82	8.90E-11
Sable	1.76 ± 0.02	1.12 ± 0.01	1.89	1.67	36.16	63.84 ± 0.69	2.73E-12
Sheep	0.67 ± 0.04	0.45 ± 0.02	1.65	1.21	33.49	66.51 ± 1.72	1.53E-07
Springbok	1.6 ± 0.04	0.96 ± 0.03	1.80	1.31	40.07	59.93 ± 0.46	5.74E-13
Tsessebe	1.32 ± 0.04	0.83 ± 0.02	1.63	1.18	37.55	62.45 ± 0.72	2.08E-13
Waterbuck	1.26 ± 0.02	1.63 ± 0	1.18	1.18	36.28	63.72 ± 0.54	1.43E-11

Table S 5: Protein AG summary statistics showing mean OD of PBS and potassium thiocyanate treated wells, mean OD of PBS and chaotrope treated wells for the goat control, percentage reduction in OD, and the mean avidity index and standard deviation (SD) and the p-value of the independent T-test comparing avidity index of each species and the cattle control. Abbreviation such as NA means Not Applicable.

Species	Mean OD ± SD PBS	Mean OD ± SD chaotrope	Mean OD cattle Control	Mean OD control chaotrope	Percentage reduction	Mean Avidity index ± SD	P- value
African buffalo	3.64 ± 0.05	0.68 ± 0.02	2.46	1.83	81.28	18.72 ± 0.63	1.14E-20
Black wildebeest	2.87 ± 0.06	1.41 ± 0.04	2.26	1.46	50.78	49.22 ± 0.69	2.96E-17
Blesbok	2.49 ± 0.1	1.02 ± 0.06	2.33	1.75	59.00	41 ± 1.04	2.76E-21
Blue wildebeest	2.53 ± 0.05	1.25 ± 0.03	2.66	1.93	50.58	49.42 ± 0.53	1.07E-15
Bontebok	3.36 ± 0.08	0.97 ± 0.02	2.43	1.83	71.20	28.8 ± 0.68	2.63E-20
Bushbuck	1.86 ± 0.03	0.52 ± 0.02	2.12	1.56	72.16	27.84 ± 0.94	7.27E-23
Cattle	2.43 ± 0.04	1.83 ± 0.03	2.43	1.83	24.49	75.51 ± 1.29	NA
Eland	2.53 ± 0.03	0.67 ± 0.01	2.63	1.92	73.59	26.41 ± 0.31	1.39E-16
Gemsbok	3.59 ± 0.02	0.95 ± 0.02	2.43	1.83	73.49	26.51 ± 0.49	2.36E-18
Giraffe	2.4 ± 0.07	1.05 ± 0.03	2.46	1.83	56.50	43.5 ± 0.71	1.88E-18
Goat	2.21 ± 0.04	1.77 ± 0.04	2.43	1.83	20.20	79.8 ± 0.9	2.43E-07
Impala	2.49 ± 0.21	0.53 ± 0.04	2.21	1.77	78.53	21.47 ± 0.66	9.31E-21
Kudu	2.77 ± 0.11	0.42 ± 0.02	2.21	1.77	84.77	15.23 ± 1.1	5.96E-25
Nyala	2.52 ± 0.09	0.85 ± 0.05	3.70	2.74	66.34	33.66 ± 1.06	1.31E-22
Plains zebra	2.66 ± 0.05	1.37 ± 0.03	2.66	1.93	48.67	51.33 ± 0.48	6.22E-15

Red hartebeest	2.19 ± 0.05	0.73 ± 0.04	2.33	1.75	66.62	33.38 ± 1.12	9.05E-23
Roan	2.78 ± 0.13	0.79 ± 0.05	2.63	1.92	71.45	28.55 ± 0.72	8.83E-21
Sable	2.46 ± 0.16	0.35 ± 0.04	2.43	1.83	85.72	14.28 ± 2.15	6.41E-20
Sheep	1.93 ± 0.05	1.05 ± 0.03	2.12	1.56	45.50	54.5 ± 1.45	3.76E-17
Springbok	2.52 ± 0.09	0.85 ± 0.05	3.70	2.74	66.34	33.66 ± 1.06	1.31E-22
Tsessebe	2.81 ± 0.03	0.81 ± 0.03	2.66	1.96	71.09	28.91 ± 1.26	2.41E-23
Waterbuck	1.89 ± 0.06	0.93 ± 0.03	2.66	1.96	50.48	49.52 ± 0.8	4.66E-18

Table S 6: Protein G summary statistics showing mean OD of PBS and potassium thiocyanate treated wells, mean OD of PBS and chaotrope treated wells for the goat control, percentage reduction in OD, and the mean avidity index and standard deviation (SD) and the p-value of the independent T-test comparing avidity index of each species and the goat control. Abbreviation such as NA means Not Applicable.

Species	Mean OD ± SD PBS	Mean OD ± SD chaotrope	Mean OD goat Control	Mean OD control chaotrope	Percentage reduction	Mean Avidity index ± SD	P- value
African buffalo	1.3 ± 0.04	0.24 ± 0.01	2.58	1.90	81.19	18.81 ± 0.75	2.85E-24
Black wildebeest	2.45 ± 0.06	1.4 ± 0.03	2.58	1.90	42.76	57.24 ± 0.88	4.11E-16
Blesbok	2.53 ± 0.03	0.66 ± 0.01	2.63	1.93	73.70	26.3 ± 0.59	7.91E-27
Blue wildebeest	1.7 ± 0.02	0.46 ± 0.02	2.14	1.59	73.21	26.79 ± 0.78	5.41E-23
Bontebok	1.64 ± 0.09	0.43 ± 0.03	2.14	1.59	73.46	26.54 ± 1.03	1.77E-19
Bushbuck	1.94 ± 0.02	0.54 ± 0.01	2.16	1.58	72.19	27.81 ± 0.38	1.13E-30
Cattle	1.36 ± 0.03	0.75 ± 0.02	2.14	1.59	45.14	54.86 ± 0.55	8.68E-23
Eland	2.15 ± 0.03	0.98 ± 0.02	2.42	1.75	54.71	45.29 ± 0.82	8.02E-20
Gemsbok	1.52 ± 0.11	0.28 ± 0.02	2.14	1.59	81.76	18.24 ± 1.37	1.48E-17
Giraffe	2.62 ± 0.06	1.16 ± 0.04	2.58	1.90	55.50	44.5 ± 1.03	2.87E-17
Goat	2.14 ± 0.03	1.59 ± 0.02	2.14	1.59	25.47	74.53 ± 0.41	NA
Impala	2.89 ± 0.08	0.68 ± 0.01	2.14	1.59	76.48	23.52 ± 0.56	7.61E-28
Kudu	2.82 ± 0.09	0.66 ± 0.02	2.14	1.59	76.39	23.61 ± 0.99	3.19E-20
Nyala	1.61 ± 0.08	0.47 ± 0.04	2.16	1.58	70.94	29.06 ± 2.45	3.85E-13
Plains zebra	1.08 ± 0.09	0.19 ± 0.01	2.14	1.59	82.01	17.99 ± 1.09	1.14E-19

Red hartebeest	2.54 ± 0.04	0.76 ± 0.03	2.63	1.93	70.20	29.8 ± 1.01	3.36E-17
Roan	2.06 ± 0.03	1.02 ± 0.02	2.42	1.75	50.80	49.2 ± 0.23	1.66E-23
Sable	1.16 ± 0.08	0.19 ± 0.01	2.16	1.58	83.42	16.58 ± 0.71	3.17E-25
Sheep	2.15 ± 0.03	1.28 ± 0.01	2.16	1.58	40.32	59.68 ± 0.26	8.46E-22
Springbok	1.56 ± 0.03	0.55 ± 0.01	2.14	1.59	64.62	35.38 ± 0.87	1.66E-20
Tsessebe	2.41 ± 0.03	1.21 ± 0.02	2.47	1.85	49.62	50.38 ± 0.65	1.05E-21
Waterbuck	2.32 ± 0.08	0.86 ± 0.03	2.47	1.85	62.86	37.14 ± 0.47	6.05E-28

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